Effects of Physical Inactivity on Skeletal Muscle Metabolic Function

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Declaration

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I would like to dedicate this thesis to the memory of my father, Owen Kenny.

Abbreviations

ADP	Adenosine Diphosphate
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
AVO_2 diff	Atrial venous oxygen difference
BCAA	Branched chain amino acids
BIS	Bioelectrical impedance spectrocscopy
BMI	Body mass index
BSA	Bovine serum albumin
CHO	Carbohydrate
CO	Cardiac output
COX IV	Cytochrome C Oxidase subunit IV
COX5a	Cytochromc C oxidase subunit 5a
CS	Citrate synthase
CSA	Cross sectional area
CVD	Cardiovascular disease
DAG	Diacylglycerol
DCF-DA	2', 7' – dichlorofluorescein diacetate
DEXA	Dual energy X-ray absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EAA	Essential amino acids
ECF	Extracellular fluid
ECL	Enganced chemiluminescence
EGTA	Eathylene glycol tetraacetic acid
EMG	Electromyographic
ERRα	Estrogen related receptor alpha
ETC	Electron Transport Chain
ETF	Electron transferring flavoprotein

ETS	Electron transport system capacity
FA	Fatty Acid
FABP	Fatty acid binding protein
FADH	Flavin adenine dinucleotide
$FADH_2$	Reduced form of Flavin adenine dinucleotide (FAD)
$FADH_2$	Reduced for of flavin adenine dinucleotide
FATP	Fatty acid transport proteins
FCCP	Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone
FCS	Foetal calf serum
Fe-S	Iron sulphur cluster
FFM	Fat free mass
FM	Fat mass
FOX 01	Forkhead transcription factor, O-box subfamily, 1A
FW	Flywheel
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glut4	Glucose transporter type 4
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HDT	Head down tilt
HO·	Hydroxyl radical
HR	Heart rate
HRR	High resolution respirometry
IGF-1	Insulin-like growth factor 1
IKE	Isokinetic exercise training
IMF	Intermyofibrillar
IRS	Insulin receptor substrate
JC-1	5,5',6,6' – tetrachloro - 1,1',3,3' – tetraethylbenzimodazolcarbocyanine iodide
$KHCO_3$	Sodium Bicarbonate
LBNP	Lower body negative pressure
LCFA-CoA	long chain fatty acyl-CoA
LPL	Lipoprotein lipase
MAFbx	Muscle atrophy F-box
MHC	Myosin heavy chain

MiR05	Mitochondrial respiration medium
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
MTG	Mito Tracker Green
mTOR	Mammalian target of rapamycin
MuRF-1	Muscle RING finger-1
MVC	Muscle voluntary contraction
MW	Molecular weight
NADH	Reduced form of nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NF-kB	Nuclear factor-kB
NRF	Nuclear respiratory factor
O_2	Oxygen
$O_2^{-}\cdot$	Superoxide anion
OXPHOS	Oxidative phosphorylation capacity
PAL	Physical activity level
PBS	Phosphate buffer saline
PDK-1	Phosphoinositide-dependent kinase-1
PFi	Permeabilized fibres
PGC1-α	Peroxisome proliferator receptor gamma co-activator -1 alpha
PI	Protease Inhibitor
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-trisphosphate
$PI(4,5)P_2$	Phosphatidylinositol-4-5-bisphosphate
PI3-K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl fluoride
PTB	Phosphotyrosine
PVDF	Polyvinylidene difluoride
RER	Respiratory exchange ratio
RM	Repetition Maximum
RMR	Resting metabolic rate
ROS	Reactive oxygen species
ROX	Residual oxygen consumption
RQ	Respiratory quotient

RVE	Resistive vibration exercise
S6K1/p70^{S6K}	70 KDa ribosomal S6 protein kinase
SaO_2	Oxygen saturation
SDH	Succinate dehydrogenase
SDS PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNAs	Small interfering RNA
SOD	Superoxide dismutase
SS	Subsarcolemmal
SUIT	Substrate uncoupler inhibitor titration
SV	Stroke volume
T2DM	Type 2 diabetes
TBD-t	Tris buffer saline with tween
TBS	Tris buffer saline
TBW	Total body water
TCA	Tricarboxylic acid cycle
Tfam	Mitochondrial transcription factor
TG	Triglyceride
TMPD	N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride
Ub	Ubiquitin
UCP3	Uncoupling protein 3
VCO_2	Carbon dioxide production
VDAC	voltage-gated ion channels
VE	Ventilation
VL	Vastus lateralis
VLDL	Very low density lipoprotein
VO_2 max	Maximal oxygen consumption
vQCT	Volumetric qualitative computed tomography
XO	Xantine oxidase

Abstract

Effects of Physical Inactivity on Skeletal Muscle Metabolic Function

Being confined to bed rest has negative physiological consequences. Bed rest represents a unique model of physical inactivity and results in changes in cardiovascular, neural and metabolic function. In particular, reduced skeletal muscle mitochondrial capacity and a reduction in intrinsic mitochondrial function in response to physical inactivity may have broad implications for human disease.

The aim of the present study was to test the hypothesis that physical inactivity, through the model of bed rest, will decrease mitochondrial function, insulin sensitivity and muscle mass in healthy young men.

A reduction in lean muscle mass was evident after bed rest ($p<0.05$). This was accompanied by a reduction in skeletal muscle insulin sensitivity ($p<0.05$). Citrate synthase as a marker of mitochondrial content was reduced after bed rest ($p<0.05$). O_2 flux expressed per mg of wet weight of muscle tissue was lower during LEAK respiration following 21 days of BR ($p<0.05$). OXPHOS and ETS capacity, indicative of maximal capacity, were increased following bed rest when normalized to citrate synthase activity ($p<0.05$). Mitochondrial proteins, COX5a, complex III core protein 2 and SDHb were down regulated.

A reduction in CS in the present study suggests that there are fewer actively respiring mitochondria, however no change in mitochodnrial respiration was noted. A reduction in LEAK respiration as an indicator of reduced mitochondrial uncoupling may be associated with increased ROS production. A significant positive correlation between the reduction in maximal ETS and insulin sensitivity suggests a relationship between the two. The mechanisms involved in disuse muscle atrophy require further clarification, as do the mechanisms linking insulin sensitivity, mitochondrial function and muscle atrophy.

Chapter I

Introduction

1.1 Introduction

Despite the well documented benefits of physical activity participation, only 5% of adults in the USA adhere to the minimal exercise guidelines of 30 minutes of moderate intensity physical activity per day (Troiano, Berrigan et al. 2008). This is surprising as, it has been recently reported that physical inactivity is the 10th leading contributor to the global burden of disease (Lim, Vos et al. 2013). The role of physical inactivity in the aetiology of chronic disease has only recently been recognised. This has resulted from scientific evidence demonstrating a direct effect of exercise/physical inactivity on risk factors including obesity, insulin resistance, dyslipidemia, inflammation and hypertension (Booth and Lees 2007). In addition, epidemiological evidence estimates that physical inactivity causes 6 -10% of global coronary heart disease, type 2 diabetes and breast and colon cancers (Lee, Shiroma et al. 2012) and 9% of premature mortality or more than 5.3 million deaths in 2008 (WHO. Global Health Observatory Data Repository. 2011. apps.who.int/ghodata).

In contrast to the evidence supporting the benefits of acute and chronic exercise, relatively little is known about the mechanisms underlying the physiological responses to physical inactivity (Hamilton, Hamilton et al. 2007). The physical activity guidelines recommending 150 minutes of moderate intensity exercise on five or most days of the week is largely accepted as sufficient to reduce the risk of chronic disease (ACSM 2010). The literature also demonstrates an inverse relationship between physical activity participation and all-cause mortality (Thyfault and Booth 2011). However, while the epidemiological data supports an association between physical inactivity and chronic disease development it is not sufficient to identify a cause and effect relationship.

The 'diseaseome of physical inactivity' presented by Pedersen (2009), suggests that physical inactivity is a strong independent risk factor for the accumulation of visceral fat and the subsequent activation of inflammatory pathways that lead to insulin resistance and cardiovascular disease (Pedersen 2009). Physical inactivity and the resulting fat accumulation, especially ectopic fat, can cause unfavourable metabolic and cardiovascular changes (Pedersen 2009). The health consequences of fat accumulation and physical inactivity are similar and it is possible the underlying mechanisms are shared. In support of this, the concept of metabolically inflexibility, first proposed by Kelley et al. (2002), has shown that dysregulation of lipid metabolism is common to

several metabolic diseases such as obesity, insulin resistance and type 2 diabetes and has also been reported in bed rest models of physical inactivity. The effects of physical inactivity on the development of metabolic dysfunction, obesity and cardiovascular disease are incompletely understood as the physiology of inactivity has been poorly investigated to date (Bergouignan, Rudwill et al. 2011), therefore there is a need for further research to specifically address such important questions.

1.2 Statement of the problem

One of the biggest barriers to combating the development of chronic diseases is the incomplete understanding of the relationship between physical inactivity and risk factor development at a cellular and molecular level. Our knowledge of the detrimental effects of physical inactivity is somewhat indirect and is mainly based on the positive effects of exercise training on the sedentary population (Booth, Chakravarthy et al. 2002). It is widely accepted that exercise has positive physiological benefits (Holloszy and Coyle 1984, Kirwan, del Aguila et al. 2000, O'Gorman, Karlsson et al. 2006, Coen, Tanner et al. 2015). However, the role of metabolism and specifically mitochondrial function with physical inactivity has not been studied. Conflicting reports exist regarding the role of mitochondria in the development of insulin resistance. In fact many studies report a dissociation between mitochondrial function and insulin resistance (Turinsky, Bayly et al. 1990, Bandyopadhyay, Joseph et al. 2006, Holloway, Thrush et al. 2007, Trenell, Hollingsworth et al. 2008, Lanza and Nair 2009) while others have suggested a positive relationship between mitochondrial dysfunction and insulin resistance (Kelley, He et al. 2002, Petersen, Befroy et al. 2003, Short, Vittone et al. 2003, Short, Bigelow et al. 2005). Published reports relating to mitochondrial capacity and insulin resistance lack consistency and this may be partly due to different outcome parameters being measured and variations in subject selection. Furthermore, gaps in our knowledge on physiological impact of physical inactivity may be due to the difficulty implementing longer term physical inactivity interventions in healthy subjects (Bergouignan, Rudwill et al. 2011).

The model of bed rest has recently been used to study the physiological adaptations associated with physical inactivity. It is well documented that bed rest causes metabolic dysfunction (Bergouignan, Rudwill et al. 2011). Independent of energy balance, bed rest

has been shown to induce muscle atrophy, a shift in muscle fibre type towards fast – twitch glycolytic type fibres, a reduced ability to use fat as substrate and insulin resistance. However, to date, no physical inactivity study has directly measured mitochondrial function. Nor has the role of mitochondrial function in the development of insulin resistance resulting from physical inactivity been clearly elucidated.

While the degree of physical inactivity experienced during bed rest appears extreme compared to physical inactivity in the general population, it should be noted that the physical activity level (PAL) experienced in bed rest is close to that measured in the general ambulatory population (PAL of 1.4 – 1.5) (Blanc, Normand et al. 1998, Bergouignan, Momken et al. 2010). An important aspect of bed rest studies is to assess the effects of countermeasures and their ability to mitigate the negative consequences of bed rest. It is widely accepted that interventions combining physical activity and nutrition are likely to be most efficacious but success to date has been limited. Protein supplementation has been shown to increase muscle protein synthesis after exercise, thereby enhancing the hypertrophic effect of exercise (Pennings, Koopman et al. 2011). Additionally, while exercise is a known moderator of insulin action (Helmrich, Ragland et al. 1991), protein supplementation appears to also have an insulinotropic effects (Pal, Ellis et al. 2010).

These studies suggest that progress is being made to mitigate the effects of physical inactivity but there is a need to further investigate and optimize countermeasures. In order to achieve this, it is imperative that we understand the physiological responses to physical inactivity and if the countermeasures are having an impact at the cellular level. Thus, bed rest studies provide not only a simulation model to test potential countermeasures but also provide a platform that can allow us to investigate human physiology in the setting of physical inactivity. In conclusion, there is a gap in our knowledge of the impact of physical inactivity on skeletal muscle mitochondrial function and the subsequent changes in whole body metabolism and insulin resistance. This information could help the design and implementation of more effective countermeasures to mitigate the effects of physical inactivity. This thesis will address the problem by investigating changes in mitochondrial function following a 21-day bed rest study in healthy young men.

1.3 Aim, Objectives, Hypotheses

Aim: The aim of this study is to determine the impact of 21-day bed rest induced physical inactivity on whole body and skeletal muscle metabolic function in healthy young men.

Objectives

1. To measure changes in insulin sensitivity, body composition and mitochondrial function before and after 21-days bed rest.
2. To identify inter-relationships, if any, between the changes in mitochondrial function, insulin sensitivity and muscle mass.
3. To determine if exercise alone or exercise and whey protein (with an alkaline salt) supplementation can counteract the negative consequences of bed rest.

   Exploratory objectives :

4. To determine if a model of metabolic stress in C2C12 mouse myotubes would have a negative impact on mitochondrial function.
5. To determine if COX5a knock down would have any effect on mitochondrial respiration.

Hypotheses:

1. Physical inactivity, through the model of bed rest, will decrease mitochondrial function, insulin sensitivity and muscle mass in healthy young men.
2. The supplementation with whey protein supplementation (with alkaline salt) and exercise will mitigate the effect of bed rest on insulin sensitivity and muscle mass and strength.
3. Treatment of C2C12 with high insulin/glucose or palmitate will decrease mitochondrial function, enhance reactive oxygen species (ROS) production and reduce mitochondrial membrane potential.
4. COX5a transfection will reduce mitochondrial respiration.

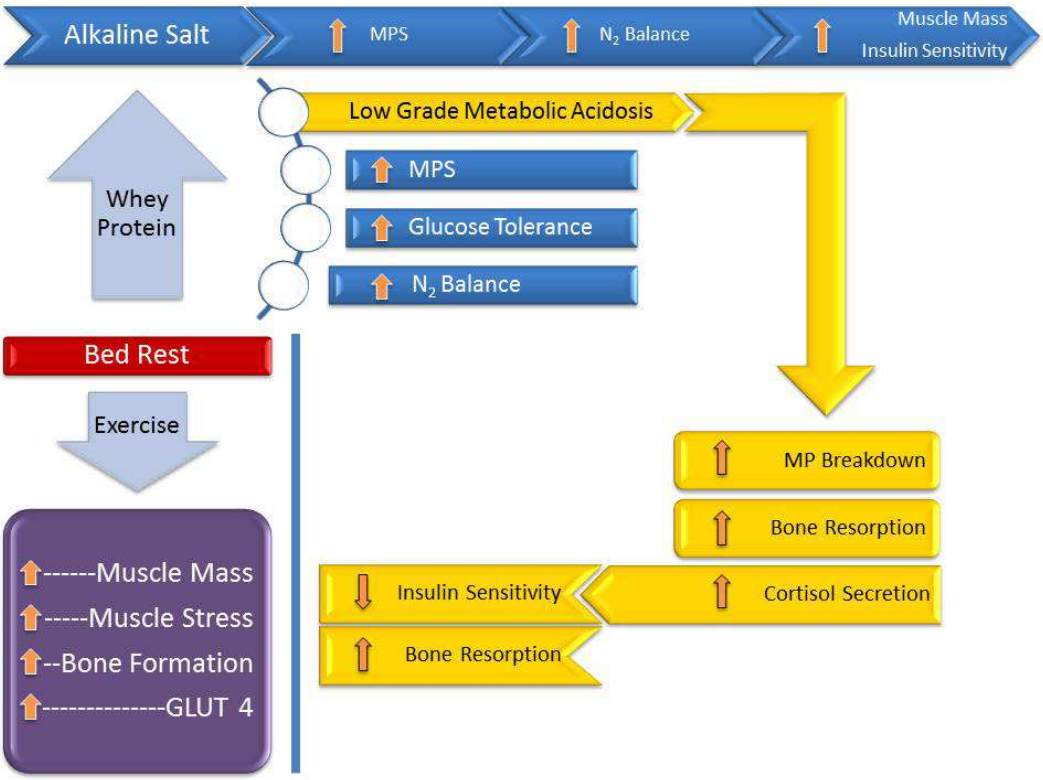


Figure 1.1 Scheme of Hypotheses. Bed rest leads to loss in muscle mass and a reduction in insulin sensitivity. Whey protein leads to increased muscle protein synthesis leading to a positive nitrogen balance and increased muscle mass. It can also cause low-grade metabolic acidosis which in turn can cause muscle protein breakdown, bone resorption and cortisol secretion which could further decrease insulin sensitivity and increase bone resorption. However, the addition of an alkaline salt can compensate for the low-grade metabolic acidosis induced by whey protein and further increase muscle protein synthesis and insulin sensitivity.

1.4 Experimental overview

In order to test the hypotheses a 3-arm randomly assigned, cross-over, 21-day bed rest study was designed. The 3 trials were (i) a control group (CONT) that consisted of 21-days bed rest; (ii) the same bed rest protocol as the CONT trial but with 2 resistive vibration exercise sessions per week (RVE) and (iii) the CONT protocol with resistance vibration exercise and a nutritional supplement (whey protein with an alkaline agent) (NEX). A number of detailed tests were performed before and after the intervention. All activities and test measurements were conducted in the head down tilt position. Energy balance was monitored throughout and energy intake was altered so that subjects would remain in energy balance. Muscle biopsies were obtained before and after each bed rest period and skeletal muscle analysis allowed to measure mitochondrial function

using high resolution respirometry. Each bed rest protocol was separated by a 4-month washout period.

Lipid over-supply has been suggested to desensitize the tissue to the catabolic effects of insulin. Palmitate treatment and high glucose/insulin of C2C12 myotubes has been previously shown to inhibit insulin stimulation of glycogen synthesis as well as inducing the accumulation of lipid metabolites, ceramides and diacylglycerol (DAG), both of which are known to inhibit insulin signalling in cultured cells (Chavez and Summers 2003). As bed rest is known to reduce insulin sensitivity in humans, we are interested in developing a model of metabolic stress in skeletal mouse myotubes to allow us to further investigate the effect a metabolic stressful environment would have on mitochondrial function. Additionally, there are certain aspects of cell metabolism that we will be unable to measure in our human samples and we intend to use the cell model to further investigate changes in mitochondrial membrane potential, reactive oxygen species production and mitochondrial content.

1.4.1 Delimitations

1. Bed rest period was for 21 days with 120 days washout between each bed rest trial.
2. The study included healthy male subjects only (age 20 -44 years).
3. Macronutrient composition of the diet was 30% fat, 15% protein and 55% carbohydrate.

1.4.2 Limitations

1. A limitation that is common to bed rest studies is that there are many other experiments being conducted on the same subjects simultaneously. While every effort is made to reduce any crossover effect, it may be suggested that this is a potential limitation.
2. The current bed rest study used a crossover design. For the wash out period, subjects had a 4 month period of which the researchers had no input or control over.

3. The clamp was conducted before the first bed rest period only and after all three campaigns. Therefore we have only one baseline measurement for insulin sensitivity. This was due to logistical purposes.

1.5 Conclusion

This thesis was carefully designed to investigate the impact of physical inactivity, induced by bed rest, on whole body and skeletal muscle metabolism. The study was conducted with a number of collaborators with specific expertise. Prof Stephane Blanc (CNRS Strasbourg, France) has expertise in whole body metabolism, in particular the role of stable isotopes. Prof. Martina Heer (Profil Institute, Cologne) was the lead investigator on the nutritional intervention and her team performed the euglycemic-hyperinsulinemic clamp. The design of the present bed rest study allows us to have control over energy regulation and timing of test measurements, thus providing us with a well-controlled study to investigate skeletal muscle physiology. In order to establish the rationale for this thesis a detailed review of the existing literature was undertaken.

Chapter II

Review of Literature

2.1 Introduction

There are several lines of evidence for a relationship between physical inactivity, obesity and early mortality. While the paradigm of physical inactivity is more recent, a landmark study by Jeremy Morris and colleagues (1953), reported the effects of sitting time on the health profile of London bus drivers. Men whose jobs involved sitting for prolonged periods had a 2 fold increased risk for cardiovascular disease (Morris and Crawford 1953). The first study to quantify the amount of time spent in sedentary pursuits reported that U.S. children and adults spent about 55% or 7.7 hours per day in activities requiring very low levels of energy expenditure (Matthews, Chen et al. 2008). It has also been suggested that time spent viewing television is associated with an increased risk for all-cause and cardiovascular disease (CVD) mortality. In fact, every one hour increment in TV viewing was related to an 11% and 18% increased risk of all-cause and CVD mortality, respectively (Dunstan, Barr et al. 2010). Therefore, it is important that we gain a better understanding of the early physiological changes that occur with physical inactivity which contribute to such chronic diseases states.

2.2 Models of Physical Inactivity

Several experimental models exist to mimic physical inactivity or exposure to microgravity and to assess muscular adaptation in both humans and in animal models (Appell 1990). Immobilization can be achieved by casting the limb, including all joints on which the muscle is acting. The pinning technique has been previously used to immobilize the calf muscle of the rat (Max 1972, Jaffe, Terry et al. 1978). The aim of this technique is to mimic the effects of casting with the additional benefit of preventing any impairments to blood flow through the muscle (Appell 1990). Another animal model that has been used is hind limb suspension whereby the rats are suspended so that their rear legs are non-load bearing. It appears, the effects of hind limb suspension on muscle are similar to those produced by casting or pinning (Fitts, Metzger et al. 1986). In 2003, Fadia & Haddad characterized a novel rodent model whereby the spinal cord is severed at the mid-thoracic level and the upper sacral level. As a result, all sensory information is eliminated by deafferentation while keeping the ventral motor units intact (Brooks 2005). Animal models have been significant in the study of muscle atrophy and allow for a more comprehensive collection of data than human studies (Appell 1990). However,

many models of physical inactivity have also been developed in humans. Reduced ambulatory activity from >10,000 steps to < 2,000 steps per day has been employed in healthy young men to assess the impact of reduced energy expenditure on metabolic health (Krogh-Madsen, Thyfault et al. 2010). The European Space Agency (ESA) use the ground based model of anti-orthostatic bed rest (BR) to simulate the physiological changes in microgravity (Buehlmeier, Mulder et al. 2014). This model is unique and allows us to study human physiology in a controlled environment. Physiological adaptation to physical inactivity and space flight induces metabolic adaptations. These changes are simulated during ground based bed rest studies (Bergouignan, Rudwill et al. 2011).

One important aspect of a bed rest study is to test the efficacy of nutrition and exercise countermeasures. The model of -6˚ head down tilt bed rest is now accepted as the best simulation model to study humans response to simulated microgravity and to evaluate countermeasures to the physiological effects of space flight (Pavy-Le Traon, Heer et al. 2007). While this model is currently the best available for the study of physiology in space, it is also an extreme model of physical inactivity and thus provides us with an opportunity to further investigate the deconditioning effects of disuse. While physical inactivity encountered during bed rest appears to be extreme compared to that seen in the normal ambulatory population, physical activity level (PAL) measured during bed rest studies is close to that measured in sedentary individuals, i.e., PAL of 1.4 – 1.5 (Bergouignan, Momken et al. 2010).

For centuries, physicians have prescribed bed rest to treat disease and disability (Brower and Hicks 1972). In fact, during World War II, it was the need for a quick recovery of fighting troops that caused the medical leaders to question the real value of prolonged bed rest and immobilization. It then became apparent that the effects of bed rest in treating illness may be more harmful and the consequences of bed rest may be more serious than the initial illness (Brower and Hicks 1972). These findings support the theory that weight bearing may be an important stimulus for the maintenance of musculoskeletal function (Berg and Tesch 1996). The physiological response to bed rest depends on the duration of the study and the angle of the tilt. The earliest bed rest studies were performed with subjects in a horizontal position. Then in the mid - 1970s, Kakurin et al. (1976) demonstrated that an anti-orthostatic head down tilt position of -4 to -16˚ induced body fluid shift and cardiovascular responses similar to those observed

during space flight deconditioning. In comparison to horizontal bed rest, subjects undergoing -6˚ head down tilt exhibit changes in fluid and electrolyte parameters which appeared to occur at an accelerated rate (Fortney, Schneider et al. 1996). This angle is therefore used in all bed rest studies designed to study the cardiovascular responses to microgravity. One study compared the body fluid responses of two groups of five men during 7 days of -6˚head down tilt bed rest (HDTBR) and horizontal bed rest. While both groups had diuresis and naturesis resulting in a net fluid loss, the HDTBR group had 1.5 times greater fluid loss and it occurred more rapidly versus the horizontal BR group (Noskov, Kozyrevskaia et al. 1985). It appears that the magnitude of the hydrostatic gradient may be a prime determinant of total fluid loss during bed rest (Fortney, Schneider et al. 1996). However, fluid loss is only one physiological component that is affected by bed rest or space flight.

2.3 Physiological Changes with Bed Rest

Bed rest and the resulting physical inactivity can induce a reduction in muscle and bone mass, cause strength decrements, cardiovascular and sensiormotor deconditioning, hormonal and metabolic alterations (Fortney, Schneider et al. 1996). When a human is in the upright position, physiological responses counter the action of gravity. However, with weightlessness, the body adapts in an attempt to function optimally by modifying afferent signalling from the various stressors and inducing an adaptive response (Fortney, Schneider et al. 1996). Such adaptations include a reduction in hydrostatic pressure of the cardiovascular system along with the elimination of bone compression known as hypogravia. Another significant metabolic adaptation that occurs is a reduction in energy metabolism due to confinement, known as hypodynamia (Greenleaf and Kozlowski 1982).

2.3.1 Cardiovascular Alterations

Following the withdrawal of orthostatic stress with the implementation of -6˚ head down tilt bed rest (HDTBR), a number of cardiovascular (CV) alterations occur in response to this new posture. The sudden shift of blood towards the head can initiate a neuro-humoral response leading to increased natriuresis and diuresis (Norsk 1992). The resulting drop in filling pressure to the heart, triggers remodelling of cardiac tissue and

structure (Perhonen, Franco et al. 2001, Perhonen, Zuckerman et al. 2001). Importantly, the cardiac consequences of a prolonged reduction in pre-load results in reduced exercise capacity and orthostatic tolerance after head down tilt bed rest (Fortney, Schneider et al. 1996). This has implications for skeletal muscle oxygen uptake. Maximal oxygen consumption (VO_2 max) is reduced in healthy individuals in response to bed rest and immobilization (Stremel, Convertino et al. 1976, Convertino 1997, Kortebein, Symons et al. 2008, Krogh-Madsen, Thyfault et al. 2010, Lee, Moore et al. 2010). Oxygen uptake is the product of systemic blood flow (cardiac output) and systemic oxygen extraction (arterial venous oxygen difference). Therefore, the determination of change in maximal oxygen consumption that occurs with bed rest or physical inactivity provides a reasonable measure of the cardiovascular consequences as it represents both central (heart rate (HR) & stroke volume (SV)) and peripheral factors (arterial venous difference (AVO_2 diff)) (Convertino 1997). A reduction in VO_2 max has been reported following 15 days of bed rest with a concomitant increase in maximal HR. This HR response may be due to a decrease in cardiac vagal tone and increased catecholamine secretion in conjunction with increased beta receptor sensitivity. Despite a compensatory elevation in HR during exercise, VO_2 max decreased due to an apparent decline in maximal SV. A decrease in SV may be primarily due to a lower venous return which can be associated with a lower circulating blood volume (Coyle, Hemmert et al. 1986, Convertino 1997). Using a less extreme model of physical inactivity, reduced ambulatory activity for 2-weeks was reported to cause a 7% decrease VO_2 max (Krogh-Madsen, Thyfault et al. 2010). Such a rapid decline in cardiovascular fitness was surprising. However, due to the strong association between low CV fitness and increased mortality (Sui, LaMonte et al. 2007), these findings are clinically relevant. By determining the change in VO_2 max, we get an indication of the cardiovascular consequences of training cessation, sedentary behaviour and bed rest (Convertino 1997). While the current belief is that maximal cardiac output is the principle limiting factor for VO_2 max during whole body measurements such as bicycle or running tests (Bassett and Howley 2000), oxygen carrying capacity, blood flow changes, peripheral diffusion gradients, capillary density and mitochondrial enzymes should also be considered. Extensive work has been undertaken to examine whether mitochondrial enzymes are a limiting factor for VO_2 max. The final step of the electron transport chain in the mitochondria is where O_2 is consumed. In theory, if you increase mitochondrial content, an increase in O_2 uptake should be evident. However, this does not appear to be the case in human studies that

have shown that there is only a modest increase in VO_2 max (20-40%) despite a 2.2 fold increase in mitochondrial enzymes (Saltin, Henriksson et al. 1977). This is consistent with the view that VO_2 max is limited by oxygen delivery and not mitochondrial function. Holloszy and Coyle (1984) addressed this issue in a landmark study and argued that as a consequence of increase in mitochondria with exercise training, exercise at the same work rate causes less disturbance in homeostasis in the trained muscle (Holloszy and Coyle 1984). While O_2 uptake is dependent on mitochondria, it is not known if a reduction in VO_2 max with bed rest contributes to mitochondrial dysfunction.

2.3.2 Bone Health and Bed Rest

Human models of disuse osteoporosis namely bed rest, immobilization and spinal cord injury have reported findings such as a negative calcium balance, alterations in biochemical markers of bone turnover and a resultant loss of bone mineral primarily in the lower limbs all indicative of increased bone resorption or reduced bone formation. In a review by Bloomfield et al (1997), they suggested that the key elements of bed rest contributing to changes in the musculoskeletal system are the lack of usual weight bearing forces and longitudinal compression which normally act on bones in the lower limbs in the vertical position. Another major contributing factor is the decrease in the number and magnitude of muscle contractions during periods of disuse (Bloomfield 1997). Bones of the lower limbs are normally exposed to frequent longitudinal compressive loading with weight bearing in 1 g and it is the lower extremity bones that will experience the greatest decrease in daily loading with bed rest, whereas the use of upper limb bones may not change significantly (Bloomfield 1997). Lang et al. (2004) provided data from DEXA measurements, volumetric qualitative computed tomography (vQCT) and ultrasound from crew members on flights of 130 to 197 days in duration. They reported a loss in bone at rates of 0.8 – 0.9% per month from the lumbar spine and 1.2 – 1.5% per month from the hip. vQCT allowed for the examination of loss in both trabecular and cortical fractions of bone. This data confirmed the large loss in the spine and proximal femur. Furthermore, they indicated that the rate of loss in bone mineral content of trabecular bone in the proximal femur was almost twice that of cortical bone loss (Lang, LeBlanc et al. 2004). This raised a major concern for bone health since trabecular bone cannot be replaced after loss of trabecular continuity (Langton, Haire et al. 2000). This study by Lang et al (2004) is the first study to use vQCT to map subregional bone loss at the two most critical skeletal sites; the spine and the hip.

Additionally, it was the first study to show differential patterns of cortical and trabecular bone mineral loss at the hip. While the focus of the present thesis is not on bone health, it is important to stress that bed rest affects all physiological systems.

2.3.3 Skeletal Muscle Response and Regulation

The gravity dependent load of the human body is fundamental to the maintenance of skeletal muscle function. Reduced weight bearing activity such as cast immobilization, lower limb unloading, bed rest or an extended stay in weightlessness can result induce muscle dysfunction in only a few weeks. Similar to the bed rest model, multiple functional alterations have been reported with space flight. Such examples include strength decrements, fibre type changes, whole-muscle cross sectional area and fibre cross sectional area reductions. A reduction in muscle strength is commonly reported following bed rest. A 15% decrease in knee extension torque was detected after 14 days of bed rest (Bamman, Clarke et al. 1998) and 20% after 20-35 days (Duvoisin, Convertino et al. 1989, Funato, Matsuo et al. 1997). Similarly, 35 days of bed caused ~25% strength decrements in the gastrocnemius and soleus muscles (Gogia, Schneider et al. 1988, LeBlanc, Gogia et al. 1988). Berg et al. (1997) have also reported that after 6 weeks of bed rest, there was a reduction of knee extensor torque of ~29% (Berg, Larsson et al. 1997). This amounts to 4-5% strength loss per week which can be attributed to changes in the size and altered contractile properties of the muscle.

The most common methods used to measure muscle size are MRI, CT scans and ultrasound. A 3% decrease in thigh muscle volume was reported after 7 days of bed rest (Ferrando, Stuart et al. 1995). Many other bed rest studies have reported a decrease in thigh and knee extensor muscle size, ranging from ~6-11% after 20 days of bed rest (Akima, Kuno et al. 1997, Tabata, Suzuki et al. 1999, Kawakami, Akima et al. 2001), ~ 5-11% after 30 days bed rest (Convertino, Doerr et al. 1989, Duvoisin, Convertino et al. 1989). With 42 days bed rest, 14-17% size reduction has been reported in knee extensor muscles (Berg, Larsson et al. 1997). Finally, 120 days bed rest caused a 15% decline in thing muscle volume (LeBlanc, Schneider et al. 1992). This suggests that thigh muscle volume loss occurs quite early in bed rest. With regard to the calf muscle size, the rate of loss of muscle size appears to be similar to the thigh muscle, reporting ~6-12% after 30-35 days (LeBlanc, Gogia et al. 1988, Convertino, Doerr et al. 1989, Duvoisin, Convertino et al. 1989). Most bed rest studies have reported similar rates of loss of

muscle size in both thigh muscle and calf muscle. One study by LeBlanc et al (1992) reported that calf muscle size decreased ~30% after 120 days bed rest, twice that seen in thigh muscle. Taken together, the bed rest data show that much of the loss of lower limb anti-gravity muscle size occurs in the first few weeks (2 – 3 weeks). The pattern of muscle size change with bed rest are similar to that seen with space flight (Adams, Caiozzo et al. 2003). The rate loss of muscle CSA in all models are faster in the first 30 days with an average loss of 0.6% per day (Phillips, Glover et al. 2009), after which the rate of loss plateaus in the quadriceps femoris and soleus (Adams, Caiozzo et al. 2003). Interestingly, muscle fibre CSA rate of loss is greater than whole muscle CSA (1% per day) (Bamman, Clarke et al. 1998, Trappe, Creer et al. 2007, Trappe, Creer et al. 2008).

Which muscle fibre type is more susceptible to atrophy is another factor to consider. A spinal cord isolation study suggested that MHC proteins and in particular the slower isoforms are selected for rapid depletion (Haddad, Roy et al. 2003). In another study by Trappe et al. (2004), they reported a reduction in myosin heavy chain (MHC) I fibres. In the same study, they reported that hybrid fibres (MHC I/IIa/IIx) increased 16% after bed rest. This finding was unique as this muscle fibre type is rarely observed in human skeletal muscle. The findings that type I muscle fibres are most susceptible to atrophy is supported by animal data showing that the anti-gravity muscles composed primarily of type I fibres, such as the soleus muscle, are most effected by unloading compared to muscles containing fast twitch fibres (Fitts, Riley et al. 2000). In the same study by Trappe et al (2004), single muscle fibre power was reduced 60% in MHC I and by only 25% in MHC IIa fibres suggesting that the presence of gravity may play a more important role in preserving these muscle fibres.

2.4 Metabolic Changes with Bed Rest

2.4.1 Normal Glucose metabolism

Skeletal muscle uses both glucose and free fatty acids as its primary fuel sources for energy production. Plasma glucose concentrations remain within a very tight range (55 - 165mg/dL) in normal healthy individuals, despite periods of fasting and feeding (Shrayyef and Gerich 2010). This narrow range that defines normoglycemia is maintained through an intricate regulatory and counter-regulatory neuro-hormonal system. Even a small decline in plasma glucose of 20 mg/dL, for example from 90 to 70

mg/dL will supress insulin release from the pancreas and reduce glucose uptake by the hypothalamus, where glucose sensors are located. This activates a counter regulatory response through the sympathetic nervous system and stimulates the release of glucagon, catecholamines, cortisol and growth hormone allowing a greater release of glucose into the blood and at the same time, reducing its removal (Gerich 1988). Tight regulation of plasma glucose regulation is therefore extremely important.

Insulin, produced in the β cells of the pancreas, plays a key role in the transport and uptake of glucose in muscle and fat tissue concomitantly inhibiting hepatic glucose production and therefore plays a primary role in the regulation of glucose homeostasis. The main regulator of insulin secretion is plasma glucose concentration (Mitrakou, Ryan et al. 1991). In fact, insulin as an anabolic hormone has many roles. It stimulates cell growth and differentiation and promotes the storage of substrates in fat, liver and muscle by promoting lipogenesis, glycogen and protein synthesis and inhibits lipolysis, glycogenolysis and protein breakdown. Any defect in insulin action will lead to profound dysregulation of these processes and could allow for elevated fasting and postprandial glucose and lipid levels (Saltiel and Kahn 2001). The inability of insulin to continuously regulate glucose homeostasis is evident in type 2 diabetes, obesity, cardiovascular disease and the metabolic syndrome, all of which are linked to insulin resistance. This is relevant in bed rest studies as numerous bed rest studies of varying durations showed a decrease in insulin sensitivity.

2.4.2 Physical Inactivity and Insulin Resistance

Previous studies have shown that bed rest causes reduced insulin sensitivity (Bergouignan, Rudwill et al. 2011). Early reports from the 1970s were among the first to observe decreased glucose tolerance and hyperinsulinemia following an oral glucose tolerance test after 10 – 14 days of bed rest due to an impairment in peripheral glucose uptake and not insulin deficiency (Lipman, Schnure et al. 1970, Lipman, Raskin et al. 1972, Dolkas and Greenleaf 1977). In more recent years, these findings have been confirmed in both men and women (Blanc, Normand et al. 2000, Bergouignan, Momken et al. 2010). To further elucidate the mechanisms involved in the development of insulin resistance Mikines et al. (1991) assessed insulin action on both whole body glucose uptake rate and leg glucose uptake using the euglycemic clamp technique before and after 7 days bed rest. The whole body and leg glucose uptake rates were markedly

reduced after bed rest. Tabata et al. (1999) also demonstrated a 16% reduction in glucose transporter (GLUT4) concentration in the vastus lateralis after 19 days of bed rest. The magnitude of decrement in GLUT4 appears to be modest, however, is comparable with that reported for insulin resistant obese subjects (18%) (Dohm, Elton et al. 1991).

Reduced ambulatory activity is another model that has been used to more closely replicate the behaviour of a sedentary individual. A novel study by Krogh-Madsen et al. (2010) provided evidence that acutely reducing ambulatory activity negatively impacts insulin sensitivity. Detraining in endurance athletes has had a similar impact. Heath et al. (1983) showed that 10 days of exercise cessation in 8 endurance athletes dramatically increased their insulin response to a 100 g glucose load. Similar findings have been reported using the rodent model. After one day of hind limb suspension, female rats had reduced glucose tolerance and reduced stimulated glucose uptake (O'Keefe, Perez et al. 2004). The wheel lock model has been developed which better simulates habitual physical inactivity. Using this model, Kump and Booth (2005), showed that rats display rapid losses of insulin stimulated glucose uptake and insulin signalling when their voluntary running was inhibited by wheel lock for 2 days or 53 hours. Clearly, the evidence suggests that detraining, reduced ambulatory activity and bed rest leads to a rapid decline in skeletal muscle insulin sensitivity in human and animal models. Physical inactivity remains an underused model to study the initial declines in insulin sensitivity that likely precede overt insulin resistance (Krogh-Madsen, Thyfault et al. 2010).

2.4.3 Metabolic Flexibility

Plasma free fatty acids (FFA) play an important physiological role in skeletal muscle as they constitute an important energy source (Boden and Shulman 2002). However, chronically elevated plasma FFAs, as in an obese state, appears to have pathophysiological consequences linking them to the insulin resistance. The dynamics of lipid oxidation, FA availability & uptake and intramyocellular triglyceride turnover, may be important to avoid the accumulation of toxic lipid intermediates. This is especially relevant in situations where energy demand is not sufficient to challenge the fat oxidative capacity of skeletal muscle. The obese, type 2 diabetic and insulin resistant phenotype is associated with impaired fat oxidation when fasting and an impaired

switch from fat to glucose oxidation after a meal (Kelley, Goodpaster et al. 1999, Corpeleijn, Mensink et al. 2008).

In 2002, Kelley and Mandarino coined the term metabolic flexibility, referring to the capacity to switch from predominantly fat oxidation to increase glucose uptake, oxidation and storage under insulin stimulated conditions. This interplay between fatty acids and glucose oxidation *in vivo* has been of interest for many years. Over 50 years ago, Randle and colleagues proposed the glucose fatty acid cycle whereby an increase in FFA availability would lead to an increase in FFA oxidation while inhibiting phosphofructokinase and pyruvate dehydrogenase. This would lead to an accumulation of glucose-6-phosphate which inhibits hexokinase activity resulting in an increase in intracellular glucose concentration resulting in a negative feedback for glucose uptake (Randle, Garland et al. 1963). Their seminal work stimulated great interest in a hypothesis that substrate competition is a key mechanism in the development of insulin resistance (Kelley and Goodpaster 2001). These findings have been substantiated, however, with modifications. It has become clear that FA can impair glucose metabolism during insulin stimulated conditions (Kelley, Mokan et al. 1993, Boden, Chen et al. 1994, Boden and Chen 1995, Roden, Price et al. 1996). Another interesting concept that has emerged suggesting that glucose can inhibit the oxidation of lipids. It has also been demonstrated in both human and animal models of type 2 diabetes and obesity that skeletal muscle can have a reduced reliance on FA during fasting conditions (Kelley, Mokan et al. 1993, Kelley and Simoneau 1994). This impairment of post absorptive FA oxidation in skeletal muscle may be due to glucose inhibition of FA utilization, known as the reverse Randle cycle (Kelley and Mandarino 1990, Sidossis and Wolfe 1996, Turcotte, Swenberger et al. 2002). Reduced reliance on FA as fuel in the post absorptive phase over time may result in increased storage of toxic lipid intermediates.

2.4.4 Conclusion

Head down tilt bed rest is a useful and reliable simulation model for most physiological effects of spaceflight. Bed rest is characterized by immobilization, inactivity and confinement which induce unloading of the body's upright weight, upward fluid shift, reduced energy expenditure and reduced cardiac performance. Additionally, body weight, muscle mass and strength is reduced as is the resistance of muscle to insulin. Therefore, it is a useful model to study the effects of physical inactivity. An important

aspect of many bed rest studies in humans is to assess the effectiveness of countermeasures such as exercise (resistance, aerobic, vibration) and nutrition supplementation.

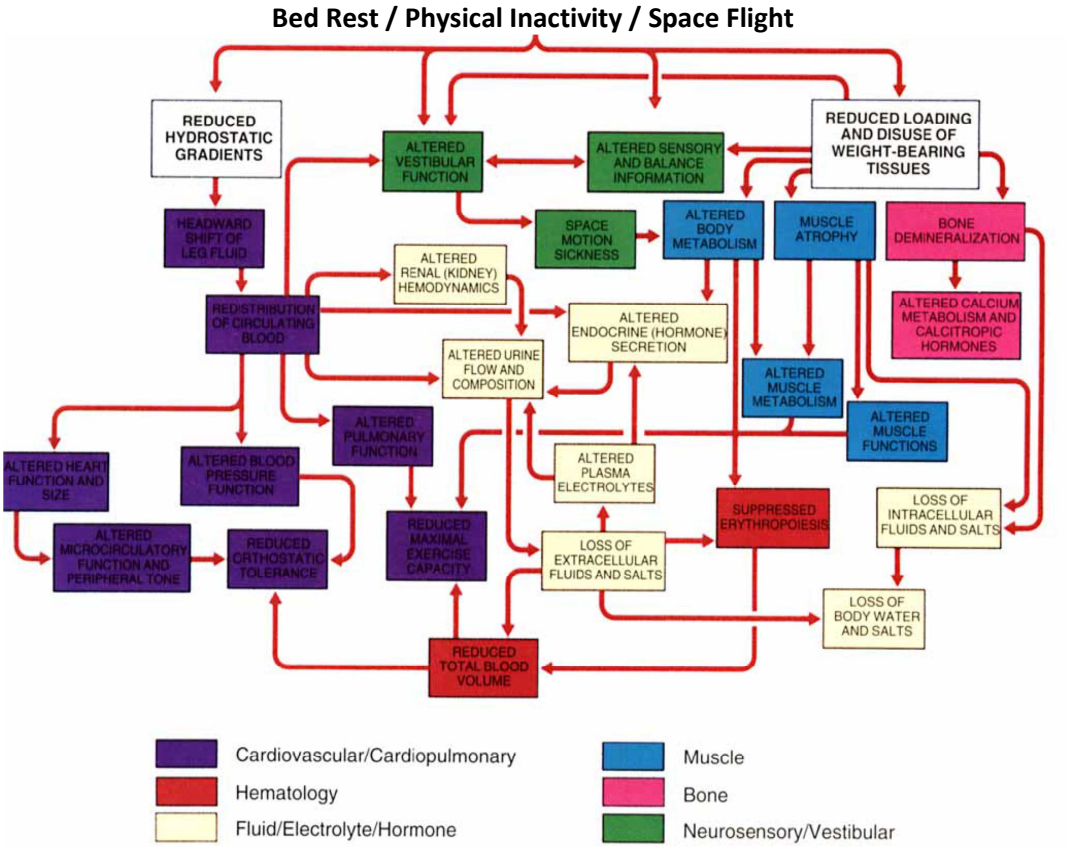


Figure 2.1 Interrelationships of physiological response to bed rest, physical inactivity and space flight. Adapted from Vernikos et al. 1996.

2.5 Countermeasures and Bed Rest

A countermeasure refers to the application of procedures or therapeutic (physical, chemical, biological or psychological) means to maintain physiological balance, maintain health and reduce risk and ensure the safety of the human during and after spaceflight (Clément 2011). The optimal countermeasure for the prevention of space flight or bed rest induced deconditioning would use a treatment modality that could maintain the body systems in a similar condition if the subjects were at normal gravity or upright. Together with on-going efforts to decipher the cellular and molecular basis of disuse

atrophy, it is crucial to concomitantly identify appropriate countermeasures. Many varied approaches have been investigated that can be mainly grouped into exercise and nutrition (Chopard, Hillock et al. 2009). The effects of a variety of potential countermeasures have been tested and in many cases have not sufficiently prevented or delayed the physiological changes (Rittweger, Beller et al. 2000).

2.5.1 Aerobic Exercise

Reduced aerobic capacity is well documented in bed rest. The issue of a central or peripheral limitation has been a widely debated (Noakes 2005, Saltin and Calbet 2006). Many have suggested that main factor limiting VO_2 max in whole body exercise is maximal cardiac output. The reduction in maximal cardiac output and maximal aerobic capacity appear to parallel in bed rest studies of less than 30 days (Hung, Goldwater et al. 1983, Capelli, Antonutto et al. 2006). However, maximal cardiac output declines rapidly in the first few weeks of bed rest and changes minimally thereafter (Capelli, Antonutto et al. 2006) suggesting that peripheral factors at the level of the muscle may be responsible for any further decrements in VO_2 max. The need for a countermeasure to address the reduction in aerobic capacity is warranted. However, the optimal aerobic exercise countermeasure has yet to be defined. Moderate intensities and durations of aerobic exercise do not prevent loss of aerobic capacity (Stremel, Convertino et al. 1976, Suzuki, Kashihara et al. 1994). With 2 weeks bed rest and an exercise intervention involving 2x30 minute bouts of supine cycle ergometry daily at 68% of pre bed rest maximal aerobic capacity, subjects still had plasma volume reduction (-8%) and reduced aerobic capacity (-9%) (Stremel, Convertino et al. 1976). In contrast, aerobic capacity was maintained with longer duration and higher intensity exercise. Greenleaf et al. (1989) demonstrated that higher intensity interval exercise (90% of VO_2 max) prevented loss of aerobic capacity after 30 days of bed rest. These findings were successfully repeated in more recent bed rest studies (Lee, Bennett et al. 1997, Watenpaugh, Ballard et al. 2000, Katayama, Sato et al. 2004). Protection against loss of aerobic capacity is only one countermeasure that is beneficial. Other exercise interventions have been designed in order to prevent muscle mass loss.

2.5.2 Resistance Exercise Training

While aerobic exercise may be effective in counteracting cardiovascular deconditioning, high intensity muscle loading (resistive or explosive) appears to particularly promising in the prevention of muscle atrophy (Akima, Kubo et al. 2000, Akima, Ushiyama et al. 2003). Resistance exercise is known to stimulate the rate of muscle protein synthesis (Kumar, Selby et al. 2009). Studies to date that have assessed resistive exercise training as a countermeasure have demonstrated only partial success. In comparing two modes of exercise, during 30 days of bed rest Ellis et al (1993) showed that intensive, alternating, isotonic (concentric and eccentric contractions) cycle ergometer exercise training in seven subjects was as effective as intensive, intermittent, isokinetic exercise training (torque ergometer) in another seven subjects in terms of maintaining muscle mass of the thigh muscles versus non training control subjects. Germain et al. (1995) investigated the effects of a combination of both isometric and isokinetic leg exercise (30-45 minutes/day) on quadriceps muscle strength during 28 days HDTBR. In this study, physical training was combined with lower body negative pressure. A significant reduction in muscle strength was reported in the control subjects and no change was noted in the exercise intervention group.

Resistance training has proven to be somewhat beneficial in preventing atrophy. Dynamic leg press and plantar flexion training used by Akima et al (2001, 2003) during 20 days HDTBR maintained muscle size and function. Shinohara et al. (2003) examined torque fluctuations of the extensor muscles of the ankle and knee employing dynamic calf raises and leg press exercises with maximal contractions and submaximal isometric contractions. The novel finding from this study was that torque fluctuations increased following bed rest only and were minimized with resistance training counteracting any decline in performance. Constant resistance exercise (5 sets of 6-10 repetitions to failure of constant resistance concentric/eccentric plantar flexion every second day) performed throughout 14 days HDTBR prevented plantar flexor performance deconditioning as seen in bed rest only control subjects (Bamman, Hunter et al. 1997). Over a 17 week period of horizontal bed rest, intensive resistive exercise only partially prevented the decline in volume of the soleus and gastrocnemius (Shackelford, LeBlanc et al. 2004). The variation in bed rest duration makes it difficult to directly compare.

Skeletal muscle inherently possesses greater mechanical efficiency and ability to generate force while lengthening (eccentric) than shortening (concentric). This is evidenced by studies that report lower electromyographic (EMG) amplitude while lowering (eccentric) than lifting (concentric) a given weight. This indicates less motor unit involvement while a greater load is placed on each active muscle fibre in eccentric contraction (Norrbrand, Pozzo et al. 2010). Some bed rest studies have employed the use of a gravity-independent flywheel ergometer which calls for greater eccentric muscle activation. Alkner & Tesch (2004) studied the effects of plantar flexor and knee extensor resistance exercise (supine squat and calf press every third day employing flywheel) on muscle size and function. While both knee extensors and plantar flexors displayed muscle atrophy with bed rest, more marked atrophy was noted in the plantar flexors compared to knee extensors. The use of gravity independent resistance exercise prevented knee extensor loss and attenuated the more pronounced plantar flexor muscle loss. In this same study, Trappe et al (2004) examined contractile properties of the muscle fibres in response to the same resistance exercise protocol. The single muscle fibre findings were intriguing as they appeared to contrast with whole muscle findings. The resistance group had a reduction in cell functional parameters despite apparent maintenance of whole muscle size and function. Myosin heavy chain I fibres maintained size but showed a decline in measures of strength, speed and power. An unusual finding in this analysis was that there was a large increase in hybrid muscle fibres in both groups (13 to 49% in bed rest only group and 14 to 31% in resistance exercise group) showing a directional shift from slower to faster contracting fibres in both groups. This shift is in agreement with previous unloading investigations using animals (Caiozzo, Baker et al. 1994, Caiozzo, Haddad et al. 1996) and humans (Zhou, Klitgaard et al. 1995, Ohira, Yoshinaga et al. 1999). In bed rest, while resistance training has been shown to maintain whole muscle size and function, the negative consequences of bed rest are still evident at the level of a single muscle fibre.

2.5.3 Vibration Exercise

Vibration exercise is a neuromuscular training method that generates sinusoidal vibration at a frequency up to 40 Hz. The mechanical stimuli elicit a sensory receptor response, most likely to be muscle spindles. This leads to the activation of the alpha motor neurons and initiation of muscle contractions (Delecluse, Roelants et al. 2003).

Vibration applied to a muscle induced a non-voluntary muscular contraction which is termed the 'tonic vibration reflex' (Eklund and Hagbarth 1966). The intensity of the vibration is determined by the amplitude (peak to peak displacement, in mm) and the frequency (measured in Hz) of the oscillations. Typically, the frequencies used for exercise range from 15 to 44 Hz and the amplitude range from 3 to 10mm. Such vibrations provide perturbations of the gravitational field during the vibration exercise. In fact, the acceleration values range from 3.5 to 15g, where g is the earth's gravitational field or $9.81.ms^{-1}$ (Cardinale and Bosco 2003). Vibration exercise was initially applied by Russian scientists who suggested that, as a mode of exercise, it would enhance strength in well trained subjects (Nazarov and Spivak 1987). Its recent popularity is based on the combined effect on neuromuscular and neuroendocrine systems (Cardinale and Bosco 2003).

Frequency-dependent vibration forces have been successfully used to increase muscle force and power. Bosco et al. (1999, 2000) reported an increase in force-velocity, force-power and vertical jump performance after one whole body vibration session. The same authors examined the benefits of a 10 day training programme using vibration exercise (5 x 90 seconds) of vertical sinusoidal vibrations at a frequency of 26 Hz. They found a significant improvement in height and mechanical power during the 5-s-continuous-jumping-test (Bosco 1998). Whole body vibration using a vibration plate or platform has also been shown to enhance vertical jump ability by up to 3.8% and mechanical power output during a horizontal leg press increased 7% and increase maximal power output (Issurin and Tenenbaum 1999).

A potential field of application for vibration exercise is space flight, where microgravity induced immobilization induces skeletal muscle deconditioning (Pavy-Le Traon, Heer et al. 2007). The efficacy of vibration exercise has been tested in bed rest studies but the effects on muscle atrophy were disappointing. During a 14 day HDTBR study, whole body vibration exercise was conducted twice daily in 5x1 minute bouts in an upright position, standing on a vibration platform at a frequency of 20 Hz and amplitude of 2-3 mm. This intervention failed to prevent the muscle atrophy induced by bed rest (Zange, Mester et al. 2009).

In contrast, the combination of resistive and vibration exercise, tested in the Berlin Bed Rest (BBR), had positive effects on muscle and bone loss during 56 days of BR

(Rittweger, Belavy et al. 2006). Throughout this period, a very demanding vibration exercise regime was employed (frequency= 19-25 Hz and amplitude was variable – side altering). Exercise was performed 11 times per week. Amplitude and frequency were altered to account for exercise progression. The findings show that atrophy of the plantar flexor and knee extensors was attenuated by the vibration exercise protocol (Blottner, Salanova et al. 2006, Mulder, Stegeman et al. 2006). Furthermore, myofibre size in both the vastus lateralis and the soleus muscles were maintained compared to the bed rest only group (Blottner, Salanova et al. 2006).

It has been suggested that enhanced central nervous motor neuron excitability is responsible for the exercise adaptation. This is supported by the findings of Mulder et al. (2009) who reported increased electromyography amplitude in the exercise group of the BBR study. Data available from the BBR suggest that the combination of resistive and vibration exercise was highly effective in preserving muscle volume and function. An ideal countermeasure to inactivity adaptations would allow simultaneous preservation of muscle mass and function, cardiovascular health and bone mass. It would also prevent the metabolic changes that occur in response to changes in body composition and endocrine regulation. The broad physiological approach presented highlights the necessity for multidisciplinary countermeasure research (Buehlmeier, Mulder et al. 2014).

2.5.4 Energy Regulation and Bed Rest

The regulation of energy intake is an important consideration in bed rest studies and many are limited due to lack of dietary control. Adequate nutritional supply is crucial to accurately decipher the true effects of bed rest. If the subjects are in a state of positive energy balance due to overfeeding, this could introduce a confounding factor that may exaggerate the deleterious effects of physical inactivity (Biolo, Agostini et al. 2008). Energy intake should therefore be adapted to avoid the positive balance and the resulting preferential deposition of fat (Olsen, Krogh-Madsen et al. 2008). In fact many of the previous bed rest studies have shown that when energy intake was not controlled, this resulted in an increase in fat mass in parallel to lean muscle mass catabolism (Barbe, Galitzky et al. 1999, Shackelford, LeBlanc et al. 2004, Agostini, Heer et al. 2008). In more recent studies in both men and women, diet was tightly controlled in order to clamp fat mass (Bergouignan, Trudel et al. 2008, Bergouignan, Momken et al.

2009). Clamping fat mass as opposed to weight (Alibegovic, Hojbjerre et al. 2009, Alibegovic, Hojbjerre et al. 2010) is more ideal as a weight clamping approach in bed rest leads to muscle atrophy and an increase in fat mass (Zahariev, Bergouignan et al. 2005). In bed rest studies that controlled energy intake to more closely match energy expenditure, changes in body composition were due to reduced muscle mass with fat mass globally unchanged.

2.5.5 Protein Supplementation

A reduction in muscle protein synthesis is reported in bed rest. Ferrando et al. (1996) observed a 50% drop in protein synthesis with no change in protein breakdown in the vastus lateralis of subjects following 14 days of bed rest. With long duration spaceflight (>3 months) astronauts and cosmonauts showed a 45% drop in whole body protein synthesis and a reduction in protein breakdown, however not to the same extent (Stein, Leskiw et al. 1999). The importance in optimal nutrition in weightlessness has been demonstrated by Paddon-Jones et al. (2006) who reported that essential amino acids stimulated muscle protein synthesis and prevented loss of lower body mass. Previous reports have demonstrated that protein ingestion is effective in stimulating muscle protein synthesis after exercise (Gautsch, Anthony et al. 1998).

Whey protein, in particular, is known for its therapeutic effects. Whey is a by-product of cheese manufacturing and was once considered a waste product. However, the discovery of whey as a functional food with nutritional applications made it a co-product of cheese production (Walzem, Dillard et al. 2002). In fact, whey protein has all of the essential amino acids and in a higher concentration compared to other vegetable sources such as soy, corn and wheat gluten (Walzem, Dillard et al. 2002) and are more efficiently absorbed and utilized (Daenzer, Petzke et al. 2001). Whey protein also contains a higher concentration of branched chain amino acids (BCAA) like leucine, isoleucine and valine. Several studies indicate that leucine, especially, functions as a nutritional signalling molecule that has a role in protein synthesis in skeletal muscle following food intake.

Countermeasures such as exercise, as previously mentioned, provide a potent anabolic stimulus (Phillips, Tipton et al. 1997, Phillips, Parise et al. 2002) which are also effective during bed rest (Ferrando, Tipton et al. 1997, Bamman, Clarke et al. 1998). However, it

may not always be feasible to exercise due to physical or environmental constraints. It has been suggested that less invasive strategies, such as dietary manipulation, be tested during bed rest to try and ameliorate the deconditioning effects of physical inactivity. A study using unilateral hind limb immobilization has suggested that oral administration of an amino acid (leucine 2.7 g kg^{-1} day^{-1}) supplementation could attenuate the loss of soleus muscle mass, fibre cross-sectional area and strength after 7 days in rodents (Baptista, Leal et al. 2010). While the animal model is invaluable to our understanding of protein metabolism, it is important to recognize its limitations with regard to its application to humans.

Rodents have a much higher rate of protein turnover and a disproportionately large amount of a rodent's life is spent in the growing phase and the majority of studies are conducted during this phase. In terms of protein supplementation, this may be relevant as muscle protein synthesis in both humans and rodents is insulin sensitive during growth but lost during adulthood in humans (Lobley 1993). To prove the benefit of protein supplementation in humans, previous studies have demonstrated that ingesting or infusing essential amino acids provides an anabolic stimulus (Volpi, Ferrando et al. 1998, Paddon-Jones, Sheffield-Moore et al. 2003). An increase in dietary protein from 0.6g protein/kg/day to over 1g protein/kg/day has been shown to maintain nitrogen balance in ambulatory humans (Stuart, Shangraw et al. 1990). However, it does not seem sufficient to prevent of muscle loss with bed rest (Ferrando, Lane et al. 1996). This may be due to the difficultly comparing studies when the total energy intake varies.

For example, there were promising improvements in muscle mass and strength after 28 days bed rest in healthy male subjects using a combination of 16.5 g of essential amino acids and 30 g sucrose taken three times per day in between meals (total leucine dose of 9.3 g per day) (Paddon-Jones, Sheffield-Moore et al. 2004, Fitts, Romatowski et al. 2007). While the intervention group received 3000 kcal/day and managed to maintain their body weight, the control group were given 2500 kcal/day and lost 2.4 kg over 28 days (Paddon-Jones, Sheffield-Moore et al. 2004, Fitts, Romatowski et al. 2007). The implication of this limitation was investigated by Biolo et al. (2007) who showed that calorie restriction enhanced the catabolic response to inactivity (Biolo, Ciocchi et al. 2007). Therefore, matching energy intake between groups is necessary.

In a 60 day bed rest study in female subjects, Trappe et al. (2007) tested the efficacy of aerobic and resistance exercise programme in preserving the size and contractile function of slow and fast twitch fibres. Another objective was to test the hypothesis that a leucine-enriched-high-protein diet would partially attenuate the loss of function of single muscle fibres as a result of bed rest. The exercise intervention prevented the 15-20% reduction in fibre diameter and the 20-40% reduction in force and power in both type I and IIa fibres. In contrast, the diet protocol (1.6 g kg^{-1} day^{-1} including 3.6 g day^{-1} of free leucine, 1.8 g day^{-1} of free valine and 1.8 g day^{-1} of free isoleucine spread over three meals) failed to provide a protective role. Ham et al. (2014) has suggested that given the synergistic relationship between protein intake and exercise, is was disappointing that a 'leucine enriched diet plus exercise' group were not included in this study. However, Brooks et al. (2008) studied the combined effects of exercise and amino acid supplementation (15 g daily) with an energy deficit of 8%. They observed that with a negative energy balance, protein supplementation in combination with exercise had no additional effect when compared to exercise alone (Brooks, Cloutier et al. 2008).

To date, evidence from iso-caloric trials in humans fail to support the notion that increasing essential amino acid intake can counteract muscle wasting (Ham, Caldow et al. 2014). Based on the positive metabolic effects of protein supplementation, it appears to be an attractive countermeasure strategy to mitigate the reduction in protein synthesis in bed rest. Furthermore, it is easy to administer, inexpensive and can easily be used in conjunction with other countermeasures such as exercise.

2.5.6 Amino acids as a Modulator of Protein Synthesis

A landmark study by Buse and Reid (1975) suggested a unique role for the essential BCAA leucine in the modulation of skeletal muscle metabolism. Many animal studies have reported an increase in protein synthesis with protein supplementation. For example, Garlick & Grant (1988) reported an increased rate of muscle protein synthesis *in vivo* in tissues (muscle, heart and plasma) of post-absorptive rats following an intravenous infusion of mixed amino acids or BCAA (9 mg leucine, 7.5 mg isoleucine and 7.3 mg valine). Of the three BCAA, it has been suggested that leucine is the most potent in terms of protein synthesis stimulation when orally administered in food deprived rats, when administered at a concentration of 1.35 mg/kg body weight (Anthony, Yoshizawa et al. 2000). These findings are supported by numerous other rodent studies and suggest

that leucine administered alone (Crozier, Kimball et al. 2005), in a mixed meal (Dardevet, Sornet et al. 2002) or in leucine rich protein (Rieu, Balage et al. 2007), stimulates muscle protein synthesis.

Studies in humans have also shown promise with regard to the beneficial effects of protein supplementation. Reports suggest that protein supplementation increases protein synthesis in both elderly men (Rieu, Balage et al. 2006) and younger men when they ingested a mixed meal supplemented with leucine (5 g) (Churchward-Venne, Breen et al. 2014). The reasons for this increase in protein synthesis could be multifactorial including increased substrate availability and increased insulin secretion (Ham, Caldow et al. 2014).

2.5.7 Conclusion

There have been many bed rest studies investigating the effects of exercise and nutrition regimes on the physiological changes. The study designs and outcomes are highly variable making it difficult to compare studies. While they have contributed to our knowledge and understanding of preventing the physiological changes associated with bed rest there is relatively little data investigating the effect of countermeasures on intracellular regulation of metabolic health. Further refinement of countermeasure protocols is necessary in order to mitigate the negative consequences of bed rest. In order to develop appropriate countermeasures, a better understanding of the mechanisms involved in skeletal muscle adaptations to bed rest may be necessary.

2.6 Intracellular Regulation of Energy Metabolism: the Role of Mitochondria

Skeletal muscle is strongly dependent on oxidative phosphorylation for the generation of ATP. Reduced glucose transport and glycogen synthesis (Shulman, Rothman et al. 1990) is evident in insulin resistance in addition to abnormal fatty acid metabolism which results in triglyceride accumulation as well as reduction in lipid oxidation in fasting and insulin stimulated states (Blaak, Wagenmakers et al. 2000, Goodpaster, Theriault et al. 2000). It is likely that there are multiple factors that contribute to perturbations in both fatty acid and glucose oxidation. As the electron transport chain is responsible for

the generation of ATP, it is plausible to consider the role of the mitochondria in abnormal energy metabolism.

Mitochondria are abundantly present in all mammalian cells with the exception of mature erythrocytes. The essential role of mitochondria in cellular functions was documented over a century ago by Altmann who initially named the cell organelle "bioplasts" (Altmann 1890). These organelles were soon renamed due to their threadlike appearance to mitochondria (mito=thread and chondros=granule) (Galluzzi, Morselli et al. 2010). In skeletal muscle, mitochondria exist as a reticular membrane network in distinct subcellular regions. Intermyofibrillar mitochondria are located in close proximity to the myofibrils and subsarcolemmal mitochondria are located just beneath the sarcolemmal membrane and account for 10 – 15% of the mitochondrial volume. Intermyofibrillar mitochondria are not as adaptable as subsarcolemmal mitochondria but have higher rates of protein synthesis, enzyme activities and respiration (Takahashi and Hood 1996). Furthermore, in obese insulin resistant subjects, intermyofibrillar mitochondrial content is decreased while subsarcolemmal content was unaffected relative to insulin sensitive lean controls (Chomentowski, Coen et al. 2010). These findings suggest that subsarcolemmal and intermyofibriallr mitochondria may have different roles.

The mitochondria are a double membrane cell organelle with most of the metabolic processes taking place in the matrix. The inner membrane is highly folded, forming cristae and embedded in the inner membrane are five complexes that make up the respiratory chain where oxidative phosphorylation takes place (Nourshahi, Damirchi et al. 2012). The most important biochemical cascade confined to the mitochondria is oxidative phosphorylation, a coordinated series of redox reactions catalysed by five multi-subunit complexes embedded in the inner mitochondrial membrane and two soluble factors that function as electron shuttles in the mitochondrial intermembrane space (Galluzzi, Morselli et al. 2010). The generation of the proton gradient across the inner membrane is coupled to ATP synthesis (Lowell and Spiegelman 2000). While oxidative phosphorylation is the primary role of the mitochondria, it is well known that they are also responsible for the production of reactive oxygen species (ROS). Additionally, mitochondria are a unique organelle as they contain their own DNA which encode for 13 of the respiratory proteins (Falkenberg, Larsson et al. 2007). Mitochondria are a complex organelle involved in many cellular processes.

2.6.1 Mitochondrial Biogenesis

A well-defined network of transcription factors and co-regulator proteins are responsible for regulating skeletal muscle phenotype in response to physiological stimuli such as exercise. It is well known that endurance training increases mitochondrial content, ranging from 50 – 100% over a 6 week period, depending on the duration, frequency and intensity. This results in improved endurance performance independent of the much smaller increase in VO_2 max (Fitts, Booth et al. 1975, Davies, Packer et al. 1981). The marked improvement in endurance performance is as a result of mitochondrial biogenesis. A fascinating aspect of mitochondrial biogenesis is that it requires the cooperation and coordination of both nuclear and mitochondrial DNA. In response to exercise training, up regulation of peroxisome proliferator receptor gamma co-activator -1 alpha (PGC1-α) is proposed to be the "master regulator" of mitochondrial transcription factors involved in mitochondrial biogenesis.

PGC-1α acts as a transcriptional co-activator of multiple transcription factors that regulate skeletal muscle gene expression, including nuclear respiratory factor (NRF-1 and NRF-2), mitochondrial transcription factor (Tfam) and estrogen related receptor alpha (ERRα) (Lin, Handschin et al. 2005). Altering the activity of PGC1-α in muscle cells induces molecular adaptations that allow the cell to meet energy demands of a changing environment (Wu, Puigserver et al. 1999, Wende, Schaeffer et al. 2007). Over expression of PGC1-α in rodents drives muscle towards a more oxidative phenotype, characterized by increased mitochondrial density, respiratory capacity, ATP synthesis and antioxidant effects in type 2 fibres and overall improved exercise performance (Wu, Puigserver et al. 1999, Wende, Schaeffer et al. 2007). Conversely, in muscle specific PGC-1α knock out mice, many of these adaptations are reversed (Handschin, Chin et al. 2007, Wende, Schaeffer et al. 2007). Therefore, the expression of PGC1-α is a key regulator in modulating an adaptive response to muscle contractile activity.

While mitochondrial biogenesis is thought to be the result of cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins with exercise training (Perry, Lally et al. 2010), it has also been demonstrated that a single bout of exercise is associated with an increase in PGC1-α expression (Pilegaard, Saltin et al. 2003, Egan, Carson et al. 2010) demonstrating the sensitivity of skeletal muscle transcriptional capacity. Denervation and resulting skeletal muscle disuse causes

dramatic reduction of PGC1-α suggesting it is an important regulator that may initiate the atrophy process (Sacheck, Hyatt et al. 2007). Together, the literature strongly suggests that PGC1-α regulates mitochondrial biogenesis in skeletal muscle characterized by increased oxidative capacity and overall muscle performance.

2.6.2 Oxidative Phosphorylation

Oxidative phosphorylation refers to the process of storing energy in the form of ATP and is generated in the mitochondria. The mitochondrial electron transport chain (ETC), is composed of a series of electron carriers (flavoproteins, iron-sulphur protein, ubiquinone and cytochromes). Electrons derived from metabolic reducing equivalents (NADH and $FADH_2$) are fed into the ETC through complex I or II and eventually pass to molecular O_2 at complex IV to form H_2O (Liu, Fiskum et al. 2002). According to Mitchell's chemiosmotic theory, the oxidation of substrates is coupled to ATP production (Mitchell 1961). As electrons are transferred through the respiratory chain, protons are concomitantly ejected from the mitochondrial matrix through complexes I, III and IV to the intermembrane space, a process which establishes a proton gradient (ΔP). ΔP is dissipated when protons move back in, mainly through the large F_0/F_1 ATP synthase.

The rotary action of ATP synthase catalyzes the generation of ATP from ADP and Pi as the protons move back into the matrix (Hirono-Hara, Noji et al. 2001). For each NADH from the tricarboxylic acid (TCA) cycle, that enters the ETC, 3 pairs of protons are pumped out of the mitochondrial matrix. This process of proton pumping and ATP generation for the most part is coupled. However, proton leak back through the membrane into the mitochondrial matrix is responsible for uncoupling substrate oxidation and ATP synthesis (Brand, Affourtit et al. 2004). The net energy yield from one NADH is 2.5mol ATP and 1.5mol ATP per mole $FADH_2$. Each complex is oxidized when it passes an electron and is reduced when it receives an electron. They continue to move along the chain because each component further along the chain has a greater affinity for the electron in comparison to the previous protein. The capacity of each respiratory complex to optimally perform its function is crucial to the oxidative capacity of the mitochondria.

2.6.3 Components of the Electron Transport Chain

Electrons enter the ETC at complex I and II and are then passed to coenzyme Q (ubiquione), a lipid soluble mobile electron carrier, to Complex III (cytochrome B-C_1 complex). Complex I, also called NADH/COQ Oxidoreductase is the largest complex made up of 42 subunits (Saraste 1999). It has a binding site for both NADH and ubiquinone and accepts the electrons from NADH as NADH becomes oxidized to NAD^+. Complex I has an FMN subunit that accepts 2 electrons and then passes one electron at a time to an iron sulphur cluster (Fe-S centre) that transfers the electrons to ubiquinone. The mechanism of pumping protons at this stage is not very well understood. However, electron transfer and proton pumping occur simultaneously. For each pair of electrons transferred from NADH to oxygen, 10 protons are pumped across the inner membrane. Complex II, also called succinate dehydrogenase in the Krebs cycle, receives electrons from $FADH_2$ as FADH becomes oxidized and transfers them to the ubiquinone pool through an iron sulphur cluster. Complex II does not translocate protons and only feeds electrons to the ETC (Saraste 1999). Complex I and complex II sequentially pass their electrons to ubiquinone (CoQ) to form ubisemiquinone ($CoQH^-$) and then ubiquinol. Ubiquinol transfers its electrons to complex III (ubiquinol: cytochrome c oxidoreductase/ cyt bc1 complex) which transfers them to cytochrome c, another mobile carrier (Wallace 2005).

Electron transfer through the Cyt bc1 complex occurs through the so called “Q cycle” to another mobile carrier. Cytochrome C is a small mobile cytochrome that has reversible binding sites on complex III and IV. It carries electrons, one at a time from Complex III to Complex IV, also known as cytochrome C oxidase. Its function is to pass electrons from cytochrome c to oxygen and in doing so, pumps protons contributing to the transmembrane proton gradient. Its substrate, cytochrome c, is a water soluble protein that donates electrons to the cytoplasmic side of the inner mitochondrial membrane and are transferred to the active site that contains a heme iron and a copper. They are used to reduce O_2 to two molecules of H_2O (Saraste 1999). One O_2 requires four electrons to be reduced to two water molecules. These four electrons interact with a molecule of oxygen and eight hydrogens. Four hydrogens are used to form two molecules of H_2O and the other four are pumped to the intermembrane space (Saraste 1999).

ATP Synthase is the transmembrane enzyme that generates ATP. It is a multi-subunit enzyme that spans the width of the membrane. It has two main portions: Fo and Fi. Fo is the inner membrane part of 12 carbon portion which can rotate in the membrane. It is attached to the asymmetric shaft. Fi is the stalk and head piece which projects into the matrix. It is composed of 3 pairs of alpha/beta subunits. The beta subunits have a catalytic site for ATP synthesis. When a proton enters the proton channel, it makes the rotor turn. The channel has an opening on the intermembrane space and to the matrix. Each carbon subunit of the Fo has a carboxyl group to accept a proton. When the carbon subunit accepts a proton, it rotates the shaft inwards in the hydrophilic lipid membrane. By rotating, a proton containing subunit is exposed to the portion of the channel that opens into the matrix. Because the matrix has a much lower concentration of protons, the carboxylic acid group releases the proton into the matrix portion of the channel. The new position of the shaft alters the conformation of the alpha/beta subunits of Fi so that beta subunit will release newly synthesized ATP from ADP and Pi. Once 3 protons have entered the shaft, there is enough energy to synthesize an ATP molecule. A gradient is always maintained by the transport of electrons (Saraste 1999).

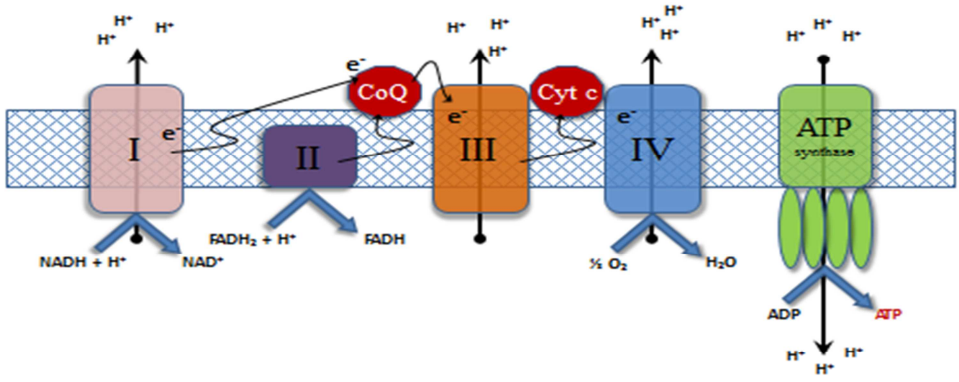


Figure 2.2 Mitochondrial Electron Transport Chain. The generation of ATP through oxidative phosphorylation.

2.6.4 Mitochondrial Function and Dysfunction

The optimal function of each of these complexes is necessary for the generation of ATP in the cell. As ATP is essential in every cellular process, optimum mitochondrial function plays a key role in metabolic health and the ultimate fate of the cell. The overall

regulation of the mitochondria is complex and incompletely understood. It involves many processes such as adaptation to a changing environment, internal metabolic conditions for example fusion/fission, mitophagy and mitochondrial biogenesis (Montgomery and Turner 2014). The diverse role of mitochondria in cellular processes and the multitude of methods used to examine 'mitochondrial dysfunction' have led to diverging definitions of mitochondrial dysfunction. It has been reported as changes in mRNA levels of mitochondrial markers, alterations of protein level, enzymatic activity, changes in mitochondrial size and shape and even the capacity of the mitochondria for substrate oxidation (Montgomery and Turner 2014). This shows the variation of measurements that can be used to describe one outcome.

The analysis of mitochondrial function has become central to basic research of mitochondria in health and disease. Proper assessment of oxidative *phosphorylation in situ* is necessary for the investigation of cellular bioenergetics (Kuznetsov, Veksler et al. 2008). Protocols for high resolution respirometryof permeabilized fibres, isolated mitochondria and intact cells offer sensitive diagnostic tests of integrated mitochondrial function. Multiple substrate-uncoupler-inhibitor titration (SUIT) protocols for the analysis of oxidative phosphorylation can improve our understanding of mitochondrial respiratory control and the pathophysiology of mitochondrial disease. Respirometry reflects the function of the mitochondria providing a dynamic measurement of metabolic flux. Mitochondrial respiration yields an integrative measure of the dynamics of complex coupled metabolic pathways. The measurement of respiratory flux in different metabolic states is necessary to evaluate the effect of changes in metabolite levels, membrane permeability and the activity of individual enzymes on oxidative phosphorylation. The high resolution respirometer has been developed for this purpose (Pesta and Gnaiger 2012).

Many studies measuring the activity of mitochondrial marker enzymes in skeletal muscle have suggested that there is a 20–40% reduction of these enzymes in type 2 diabetics versus normal healthy, aged matched control subjects. The enzymes that were measured included citrate synthase, cytochrome oxidase, $NADH_2$ oxidoreductase, carnitine palmitoyl transferase and succinate dehydrogenase (Kelley, Goodpaster et al. 1999, Simoneau, Veerkamp et al. 1999, He, Watkins et al. 2001, Kelley, He et al. 2002). An additional feature of mitochondria in type 2 diabetic skeletal muscle was that they were smaller (Kelley, He et al. 2002). These findings have led to the term 'mitochondrial

dysfunction' or 'mitochondrial impairments'. Interestingly, Holloszy (2013) points out that no measure of function was completed in these particular studies. Furthermore, when mitochondrial function was later evaluated in diabetic muscle, it appeared that the mitochondria had normal function (Boushel, Gnaiger et al. 2007, Larsen, Ara et al. 2009, Ara, Larsen et al. 2010).

Some investigations have suggested that there is reduced mitochondrial content (30%) in type 2 diabetic patients, obese insulin resistant individuals and lean insulin resistant off-spring of type 2 diabetic parents (Patti, Butte et al. 2003, Morino, Petersen et al. 2005, Befroy, Petersen et al. 2007). Therefore using the terms 'dysfunction' or 'impairment' can be misleading as they imply pathology in skeletal muscle, however, this has not been clearly established. Total mitochondrial oxidative capacity is dependent on both mitochondrial content and the intrinsic capacity of each mitochondrion. Using the term mitochondrial function may refer to either, therefore, it is important to distinguish between the two components that contribute to overall function (Toledo and Goodpaster 2013). A clearer definition of mitochondrial dysfunction would help clarify the role of mitochondria in the development of type 2 diabetes. A large body of data have implicated the role of mitochondria in the insulin resistance.

2.7 The Pathway of Insulin Signalling

Upon binding and activating its cell surface receptors, insulin triggers signalling cascades that regulate many cellular processes. Of particular interest is glucose homeostasis, as insulin suppresses hepatic glucose production and increases glucose transport into muscle and adipose tissue (Cheatham and Kahn 1995). Circulating insulin binds to the extracellular α subunit of the insulin receptor and activates the intrinisic kinase activity of the mostly intracellular β subunits leading to autophosphorylation of the adjacent β subunits (White 1997, Saltiel and Kahn 2001). The insulin receptor substrate (IRS) interacts with the phosphorylated insulin receptor through a phosphotyrosine binding domain that facilitates phosphorylation of IRS on a number of tyrosine residues and then acts as a docking site for other proteins with Src Homology 2 (SH2) domains (Cheatham and Kahn 1995). One of the proteins that dock with phosphorylated IRS is the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (Okada, Kawano et al. 1994).

PI3-K exists in the cytosol as a dimer of a p110 catalytic subunit and a p85 regulatory subunit that has two SH2 domains that interact with the phosphorylated IRS (Fruman, Meyers et al. 1998). Recruitment of the regulatory subunit, p85, brings the catalytic subunit p110 to the plasma membrane, up regulating PI3-K activity (Cantley 2002). It catalyzes the phosphorylation of the 3' position in the inositol ring of phosphoinositide (PI) lipids, converting phosphatidylinositol-4-5-bisphosphate ($PI(4,5)P_2$) to phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) (Fruman, Meyers et al. 1998, Cantley 2002). Phosphorylation of 3' position recruits and activates proteins that contain pleckstrin homology (PH) domains including the 3' phosphoinositide-dependent kinase-1 (PDK-1) and Akt/PKB by directly binding to $PI(3,4,5)P_3$ (Watson, Kanzaki et al. 2004). Association of these proteins with $PI(3,4,5)P_3$ facilitates phosphorylation of Akt by phosphoinositide dependent protein kinase -1 (PDK1).

The role of PI3-K in GLUT-4 translocation has been confirmed by many studies as specific inhibition by wortmannin almost completely abolished the insulin induced increase in hexose uptake in isolated adipocytes (Okada, Kawano et al. 1994). A similar response was seen in 3T3-L1 adipocytes treated with LY294002 where an inhibition of insulin stimulated PI3-K was evident with a concomitant reduction in GLUT-4 translocation, visualized by immunofluorescence (Ebina, Ellis et al. 1985). Conversely, overexpression of constitutively active form of the catalytic subunit, p110α, increases basal glucose uptake and GLUT-4 translocation even in the absence of insulin (Gerich 1988, Gerich, Wittlin et al. 2004). These data suggest that PI3-K is necessary for insulin stimulated GLUT-4 translocation. However, the role of $PI(3,4,5)P_3$ and downstream effector Akt and its contribution to GLUT 4 translocation and glucose uptake remain controversial (Khan and Pessin 2002).

Activation of Akt requires phosphorylation at two sites, both the threonine and serine residues (Haruta, Morris et al. 1995, Alessi and Cohen 1998). PDK1 phosphorylates Akt at Thr308, a residue located in its kinase domain activation loop. Also Ser347 in the C-terminal hydrophobic motif of Akt undergoes phosphorylation but the identity of the kinase responsible remains elusive (Cheatham and Kahn 1995). More recently, an enzyme complex consisting of mTOR (mammalian target of rapamycin) and RICTOR (rapamycin insensitive companion of mTOR) has been shown to phosphorylate Akt at site Ser347 in response to insulin stimulation (Katagiri, Asano et al. 1996). This phosphorylation of Akt results in the catalytic activity of Akt resulting in the

phosphorylation of a host of other proteins downstream. Of particular interest is its role in GLUT 4 cycling.

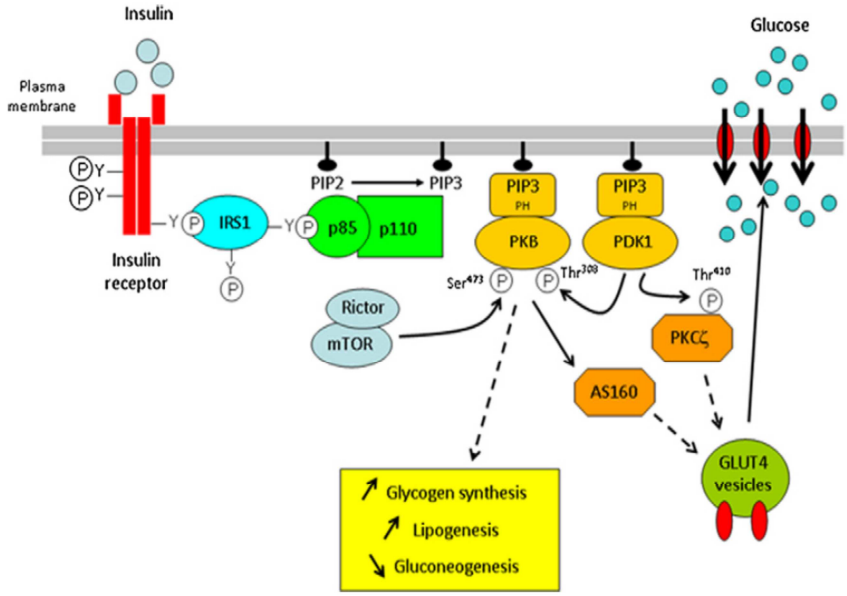


Figure 2.3 Insulin Stimulated Glucose Uptake. Circulating insulin binds to insulin receptor leading to the phosphorylation of IRS. This allows the docking of p85 subunit of PI3-K. Phosphorylation of PI3-K activates the proteins that contain pleckstrin homology (PH) domains including Akt/PKB by directly binding to PI(3,4,5)P3 facilitating the phosphorylation and activation of Akt and subsequent translocation of Glut4 to the cell membrane. Adapted from Turban et al. 2010

Glut-4 is a facilitative glucose transporter that is highly expressed in adipose tissue and striated muscle. During basal conditions, Glut-4 cycles to and from the cell surface at a much lower rate, almost entirely sequestered within the cell. Activation of the insulin receptor initiates the signalling cascade (already discussed) and substantially increases the translocation of Glut-4 containing vesicles to the plasma membrane. The rate of Glut-4 exocytosis can be increased 5-fold while the rate of endocytosis from the cell surface may be reduced 2-fold (Jhun, Rampal et al. 1992, Holman, Leggio et al. 1994). The ultimate goal is to cause a dramatic redistribution of Glut4 to the plasma membrane to allow the diffusion of glucose. Once circulating levels of glucose return to basal levels, Glut-4 is internalized through calthrin-coated pits and recycled back to their intracellular compartments.

2.7.1 Insulin Resistance

In insulin resistant states such as type 2 diabetes and obesity, insulin stimulated glucose disposal in striated muscle is impaired. Both receptor and post receptor defects have been suggested to contribute to insulin resistance. At the level of the insulin receptor, some studies have demonstrated a reduction in insulin binding in human muscle and adipocytes, however, this finding is not due to reduced binding affinity (Caro, Sinha et al. 1987, Freidenberg, Henry et al. 1987). Tyrosine kinase activity of the insulin receptor has been shown to be decreased in insulin resistant skeletal muscle in most studies (Caro, Sinha et al. 1987, Nolan, Freidenberg et al. 1994, Cusi, Maezono et al. 2000) but not all (Klein, Vestergaard et al. 1995, Krook, Björnholm et al. 2000). Further down the pathway, the ability of insulin to activate the insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation in muscle is reduced in obese insulin resistant non diabetic subjects and severely impaired in type 2 diabetics (Cusi, Maezono et al. 2000). Furthermore, the association of p85 subunit of PI3-K with IRS-1 and activation of PI3-K with insulin stimulation is suppressed in both obese non-diabetics and type 2 diabetics versus lean healthy controls (Cusi, Maezono et al. 2000, Krook, Björnholm et al. 2000). A reduction in insulin stimulated glycogen synthase was also noted in this population and reflects the impairments in the insulin signalling pathway (Kim, Nikoulina et al. 1999, Cusi, Maezono et al. 2000). Impaired association of PI3-K with IRS-1 are characteristic abnormalities of type 2 diabetes (DeFronzo 2004). The *in vivo* administration of insulin in humans stimulates Akt1/2 activity in skeletal muscle. In insulin resistant obese subjects with type 2 diabetes, insulin stimulated phosphorylation and activation of Akt are normal despite a severe impairment in IRS-1 associated PI3-K activity. The link between these cellular mechanisms that lead to insulin resistance and mitochondria require further clarification.

2.7.2 Lipid metabolism

Whole body fatty acid metabolism is regulated by a number of processes at the cellular level. Firstly, fatty acid availability is highly dependent on the rate of lipolysis in adipose tissue. Secondly, fatty acid uptake is determined by the rate of lipolysis and specific fatty acid transport proteins (FATP); fatty acid translocase and FABP. Thirdly, fatty acid storage is regulated by intracellular signalling and biochemical processes and has been a key factor in the hypothesis of lipid induced insulin resistance. Finally, fatty acid

oxidation is regulated by mitochondrial transport, β-oxidation and respiration and has been reported to be decreased in obese and insulin resistance states (Corpeleijn, Saris et al. 2009).

In the fasting state, the contribution to the lipid pool comes from lipolysis of stored triglycerides in adipocytes and lipolysis of circulating VLDL-triglycerides by lipoprotein lipase (LPL) in the capillary endothelium which can also contribute significantly to the lipid pool (Samra, Clark et al. 1996, Mead, Irvine et al. 2002). In the postprandial state FFA can also be derived from lipolysis of chylomicron-triglycerides. Lipolysis in adipose tissue is suppressed by insulin. Due to the large lipid stores in obesity, it is generally suggested that the origins of insulin resistance lie in the increased supply of FFA from increased adipose tissue mass (Kahn, Hull et al. 2006).

2.7.3 Free Fatty Acids and Insulin Resistance

More recent studies have started to identify post receptor signalling mechanisms that contribute to insulin resistance. Altered fatty acid metabolism in skeletal muscle in the presence of increased content of triglyceride within the muscle fibre has been demonstrated, identified by either biochemical extraction of lipids from a muscle biopsy (Pan, Lillioja et al. 1997), electron microscopy (Vock, Hoppeler et al. 1996), computed tomography (Kelley, Slasky et al. 1991, Goodpaster, Thaete et al. 1997) and magnetic resonance imaging (Szczepaniak, Babcock et al. 1999). In humans, it is reported that muscle lipid content is correlated with the severity of insulin resistance (Goodpaster, He et al. 2001), independent of visceral fat (Ellis, Poynten et al. 2000, Itani, Ruderman et al. 2002).

Many animal and human studies have increased muscle TG content to identify plausible mechanistic links between lipid accumulation and insulin resistance. Intramuscular lipid accumulation is evident in a variety of experimental models, including insulin resistance induced by lipid infusion in humans (Bachmann, Dahl et al. 2001, Boden, Lebed et al. 2001, Itani, Ruderman et al. 2002) and in rodents (Chalkley, Hettiarachchi et al. 1998, Griffin, Marcucci et al. 1999, Yu, Chen et al. 2002). Other rodent models of insulin resistance include genetic forms of obesity in Zucker rats, chronically glucose infused rats and high fat fed rats (Laybutt, Chisholm et al. 1997, Ellis, Poynten et al. 2000, Dobbins, Szczepaniak et al. 2001, Oakes, Thalén et al. 2001). The literature strongly

suggests that lipid accumulation within skeletal muscle is associated with insulin resistance, however, there is a paradox where skeletal muscle of highly trained athletes can contain high triglyceride content who are insulin sensitive (Goodpaster, He et al. 2001). Therefore, lipids alone may not induce insulin resistance but provide an avenue for other potentially harmful lipid metabolites including diacylglycerol, ceramides and long chain fatty acyl-CoA.

2.7.3 Lipid Metabolites

FFAs, primarily palmitate, oleate and linoleate in muscle are converted to long-chain acyl-CoAs (LCACoAs) before they are transported across the inner mitochondrial membrane by carnitine palmitoyltransferases and then metabolized by β oxidation (Schmitz-Peiffer 2000). LCACoAs content of skeletal muscle is increased in both human and animal models using lipid infusion (Chalkley, Hettiarachchi et al. 1998, Ellis, Poynten et al. 2000). It may influence insulin action by inhibiting hexokinase, the first enzyme involved in intracellular glucose metabolism, contributing to a reduction in glucose uptake into the cell (Thompson and Cooney 2000). LCACoAs may also interfere with insulin action through protein kinase C (PKC) activation either directly or through the activation of the second messenger diacylglycerol (DAG) (Nishizuka 1995).

Increased DAG content and altered activity and distribution have been observed in a number of insulin resistant states in rodents including fat feeding, inactivity, obesity, glucose infusion and type 2 diabetes (Turinsky, Bayly et al. 1990, Schmitz-Peiffer, Browne et al. 1997, Ruderman, Saha et al. 1999, Bell, Schmitz-Peiffer et al. 2000). Interestingly, exercise induced improvements in insulin sensitivity in rats is associated with a more normal pattern of PKCβ and PKCθ activity (Bell, Schmitz-Peiffer et al. 2000). Altered PKC activity has also been established in obese, insulin resistant humans both with (Itani, Pories et al. 2001) and without (Itani, Zhou et al. 2000) type 2 diabetes. There are many isoforms of PKC and differential alteration between rodents (PKCθ and PKCε) and humans (PKCβ and PKCθ) exists (Itani, Zhou et al. 2000, Itani, Pories et al. 2001). PKC induced insulin resistance may occur due to the serine/threonine phosphorylation of both the insulin receptor (Chin, Dickens et al. 1993, Itani, Zhou et al. 2000) and insulin receptor substrate-1 (IRS-1) (Ravichandran, Esposito et al. 2001) causing impaired insulin signalling.

Increased ceramide concentration was first described in skeletal muscle of insulin resistant Zucker rats by Turinsky et al. (1990) . They are a family of sphingolipids and are known to induce insulin resistance in cultured cells (Kanety, Hemi et al. 1996, Zhou, Summers et al. 1998, Schmitz-Peiffer, Craig et al. 1999, Hajduch, Balendran et al. 2001). In particular, C2C12 myotubes treated with palmitate, a precursor of *de novo* synthesis of ceramide, developed insulin resistance (Schmitz-Peiffer, Craig et al. 1999). Ceramide can also be generated by hydrolysis of sphingomyelin, a reaction catalysed by sphingomyelinase (Hannun and Obeid 2002). In insulin resistant obese humans, ceramide content was increased twofold versus lean control subjects. This was accompanied by a reduction in insulin stimulated Akt activity which could lead to a reduction in insulin stimulated glucose uptake (Adams, Pratipanawatr et al. 2004). However, the relative contribution of the various lipid species to insulin resistance remains to be defined.

2.7.4 Mitochondria and Insulin Resistance

Concurrent impairment in mitochondrial function and insulin resistance has been observed in aging (Petersen, Befroy et al. 2003, Short, Vittone et al. 2003, Short, Bigelow et al. 2005), type 2 diabetic (Kelley, He et al. 2002) and obese individuals (Simoneau and Kelley 1997). It is unclear whether mitochondrial dysfunction precedes insulin resistance or vice versa or if the co-existence of both is simply coincidental (Lanza and Nair 2009). Recent findings suggest that the mitochondria of type 2 diabetics and lean off-spring of diabetic patients may be dysfunctional. It is strongly debated whether the reduction in ATP synthesis in insulin resistance (Shulman 2000, Petersen, Dufour et al. 2005) is due to either an intrinsic defect of the mitochondria (Mogensen, Sahlin et al. 2007) or if it is due to reductions in mitochondrial number and density (Morino, Petersen et al. 2005, Rabøl, Boushel et al. 2006, Boushel, Gnaiger et al. 2007). Many studies have employed mitochondrial respiration measurements (Gnaiger 2008) to assess whether changes in oxidative phosphorylation are the result of an intrinsic defect in mitochondria and to elucidate the role of mitochondria in insulin resistance. However the results have been conflicted and the various arguments are presented in the following section.

2.7.5 Causal Role of Mitochondrial in Insulin Resistance

The interest in the causal role of mitochondria in insulin resistance came about when Kelley et al. (2002) described 'dysfunctional' skeletal muscle mitochondria in type 2 diabetics. However, this was illustrated by reduced activity of mitochondrial enzymes, NADH:O_2 reductase and citrate synthase. This hypothesis has since been supported by many studies which have reported lower mitochondrial oxidative capacity in insulin resistant subjects (Kim, Hickner et al. 2000, Kelley, He et al. 2002, Petersen, Befroy et al. 2003, Petersen, Dufour et al. 2004, Ritov, Menshikova et al. 2005, Ritov, Menshikova et al. 2010). Some explanations exist to try and explain why reduced mitochondrial function could contribute to insulin resistance. Reduced expression of regulators of mitochondrial biogenesis like proliferator-activated receptor γ coactivator 1α (PGC1α), in insulin resistance is a possible explanation (Patti, Butte et al. 2003) or an increase ratio of glycolytic to oxidative enzymes is another (Simoneau and Kelley 1997). Furthermore, proteomic analysis reported a reduction in abundance of mitochondrial proteins in skeletal muscle from type 2 diabetic patients (Hwang, Bowen et al. 2010) probably as a result of a reduction in PGC1α. In theory, this may result in a reduction in oxidative capacity and could be accounted for by a reduction in the amount of mitochondria present in the muscle (mitochondrial content) or an intrinsic defect (functional capacity per mitochondrion), a topic that is hotly debated.

2.7.6 Surrogate Markers of Mitochondrial Content

A common experimental approach in bioenergetics research is the determination of mitochondrial content, an important quantitative indicator of oxidative capacity. Mitochondrial content is often used to normalize global measures of skeletal muscle bioenergetic capacity. Two-dimensional imaging using transmission electron microscopy is considered the gold standard for measuring mitochondrial content. However, as this method is expensive and time consuming, other biomarkers of mitochondrial content are commonly used as surrogate markers. Commonly used biomarkers include citrate synthase activity, cardiolipin content, mitochondrial DNA content (mtDNA), complex I – IV protein content and complex I – IV activity. Previous studies have validated the use of citrate synthase showing a close association between the change in its activity and morphological changes in mitochondrial content in rabbit tibialis anterior muscle that was chronically electrically stimulated (Reichmann, Hoppeler et al. 1985). More recently,

Larsen et al (2012) has shown that citrate synthase has high relative concordance (R_c =0.8) with mitochondrial content.

Another interesting finding in this study was that there was a strong correlation between mitochondrial content and respiratory rate in permeabilized muscle fibres when using ascorbate and TMPD as substrates (r=0.94), substrates that regulate Complex IV activity. Using complex IV activity as a marker may be advantageous and conducted on the same experiment as other respiratory measures and on the same muscle samples avoiding any long term sample storage. However, caution should be taken when interpreting ascorbate and TMPD as the respiratory rate does not reach steady state but rather displays a spiked response and should be interpreted with caution (Larsen, Nielsen et al. 2012).

Cardiolipin, an inner mitochondrial membrane lipid was suggested to show the strongest association with mitochondrial content, closely followed by CS. Interestingly, mtDNA, which is commonly used, was found to be a poor indicator of mitochondrial content (Larsen, Nielsen et al. 2012).

Many studies have used different markers of mitochondrial content and have used these enzyme, lipid or protein markers to normalize for mitochondrial function. Studies have examined surrogate markers of mitochondrial content in insulin sensitive versus insulin resistant subjects and have reported conflicting findings. Some studies have shown a reduction in citrate synthase in obese individuals (Kim, Hickner et al. 2000, Heilbronn, Gan et al. 2007, Holloway, Thrush et al. 2007), while others report no change in obesity (Simoneau and Kelley 1997, Simoneau, Veerkamp et al. 1999, Kelley, He et al. 2002), type 2 diabetes (Mogensen, Sahlin et al. 2007) or non-diabetic subjects with a family history of T2DM (Østergård, Andersen et al. 2006). However, the use of citrate synthase appears to be the most common marker and is easy to measure. The disadvantage of using this marker is that citrate synthase may be an indicator or mitochondrial functional and not always an indicator of mitochondrial content (Larsen, Nielsen et al. 2012).

2.7.7 Intrinsic Mitochondrial Defects and Insulin Resistance

Several studies have examined if mitochondrial intrinsic defects are present in type 2 diabetes. Kelley et al. (2002) reported reduced electron transport chain capacity in T2DM and obesity. The same group showed a reduction in ETC capacity in subsarcolemmal mitochondria in T2DM versus lean subjects which could not be completely explained by a reduction in mtDNA, perhaps suggesting an additional intrinsic defect (Ritov, Menshikova et al. 2005). In support of the intrinsic mitochondrial defect, Mogensen et al. (2007) reported a reduction in function per mitochondrion in type 2 diabetic skeletal muscle in comparison to obese non-diabetic subjects. *Ex vivo* measurements of mitochondrial function have been development in muscle fibres that are manually separated and permeabilized by saponin, allowing entry of substrates to assess mitochondrial function, while leaving the cell membrane intact (Kuznetsov, Veksler et al. 2008). Using this approach, mitochondrial respiration in permeabilized muscle fibres when corrected for using mtDNA was approximately 35% lower in T2DM in comparison to BMI matched control subjects (Phielix, Schrauwen-Hinderling et al. 2008). The findings of Meex et al. (2010) further support the hypothesis that an intrinsic mitochondrial defect reduces mitochondrial respiration as they reported a reduction in basal ADP-stimulated and maximal mitochondrial respiration, independent of mitochondrial content. On the other hand, oxygen consumption was significantly reduced in muscle fibres of T2DM patients but when the oxygen flux was normalized for mtDNA content, no change was noted indicating that the lower mitochondrial capacity was linked to reduced mitochondrial content rather than to an intrinsic defect (Boushel, Gnaiger et al. 2007). These findings have been supported by numerous other studies using permeabilized muscle fibres in T2DM patients (Boushel, Gnaiger et al. 2007, Larsen, Ara et al. 2009, Rabøl, Højberg et al. 2009, Rabøl, Højberg et al. 2009, Rabøl, Boushel et al. 2010, Rabøl, Larsen et al. 2010, Larsen, Stride et al. 2011). However, these reports fail to ascribe the functional defect to a reduction in mitochondrial content or of distinct pools (subsarcolemmal or intermyofibillar), or intrinsic mitochondrial capacity or both.

Holloszy (2013) poses an important question; does a reduction in muscle mitochondria cause peripheral insulin resistance? Previously published correlative data have suggested that mitochondrial dysfunction in skeletal muscle may be a cause of

peripheral insulin resistance in patients with T2DM (Kelley, He et al. 2002, Petersen, Befroy et al. 2003, Petersen, Dufour et al. 2004, Ritov, Menshikova et al. 2005). Wredenberg et al (2006) generated muscle–specific mitochondrial transcription factor A (Tfam) knockout mice which had a progressive deterioration in respiratory chain capacity, however showed an increase in insulin action and glucose tolerance. Tfam is a transcription factor responsible for mediating the transcription of genes encoded in the mitochondrial genome which include a number of respiratory chain proteins. Similarly, ablation of the mitochondrial flavoprotein apoptosis inducing factor (AIF) in mice led to a subtle reduction in all oxidative phosphorylation genes from all respiratory chain complexes which manifested functionally with an increase in insulin sensitivity and resistance to obesity (Pospisilik, Knauf et al. 2007). These studies contradict the hypothesis that mitochondrial dysfunction causes insulin resistance, however, reports from mouse studies, while informative must be interpreted with caution.

2.7.8 Normal Mitochondrial Function in Insulin Resistance

While many studies have reported associations between diminished mitochondrial function and insulin resistance (Kelley, He et al. 2002, Mogensen, Sahlin et al. 2007, Phielix, Schrauwen-Hinderling et al. 2008, Meex, Schrauwen-Hinderling et al. 2010), others have failed to show such correlations (Bandyopadhyay, Joseph et al. 2006, Van Tienen, Praet et al. 2012). In fact, several reports suggest that muscle mitochondrial function is not impaired in obese and T2DM human subjects compared to controls (Bandyopadhyay, Joseph et al. 2006, Holloway, Thrush et al. 2007, Trenell, Hollingsworth et al. 2008, Van Tienen, Praet et al. 2012). These findings support the hypothesis of Lanza & Nair (2009) that mitochondrial function is not directly related to insulin resistance. They suggest that the correlative data suggesting a relationship between mitochondrial dysfunction and insulin resistance is a coincidence. Published reports relating to mitochondrial capacity and insulin resistance are not conclusive and this may be partly due to different outcome parameters being measured and variations in subject selection. The well documented relationship between exercise and mitochondrial function may prompt researchers to match subjects for VO_2 max, age and BMI.

2.7.9 Improved mitochondrial Function in Insulin Resistance States

Interestingly, other research groups have shown a compensatory increase in mitochondrial oxidative capacity in the presence of impaired insulin sensitivity and glucose tolerance by feeding mice a high fat diet. A simultaneous increase in fatty acid oxidation and protein content as well as increase in mitochondrial protein activity was noted. However, while there appears to be a compensatory increase in mitochondrial oxidative capacity in rodents in response to lipid overload, the magnitude was not great enough to offset ectopic fat deposition and insulin resistance at 3-4 weeks of high fat feeding (Garcia-Roves, Huss et al. 2007, Turner, Bruce et al. 2007, de Wilde, Mohren et al. 2008, Turner, Hariharan et al. 2009). Conflicting reports exist regarding the role mitochondria have in the development of insulin resistance.

2.8 Protein Turnover: The Balance of Synthesis and Degradation

The growth of skeletal muscle mass depends on protein turnover and cell turnover. Protein degradation pathways are activated by disuse which leads to muscle atrophy. Muscle hypertrophy on the other hand occurs in response to stimulation such as exercise and involves changes in myofibrillar gene expression. It also induces recruitment of satellite cells to increase myofibre number (Sartorelli and Fulco 2004). Generally, muscle hypertrophy is the result of an increase in the size of existing muscle fibres as determined by cross sectional area. In contrast, muscle atrophy is the loss of such proteins due to a reduction in muscle fibre cross sectional area. Essentially, maintenance of skeletal muscle mass is related to the dynamic balance between muscle protein synthesis and degradation.

Considerable efforts have been undertaken to characterize the signalling pathways that regulate protein synthesis and degradation. An acute bout of resistance exercise is known to increase the rate of protein synthesis for up to 24 hours post exercise (Hernandez, Fedele et al. 2000). This increase in protein synthesis correlated with Pi3K, activity, the mammalian target of rapamycin (mTOR) and the 70 KDa ribosomal S6 protein kinase (S6K1/p70^{S6K}) (Baar and Esser 1999, Hernandez, Fedele et al. 2000,

Bolster, Kubica et al. 2003). The same modality of exercise increased AKT activity (Nader and Esser 2001, Bolster, Kubica et al. 2003) and the downstream target S6K1 via mTOR (Bodine, Stitt et al. 2001). The activation of this signalling network Pi3K/Akt/mTOR/S6K1 is associated with the growth response of muscle to resistance exercise (Nader 2005). This is confirmed by studies utilizing rapamycin, a specific mTOR inhibitor which suggested that mTOR is essential for muscle growth (Bodine, Stitt et al. 2001).

As protein synthesis occurs, protein degradation is essential for the maintenance of cellular homeostasis and plays a significant role in the quality control of the cell by rapidly eliminating misfolded or damaged proteins whose accumulation would interfere with normal cell function and viability (Goldberg 2003). However, in certain situations when protein degradation exceeds protein synthesis, skeletal muscle wasting occurs. This can occur with exposure to microgravity (Adams, Caiozzo et al. 2003), aging (Singh 2002), prolonged bed rest and physical inactivity (LeBlanc, Gogia et al. 1988, Berg, Larsson et al. 1997, Bloomfield 1997) and chronic disease states such as cancer, diabetes and renal failure (Price, Bailey et al. 1996, Baracos 2001, Mitch and Price 2001). All cells contain multiple pathways for protein degradation.

The role of MuRF-1 and atrogin-1 in breaking down myofibrillar proteins in disuse in small mammals (Ikemoto, Nikawa et al. 2001) and with mechanical ventilation in humans (Levine, Nguyen et al. 2008) is well established. Activation of MuRF-1 and Atrogin-1 have been observed in human skeletal muscle disuse, at early timepoints (2-5 days) and their activation appear to be transient (de Boer, Selby et al. 2007, Urso, Chen et al. 2007). Data to support longer duration disuse and activation of MuRF-1 and atrogin-1 are lacking. This could be due to a delayed activation of translation, ie. activities of ubiquitin ligases could be higher at times when their expression is back to normal (Brocca, Cannavino et al. 2012). One exception is a study by Ogawa et al. (2006) who examined the effects of 20 days of bed rest in 20 healthy male volunteers on ubiquitin ligase gene expression. Up regulation of atrogin-1 was noted with a 4.6% decrease in quadriceps femoris cross-sectional area, suggesting that atrogin-1 mediated ubiquitination plays an important role in unloading induced muscle atrophy (Ogawa, Furochi et al. 2006). Akt has an additional role in phosphorylating FOXO transcription factors, making them unable to enter the nucleus to drive transcription of certain ubiquitin ligases like atrogin-1 and MuRF-1 (Sandri, Sandri et al. 2004).

When amino acids are infused or consumed in large doses, particularly leucine, insulin secretion from the β cells is increased suggesting an insulinotropic effect of amino acid supplementation. Many studies have shown that amino acid supplementation in combination with carbohydrate ingestion results in a large increase in plasma insulin (van Loon, Saris et al. 2000). It is well known that bed rest leads to a decrease in insulin sensitivity (Bergouignan, Rudwill et al. 2011). Therefore, a beneficial effect of amino acid supplementation may be to increase plasma insulin concentrations at the early stages of declining insulin sensitivity in bed rest, microgravity or indeed in the development of type 2 diabetes. Insulin would, in turn, stimulate protein synthesis by activating the Akt signalling cascade and, through mTOR, regulate the balance between protein synthesis and degradation.

2.8.1 Interaction between Muscle Atrophy and Insulin Resistance

The changes in skeletal muscle during bed rest or space flight are well documented and include muscle atrophy, insulin resistance, oxidative stress and fibre type changes. While these alterations tend to be studied in isolation, there is growing evidence to suggest common initiating factors. For instance, the binding of insulin to the insulin receptor activates a pleiotrophic signalling cascade that activates glucose transport and protein synthesis in skeletal muscle, as previously described. With bed rest, there is a reduction in aerobic capacity (Convertino, Bloomfield et al. 1997, Capelli, Antonutto et al. 2006), a reduction in insulin sensitivity (Alibegovic, Hojbjerre et al. 2009) and a reduction in GLUT-4 protein (Bienso, Ringholm et al. 2012). All these metabolic changes occur in skeletal muscle and it may be important to identify cellular changes that link aerobic capacity, insulin resistance and muscle atrophy.

While insulin binding is the key component in muscle glucose uptake, as an anabolic hormone it also increases Akt mediated protein synthesis by activating mTOR (Bodine, Stitt et al. 2001). Akt also supresses protein degradation by phosphorylating and promoting nuclear exclusion of FOXO1 (Sandri, Sandri et al. 2004). In a model of insulin deficiency, it was reported that there is accelerated muscle proteolysis and that this is caused by a decrease in PI3K activity (Du, Wang et al. 2004, Lee, Dai et al. 2004). Reduced PI3K was found to reduce the levels of phosphorylated Akt (pAkt) and a low pAkt has been shown enhance the expression of specific E3 ubiquitin-conjugating enzymes, Atrogin-1 and MuRF1 in muscle (Sandri, Sandri et al. 2004). As well as

enhancing the ubiquitin-proteasome proteolytic pathway, decreased PI3K activity has also been shown to activate Bax, which stimulates the activity of caspase-3 leading to muscle protein loss and substrate for ubiquitin-proteasome proteolytic pathway (Du, Wang et al. 2004, Lee, Dai et al. 2004). Another important downstream target of the PI3K/Akt pathway is FOX01. Akt blocks the function of FOXO1 leading to sequestration in the cytoplasm away from target genes (Brunet, Bonni et al. 1999). Dephosphorylation of the FOXO1 leads to nuclear entry and growth suppression (Ramaswamy, Nakamura et al. 2002) through its role in the regulation of transcription of atrogin-1 and MuRF1 (Chopard, Hillock et al. 2009). It has become clear that there is a high degree of interaction between the cellular and molecular regulators of metabolism and muscle mass.

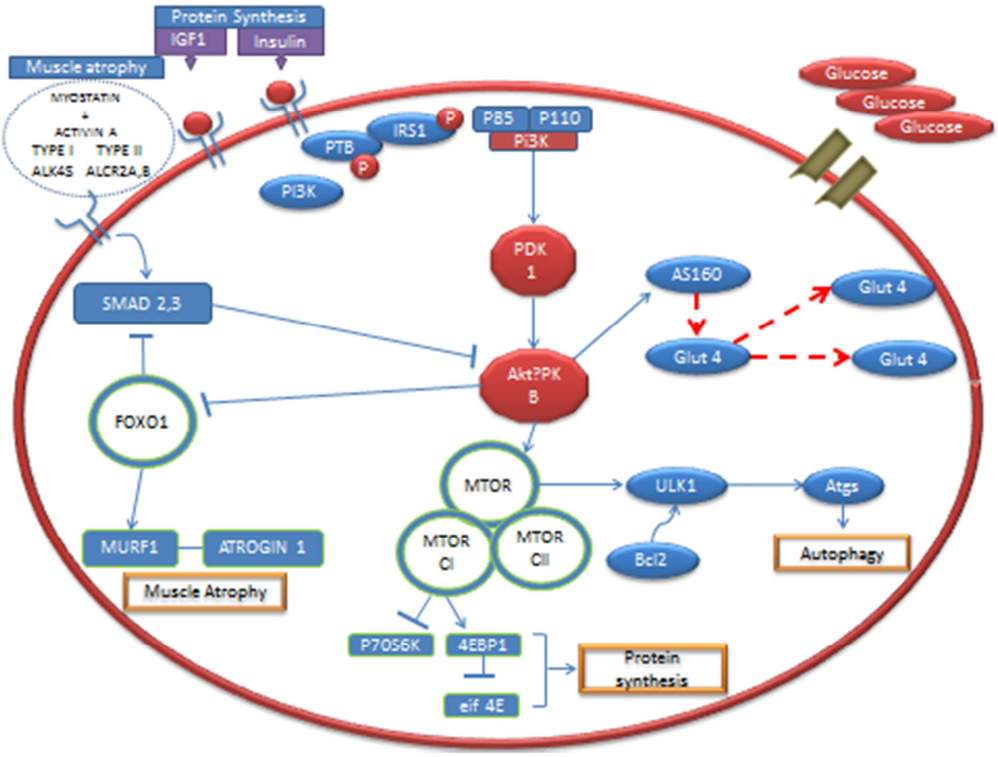


Figure 2.4 Schematic illustrating the major pathways involved in disuse muscle atrophy.

2.9 Common metabolic mechanisms regulating insulin resistance and muscle atrophy: the role of reactive oxygen species (ROS)

It was discovered that free radicals existed in biological materials over 50 years ago. In 1956, Denham Harman suggested that oxygen radicals were by-products of enzymatic reactions *in vivo* and coined the free radical theory of aging (Harman 1955, Harman 1981, Appell, Duarte et al. 1997). Convincing work by McCord and Fridovich suggested that free radicals were important in biology, triggering an interest among researchers to investigate the effects of free radicals on proteins, DNA and lipids (McCord and Fridovich 1969). Since Jensen (1966) reported that the electron transport chain produced reactive oxygen species (ROS), pioneering work has been undertaken to identify the mitochondrial production of H_2O_2 (Jensen 1966, Boveris and Chance 1973, Chance, Sies et al. 1979). Now, in the 21st century, a large body of evidence exists showing that living organisms have developed mechanisms for advantageous use of free radicals (Dröge 2002).

Initial studies did not reveal the source and type of ROS but subsequent investigation found that contracting skeletal muscle is a prominent source of ROS production (Powers, Duarte et al. 2010). Paradoxically, ROS production has been shown to increase with muscle disuse also (Kondo, Miura et al. 1991). ROS has been loosely termed free radicals. This is not entirely true as not all ROS are free radicals. A free radical is an atomic or molecular species capable of independent existence and contains one or more unpaired electrons in one of its molecular orbitals (Dröge 2002). It appears that ROS is an important cell signalling molecule but if left unchecked, it may impair cellular function through its role as a strong oxidizing agent. In this regard, ROS have been implicated in neurological diseases like Alzheimers and Parkinsons and metabolic diseases such as obesity, diabetes, metabolic syndrome, cardiovascular disease and aging (Harper, Bevilacqua et al. 2004, Chan and Harper 2006, Ando and Fujita 2009, Patten, Germain et al. 2010).

2.9.1 Mitochondrial Sites of ROS Production

Mitochondria are central to cellular bioenergetics and are a major source of ROS in the cell. Reactive oxygen species are derived from molecular oxygen and can readily oxidize

other molecules (Sena and Chandel 2012). The mitochondrial electron transport chain, as described in Section 2.6.3, is composed of a series of protein complexes that shuttle electrons and transport hydrogen ions through a series of redox reactions and terminated to water in a four electron reduction of molecular oxygen catalysed by cytochrome C oxidase (Liu, Fiskum et al. 2002, Hou, Wang et al. 2014). Molecular oxygen in the ground state is a bi-radical containing two unpaired electrons in its outer orbital. Superoxide anion ($O_2^{-}\cdot$) is the product of a one electron reduction of oxygen and is the precursor of most ROS (Turrens 2003, Sena and Chandel 2012). This reaction can occur both enzymatically and non-enzymatically. The non-enzymatic production of O_2^{-} occurs when a single electron is transferred to oxygen by reduced coenzymes or prosthetic groups like flavins or iron sulphur clusters. The mitochondrial electron transport chain contains many redox centres that leak electrons to oxygen. This constitutes the primary source of $O_2^{-}\cdot$in most tissue (Turrens 2003). Dismutation of the O_2^{-}, either spontaneously or catalysed by superoxide dismutase (SOD), produces hydrogen peroxide (H_2O_2) which can be partially oxidized to hydroxyl radical (HO·), one of the strongest oxidants in nature (Fridovich 1978, Turrens 2003). The formation of HO· is catalysed by reduced transition metals, which can by re-reduced by O_2^{-}, propagating this process (Liochev and Fridovich 1999). The dismutation of the $O_2^{-}\cdot$radical to H_2O_2 can be beneficial for the cell through its actions as a weak oxidizing agent (Mailloux and Harper 2011).

The sites with greatest contribution to electron leak are complex I and complex III of the mitochondrial electron respiratory chain (Barja 1999, Liu, Fiskum et al. 2002, Chen, Vazquez et al. 2003). Complex I (sites IF and IQ) (Treberg, Quinlan et al. 2011) produces superoxide in the matrix and complex III (site IIIQo) produces it into the matrix and the inter membrane space at about equal rates under resting conditions (St-Pierre, Buckingham et al. 2002, Han, Canali et al. 2003, Muller, Liu et al. 2004). The relative contribution of each site to $O_2^{-}\cdot$production varies from organ to organ and also depends on whether mitochondria are actively respiring (State 3) or if the respiratory chain is reduced (State 4) (Barja 1999).

2.9.2 ROS and Exercise and Physical Inactivity

The observation that muscle contraction through exercise increases the production of ROS in skeletal muscle was reported first by Davies et al (1982). Increasing evidence suggests that ROS production plays an important role in skeletal muscle cell signalling as

an adaptation to exercise and disuse (Powers, Duarte et al. 2010, Wellen and Thompson 2010). An acute and small increase in ROS production during a bout of exercise appears to play an important role in the regulation of cell signalling pathways that promotes gene expression leading to an increased oxidative phenotype of skeletal muscle (Dröge 2002, Jackson 2008). As a malleable tissue, skeletal muscle can undergo significant phenotypic change in response to repeated bouts of exercise or disuse through pathways that are sensitive to ROS. Redox sensitive pathways can increase or decrease the transcription of targeted genes. Two important redox sensitive transcription factors that are involved in muscle adaptation in response to endurance training are nuclear factor-kB (NF-kB) and PGC1α in skeletal muscle fibres (Powers, Talbert et al. 2011). NF-kB transcription factors are signalling molecules that control the expression of genes involved in a number of processes such as inflammation, cell growth, stress response and apoptosis and are maximally activated 2 hours post exercise in rats and *in vitro* by exposing C2C12 cells to 1-2mmol.L^{-1} H_2O_2 (Zhou, Johnson et al. 2001, Ji, Gomez-Cabrera et al. 2004, Ji 2007, Kramer and Goodyear 2007). Increased mitochondrial content is a well-documented skeletal muscle adaptation to endurance exercise through mitochondrial biogenesis (Holloszy 1967, Holloszy and Coyle 1984). PGC 1α plays a key role by interacting and co-activating a variety of transcription factors and nuclear receptors involved in the up regulation in both nuclear and mitochondrial encoded genes involved in organelle synthesis (Lin, Wu et al. 2002, Scarpulla 2006). It is suggested that PGC 1α is sensitive to the redox status of the cell and treatment of cells with H_2O_2 induces PGC 1α transcription (Irrcher, Adhihetty et al. 2003).

It is well established that oxidant production occurs in both inactive and contracting skeletal muscle (Kondo, Miura et al. 1991, Reid 2001). Excess ROS overwhelms antioxidant defences exceeding its capacity to buffer free radicals, leading to oxidative stress, which is reported to play an important role in the pathogenesis and progression of various disease states including cancer, type 2 diabetes, chronic kidney disease and cardiovascular disease (Urakawa, Katsuki et al. 2003, Waris and Ahsan 2006, Sachse and Wolf 2007, Loukogeorgakis, van den Berg et al. 2010). Numerous studies have demonstrated that oxidative injury occurs during periods of disuse in skeletal muscle (Kondo, Miura et al. 1991, Kondo, Miura et al. 1993, Lawler, Song et al. 2003). These studies were conducted in animal models of hind limb suspension. There are limited data in the literature on oxidative stress and its contribution to skeletal muscle atrophy

in humans. Additionally, it is unclear what ROS producing pathways are involved in response to inactivity; xantine oxidase (XO), nitric oxide, reactive iron, NAD(P)H oxidase and mitochondrial production of ROS (Powers, Kavazis et al. 2005). To further support the hypothesis that oxidative stress in inactive skeletal muscle contributes to muscle atrophy, exogenous antioxidants have been shown to retard muscle atrophy in response to physical inactivity (Appell, Duarte et al. 1997, Ikemoto, Nikawa et al. 2002, Betters, Criswell et al. 2004). Further investigation into the effects of oxidants on muscle atrophy with physical inactivity is required to develop strategies to scavenge ROS and prevent oxidative injury in the cell (Powers, Kavazis et al. 2005). Pathological conditions that cause muscle wasting are characterized by an increase in oxidative stress where the antioxidant defences are overwhelmed by oxidant production. Antioxidant enzymes include superoxide dismutase, catalase and glutathione peroxidase. Hind limb suspension over 28 days in rats showed a reduction in the antioxidant scavenging system and a reduction in catalase and glutathione peroxidase were significantly reduced in the soleus after hind limb unloading (Lawler, Song et al. 2003).

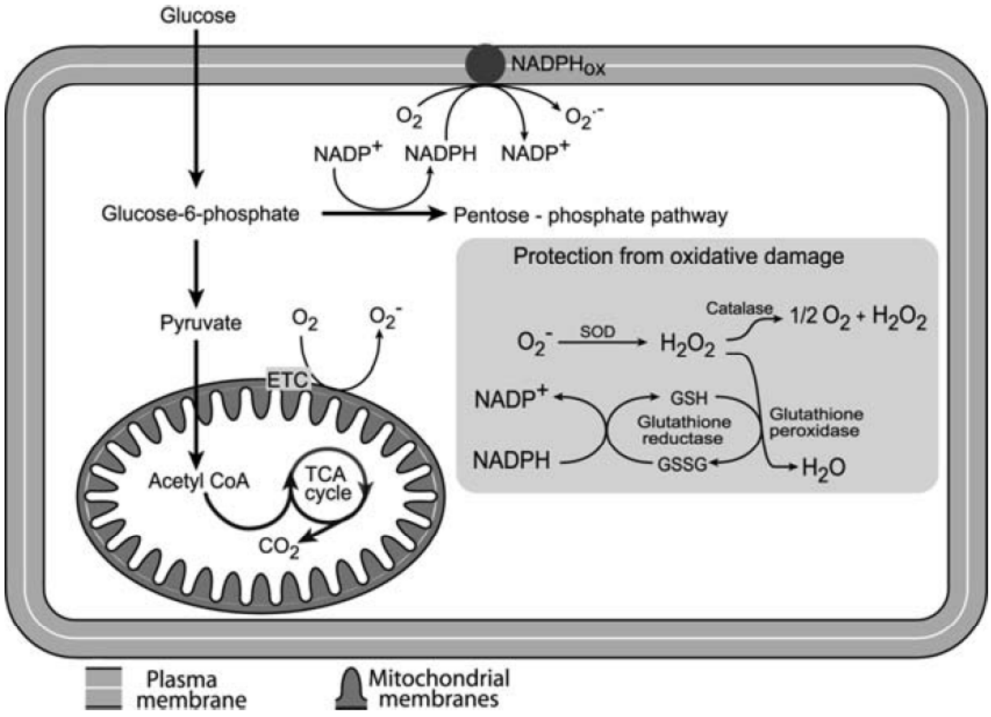


Figure 2.5Relevant sites of production of reactive oxygen species and antioxidant system. ROS can be produced through glucose metabolism in the mitochondrial electron transport chain or in the plasma membrane. The main antioxidant enzymes are superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase and catalase. Adapted from Newsholme et al. (2007).

2.9.3 Uncoupling Proteins and Reactive Oxygen Species

Uncoupling proteins have become prominent in the areas of thermogenesis, energy regulation and free radical biology. They are mitochondrial proteins that uncouple substrate oxidation from phosphorylation of ADP to ATP. There is strong evidence to suggest that they cause mild regulated uncoupling which attenuates ROS production, protects against cellular damage and diminishes insulin secretion (Brand and Esteves 2005). UCP2 and UCP3 only transport proteins when they are specifically activated (Brand, Affourtit et al. 2004, Esteves and Brand 2005). This was evidenced by the lack of effect of UCP2 and UCP3 knock out on basal proton conductance in isolated mouse mitochondria (Cadenas, Echtay et al. 2002). Such activators include reactive alkenals such as hydroxynonenal, that are produced by peroxidation of membrane phospholipids (Echtay, Esteves et al. 2003). The proton conductance in the presence of such activators is inhibited by purine nucleotides such as ATP and GDP. Fatty acids are probably required for activation to relieve the inhibition by nucleotides (Echtay, Murphy et al. 2002, Echtay, Roussel et al. 2002).

Mitchell's chemiosmotic hypothesis proposed the mechanism by which oxidation of substrates was coupled to ATP production in the mitochondria (Mitchell 1961). As electrons are transferred through the respiratory chain, protons are concomitantly ejected from the mitochondrial matrix to the intermembrane space, a process which establishes a proton gradient. This process of proton pumping and ATP generation for the most part is coupled. However, proton leak back through the membrane into the mitochondrial matrix is responsible for uncoupling substrate oxidation and ATP synthesis (Brand, Affourtit et al. 2004). Leak respiration is mediated by membrane proteins such as uncoupling proteins, adenine nucleotide translocase (ANT), glutamate carrier or complexes such as the mitochondrial permeability pore (Hunter, Haworth et al. 1976, Nègre-Salvayre, Hirtz et al. 1997, Samartsev, Smirnov et al. 1997, Brand, Affourtit et al. 2004, Brand, Pakay et al. 2005, Arvier, Lagoutte et al. 2007, Bézaire, Seifert et al. 2007). It has been suggested that these proteins are activated by ROS through a negative feedback mechanism, however, the exact mechanisms still need further investigation (Mailloux and Harper 2011).

The "uncoupling to survive" hypothesis suggests that part of the mitochondrial proton gradient used to drive ATP synthesis is leaked back into the mitochondrial matrix and

reduced mitochondrial ROS emission (Brand 2000). O_2 consumption in the absence of phosphorylation may be used as an indirect measure of proton leak activity. This is referred to as LEAK respiration (Pesta and Gnaiger 2012). During basal respiration, uncoupling accounts for 20-50% of energy consumption by the mitochondria in normal cellular metabolism (Rolfe and Brand 1996, Rolfe, Newman et al. 1999, Harper, Green et al. 2008). What appears to be a "futile" cycle of proton leak is in fact a very useful biochemical process that is induced to abate ROS formation. This is achieved by maintaining the mitochondrial inner membrane in a less polarized state.

ROS production is extremely sensitive to the protonmotive force set up across the inner membrane by electron transport (Korshunov, Skulachev et al. 1997, Starkov and Fiskum 2003). Small increases in membrane potential induce ROS production and even a slight decrease in ΔP can diminish ROS formation. Mild regulated uncoupling caused by activation of UCP2 or UCP3 can reduce the protonmotive force slightly, attenuate ROS production and protect against ROS related cellular damage without any great inhibition of oxidative phosphorylation (Arsenijevic, Onuma et al. 2000, BRAND, Pamplona et al. 2002, Brand and Esteves 2005). During fatty acid oxidation, superoxide production from the electron transport chain is high (St-Pierre, Buckingham et al. 2002). The activation of UCP2 and UCP3 is mediated by the activation of n-6-polyunsaturated fatty acyl chains of phospholipids, chiefly arachidonic acid chains by superoxide or hydroxyl radicals to form carbon centred fatty acyl radicals. Subsequent oxidation of carbon centred radicals to peroxyl radicals leads to the production of 4-hydroxynonenal and other reactive alkenals. These alkenals activate the proton conductance of UCPs, lowering the protonmotove force and attenuating the original superoxide production (Echtay, Murphy et al. 2002, Echtay, Roussel et al. 2002, Murphy, Echtay et al. 2003, Brand, Affourtit et al. 2004).

The role of ROS production in human physiology is not well understood. In theory ROS should have an important role given the physiological changes that occur during bed rest. The decrease in maximal oxygen consumption, skeletal muscle fibre size and density, bone mass and insulin sensitivity suggest a role for mitochondrial ROS production. It is not clear whether this is the result of a decrease in mitochondrial mass, efficiency or function but it is important that the role of ROS production in the development of metabolic dysfunction with physical inactivity is investigated.

2.10 Conclusion

Reduction in exercise capacity with confinement and bed rest is well established. Reduction in plasma volume reduces cardiac filling pressure, stroke volume and cardiac output. Skeletal muscle fibre size and density are reduced, as is bone mass resulting in profound changes in functional capacity. Bed rest also causes metabolic alterations, inducing insulin resistance, decreased fat oxidation, ectopic fat storage all lending to overall metabolic dysfunction. The role of mitochondria and production of ROS in the development of metabolic dysfunction with physical inactivity is under investigation. There is also a high level of interaction between the pathways involved in muscle atrophy, insulin resistance and mitochondrial function. Having a better understanding of the mechanisms involved in these pathways and how they interact will allow us to develop appropriate countermeasures to counteract the negative impact of physical inactivity.

Chapter III

Methodology

3.1 Experimental Design

3.1.1 Overview

In order to address the issue of reduced physical activity, we participated in a European Space Agency (ESA) funded bed rest study. Bed rest studies serve as an important platform in order to prepare for future human exploration in space. Additionally, they provide us with a useful experimental design to study human physiology in the setting of extreme physical inactivity. This study was a randomized, crossover design which was conducted at the MEDES clinic, Toulouse, France from June 2012 to December 2013. A total of 9 scientific teams were involved in the study, each with their own experimental protocol. Additionally, bed rest core data were obtained. These included a selection of standardized measures that are conducted in every bed rest study. A total of 12 healthy male subjects volunteered to participate in the study. Each subject attended the clinic three times for 35 days. Each 35 day period began with a 7 day ambulatory control period (BDC: Baseline data collection). During this phase, subjects were allowed to be ambulatory during the day, remaining in the clinic at all times while undergoing baseline evaluation. This was followed by 21 days head-down tilt bed rest (-6 ˚) (HDT: head down tilt). Preventative methods, known as countermeasures have been developed to eliminate the deconditioning associated with prolonged physical inactivity. During each HDT period, subjects were allocated to one of three groups:

- Control group (CONT) which underwent bed rest only
- Resistive vibration exercise (RVE) group
- Nutrition and exercise group (NEX)

Throughout bed rest, all activities of daily living including eating, body weight measurement, energy expenditure and hygienic procedures were conducted in bed. For any measurements that were carried out in another part of the clinic, subjects were transported in their beds remaining in the head down tilt position at all times. Subjects were permitted to change position in the horizontal plane once one shoulder remained in contact with the mattress. Any muscular activity of the lower body was prohibited. Passive physical therapy was applied every 3-4 days. Adherence to all study rules were

controlled by the study nurse manager. Additionally, compliance with the bed rest study requirements were monitored with 24 hour video surveillance using random real-time control. Following the bed rest and countermeasure period, subjects were required to remain in the clinic for assessment and recovery for a further 7 days. All participants were asked to return to MEDES 14 and 28 days after each period of bed rest for follow up visits.

Table 3.1 Overview of Data Collection

	BDC: Baseline Data Collection period 7 days of ambulatory controlled conditions for baseline data collection	HDT: Head Down Tilt period 3 weeks (21 days) of continuous head-down tilt bed rest (with -6° angle)	R+: Recovery Period of 7 days allowing volunteers to recover after the bed rest period and data collection
	BDC -7 to BDC -1	HDT 1 to HDT 21	R +0 to R +7
	-7 -6 -5 -4 -3 -2 -1	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21	+0 +1 +2 +3 +4 +5 +6 +7
RMR			
DEXA			
Muscle Biopsy			
VO_2 Max			
MVC			
Clamp			
RVE			
NEX			
BIS			

3.1.2 Subject Selection

Following a call for candidates via the internet on the MEDES and ESA sites and by media, 40 subjects were selected based on their application file which comprised of information on the subject's lifestyle, educational and professional experience. They also completed a medical questionnaire including personal and family medical history. The 40 potential candidates underwent a medical examination, a DEXA scan, biological tests and psychological assessment. Finally, 12 men and 2 back up subjects were selected to partake in the bed rest study. An information document was provided to each candidate and they had the opportunity to ask any questions. Once satisfied, they signed the specific Information and Consent Form. These volunteers were then notified of a selection visit at MEDES to check if they did (or did not) meet the requirements of the inclusion and non-inclusion criteria. The study was approved by the appropriate Ethics Committee (Comité de Protection des Personnes / CPP Sud – Ouest Outre-Mer I) and the French Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé).

Inclusion criteria

- Healthy male volunteer, age 20-45, BMI between 20-25 and height between 158-190cm.
- No personal nor family past record of chronic or acute disease or psychological disturbances which could affect the physiological data and/or create a risk for the subject during the experiment,
- Fitness level assessment:
    - if age < 35 years: 35 ml/min./kg < VO2max < 60ml/min./kg,
    - if age > 35 years: 30 ml/min./kg < VO2max < 60ml/min./kg
- Active and free from any orthopedic, musculoskeletal and cardiovascular disorders,
- Non smokers, no alcohol, no drug dependence and no medical treatment,
- Covered by a Social Security system,
- Free of any engagement during the three study planned periods

Exclusion criteria

- Past record of orthostatic intolerance,

- Cardiac rhythm disorders,
- Chronic back pains,
- History of hiatus hernia or gastro-esophageal reflux,
- History of thyroid dysfunction, renal stones, diabetes, migraines,
- Past record of thrombophlebitis, family history of thrombosis or positive response in thrombosis screening procedure,
- Abnormal result for lower limbs echo-doppler,
- History or active claustrophobia,
- History of genetic muscle and bone diseases of any kind,
- Bone mineral density: T-score ≤ -1.5,
- Osteosynthesis material, presence of metallic implants,
- History of knee problems or joint surgery/broken leg
- Poor tolerance to blood sampling,
- Having given blood (more than 8ml/kg) in a period of 8 weeks or less before the start of the experiment,
- Special food diet, vegetarian or vegan,
- History of intolerance to lactose or food allergy (milk proteins...)
- Positive reaction to any of the following tests: HVA IgM (hepatitis A), HBs antigen (hepatitis B), anti-HVC antibodies (hepatitis C), anti-HIV1+2 antibodies,
- Echocardiography: inappropriate thoracic acoustic window,
- Subject already participating or in the exclusion period of a clinical research,
- Refusal to give permission to contact his general practitioner,
- Incarcerated persons,
- Subject who, in the judgment of the investigator, is likely to be non-compliant during the study, or unable to cooperate because of a language problem or poor mental development,
- Subject who has received more than 4500 Euros within 12 months for being a research subject.
- Subject under guardianship or trusteeship.

3.1.3 Randomization

Each volunteer was randomly assigned to one of three groups during the first and second bed rest campaign and were assigned to the third group for the final and third bed rest period. The order of sequence was random. The wash out period in between the bed rest campaigns was for a minimum duration of 120 days to allow subjects to return to pre bed rest physical status.

Table 3.2 Countermeasure group assignment

Subject	Campaign 1	Campaign 2	Campaign 3
A	RVE	NEX	CONT
B	CONT	RVE	NEX
C	RVE	NEX	CONT
D	NEX	CONT	RVE
E	NEX	CONT	RVE
G	NEX	CONT	RVE
J	RVE	NEX	CONT
K	NEX	CONT	RVE
	CONT (n=8)	*RVE (n=8)*	*NEX (n=8)*

Campaign 2 – I: withdrawal on BDC-1 (C2)
Campaign 3 – F: withdrawal on BDC-6 (C3)
H: withdrawal of C3 before the beginning of the campaign
L: withdrawal on HDT17 (C3)

3.2 Physiological Assessment

3.2.1 Anthropometrics

Height (m) was measured to the nearest centimeter using a stadiometer and body mass (kg) was measured to the nearest 0.1 kg at baseline and the first thing every morning during bedrest on a weighing trolley. This allowed them to roll from the bed to the weighing scales.

3.2.2 Body Composition

There were two methods applied in order to obtain body composition measurements. Subjects had DEXA scan for body composition at 5 time points throughout each study campaign (BDC-7, HDT1, HDT10, HDT20 and R+3). They also had body composition measurement using bioelectrical impedance spectroscopy (BIS) every second morning during bedrest under fasting conditions. The purpose of the BIS analysis was to closely monitor body fat mass with the aim of avoiding any major changes by adjusting the diet accordingly.

3.2.3 Dual Energy X-Ray Absorptiometry (DEXA)

The measurement of body composition using DEXA is achieved from the differential absorption of x-rays of two different intensities. This calculation allows for the quantification of overlying soft tissue. It is possible to obtain values for fat and fat free mass using a whole body scan and using instrument specific algorithms (Wells and Fewtrell 2006). DEXA was used for the purpose of analysing body composition on days -7, 1, 10 and day 20 using the Hologic DXA, QDR4500C, USA. Positioning of all subjects on the scanner platform were in accordance with the manufacturers guidelines. For the total body scan, subjects were centred and squared in the middle of the table with ankles and knees taped together and the scanner laser light was positioned 3cm above the subjects head. The scanner arm moved down over the subject's body obtaining sliced images. Fat mass and fat free mass were calculated. The radiation exposure is extremely low (0.001mSv) (www.radiologyinfo.org).

3.2.4 Bio Impedance analysis (BIA)

Body composition was analyzed using bioelectric impedance spectroscopy (BIS) with the bioimpedance device SFB7 (Impedimed®) every second morning throughout the bed rest study. The BIS measurement took approximately 5 minutes per subject per morning. The device utilizes Cole modeling with Hanai mixture theory to determine total body water (TBW), extracellular fluid (ECF) and intracellular fluid (ICF) from impedance data. Fat-free mass (FFM) and fat mass (FM) are then calculated on the device. Further data analysis can be undertaken using the supporting software. Therefore, no population specific prediction equations (algorithms) were required for data analysis.

3.2.5 Resting Metabolic Rate (RMR)

Open circuit indirect calorimetry was conducted using the Deltatrac® system on days -7, 1, 5, 10, 15 and 20 for the measurement of resting metabolic rate (RMR). Upon wakening, a plastic hood with two outlets was placed over the subjects head while they remained in the lying position. The canopy consists of an inlet and an outlet valve. The subject was supplied with room air via the inlet valve at a rate of 40 L/min, while extracts of the expired air was obtained via the outlet valve. Both valves were connected to the calorimeter (Deltatrac II) via tubes. The Deltatrac II records the oxygen consumption (VO_2) and carbon dioxide production (VCO_2) from the inlet and outlet gas samples of the valves and calculates the oxygen consumption (VO_2) and carbon dioxide production (VCO_2) in ml/min by differentiation. Values for all parameters were averaged over 1 minute intervals. Subsequently, these values were used to estimate RMR using the Weir equation (Weir 1949).

3.2.6 Aerobic Fitness Assessment

The assessment of aerobic capacity was performed on a cycle ergometer in upright position at selection and before and after each bed rest period. The subjects cycled for 3 minutes at 50, 100 and 150 W each followed by an increase of 25 W every 1 minute until peak exertion was reached. The exercise test took approximately 12-16 minutes to complete. Following this test, the subject pedalled the cycle ergometer at a low work rate (~25 W) in order to allow time for adequate recovery from maximal exertion. Heart

rate and blood pressure were monitored continuously. Subjects wore a nose clip and were required to breathe through a respiratory valve that was connected via respiratory tubing to the metabolic cart (Oxycon Pro ™, Jegger™) for gas exchange determination in the breath by breath mode. VO_2, VCO_2 and VE were measured continuously with a metabolic cart in breath by breath mode. Arterial blood pressure was measured continuously with Finapres, Portapres or equivalent device. Percent oxygen saturation (SaO_2) was measured continuously with fingertip infrared photometry. The test continued until volitional fatigue was achieved. Oxygen uptake was considered maximal if two the following criteria were achieved: a levelling off of VO_2 max, maximal heart rate (HR) achieved was within 10 beats of age predicted HR max and /or if respiratory exchange ratio was >1.1.

3.2.7 Maximal Isometric Voluntary Contraction

Maximal isometric voluntary contraction (MVC) of four muscle groups; the knee extensor and flexors and plantar flexors and dorsiflexors was recorded before and after each bed rest period. A familiarization session was conducted in order to familiarize the subjects with the test equipment and the test performance. The CON-TREX isokinetic dynamometer (Medimex, France) was used for this measurement. For all tests, the dominant limb was used. The isometric knee extension and flexion were performed with the subject in a sitting position and in a prone position for ankle extension and flexion. The subject was strapped firmly to the examining chair or bed when isometric MVC was being conducted. The test protocol was similar for each muscle group: after a short warm-up phase, in neutral position, the subject was required to perform a maximum extension followed by maximal flexion with 30 seconds break in between. Each contraction lasted 5-7 seconds. Three sets of extension/flexion were recorded with a two minute rest period between each set. The total duration of the isometric MVC was 15 minutes per pair of agonist/antagonist muscle group. The measured parameters are the maximal isometric torque (units = Nm) for extension and flexion for the different tested muscle groups.

3.2.8 Hyperinsulinaemic Euglycaemic Glucose Clamp

The hyperinsulinaemic euglycaemic glucose clamp was measured at baseline before the first bed rest campaign. The clamp was then performed after each bed rest campaign. Therefore, only one pre clamp measurement was made and this was used to compare to three post clamps. We understand that this imposes a limitation, however, this was beyond our control. The evening prior to the clamp, the subjects received a standard dinner at approximately 7:00pm. The subjects remained fasting until the clamp was complete at 4:00pm the following day.

The clamp consisted of three phases:

- Baseline period (no insulin infusion, baseline blood glucose measurements, t=-30 to -1 minute, approximately 7.30 – 8.00am)
- Insulin infusion step 1 (0.25µU/kg/min, t= 0-240 minutes, approximately 8:01am – 12:00)
- Insulin infusion step 2 (1.0µU/kg/min, t=241 to 480 min, approximately 12:01 – 04:00pm)

The last 60 minutes of the two insulin infusion steps were referred to as steady state phase 1 and steady state phase 2. During the clamp, one hand was placed in a hot box which was warmed to an air temperature of 55˚C. This technique allowed sampling of arterial-like venous blood. Insulin was infused intravenously using a precision pump during blood glucose adjustment phase and during the insulin infusion steps.

For the purpose of this thesis, the hyperinsulinemic euglycaemic clamp was used to measure skeletal muscle insulin sensitivity. However, the clamp technique can also be used to assess antilipolytic action of insulin using a lower insulin infusion. This can be assessed by the reduction of glycerol rate of appearance (GLY_{RA}) from basal to steady state low dose insulin as an index of the suppression of lipolysis. This was the main purpose of stage 1 of the clamp with insulin infusion rates at 0.25µU/kg/min. In stage 2, the insulin infusion was increased to 1 µU/kg/min. The main goal of stage 2 was to

assess skeletal muscle insulin sensitivity. This was achieved by measuring the amount of glucose that was infused in order to maintain circulating plasma glucose at 5 mmol.L.

Baseline period:

The subjects were connected to the Biostator (artificial pancreas, MTB Medizintechnik, Amstetten, Germany). The Biostator performed continuous blood glucose measurements and at the same time controls a variable glucose infusion adjusted to maintain the blood glucose concentration at a constant level. This is a safe method of administering insulin under controlled conditions.

For blood glucose sampling of arterialised venous blood using the Biostator, a forearm vein of one arm was cannulated. This arm remained in the thermoregulated box at a temperature of approximately 55˚C throughout the clamp procedure. The heating of the limb results in an arterialization of the venous blood due to a reflective opening of arterio-venous shunts. A low dose heparin solution (10,000U/100 ml saline) was also infused via a double lumen catheter with the aim of preventing blood clotting in the system. A second cannula was inserted in the forearm vein of the same arm as close to the thermo regulated box as possible for sampling of serial measurements of insulin and blood glucose for safety and calibration of the Biostator. This cannula was kept open by a slow, continuous infusion of 0.9% saline. A forearm vein in the contralateral arm was cannulated with an 18-guage polytetrafluoroethylene (PTFE) catheter for the Biostator's controlled variable infusion of glucose and insulin.

The clamp level was set to a blood glucose level of 100 mg/dl (5.6mmol.L). The Biostator infused 20% glucose solution at a variable rate in order to maintain blood glucose constant at the specified clamp level. The rate of glucose delivery was adjusted by the Biostator in response to changes in blood glucose at one minute intervals for the duration of the clamp. The Biostator automatically calculates the adjustments of the intravenous glucose infusion rate using an algoritim based on the actual measured blood glucose concentration and the grade of variability in the previous 5 minutes. The glucose infusion rate was recorded every minute throughout the clamp. In the case of a high glucose infusion requirement, a part of the glucose infusion pump was taken over by an external pump (infusion-pump Midpress TE*171CW3, Terumo Corporation, Tokyo,

Japan). The glucose infusion rate provided by the external pump was added to the infusion rate of the Biostator.

Blood samples for the measurement of glucose and insulin were drawn at regular intervals. Blood glucose concentrations were measured by the glucose oxidase method, using a glucose analyser (Super GL, Hitado, Mohnesee-Delecke, Germany). The Biostator's blood glucose measurements were compared with the blood glucose measurements from the glucose analyser every 30 minutes throughout the clamp and recalibration was performed as necessary.

Insulin infusion step 1: (0.25µU/kg/min, t=0 min to 240 min)

At t=0 minutes, an intravenous insulin infusion (15 IU Actrapid® (Novo Nordisk Pharma GmbH, Mainz, Germany), 100IU/ml in 49ml saline and 1 ml of the subjects blood) was started at a rate of 0.25 µU/kg/min and maintained at this level for 240 minutes. The last 60 minutes of this period (t = 180-240) was considered steady state period 1. Measurements of blood glucose and insulin concentrations were performed every 10 minutes during the steady state period.

Insulin infusion step 2: (1.0µU/kg/min, t= 241 to 480 min)

At t = 241 min, the insulin infusion was increased to 1µU/kg/min and maintained at this level for another 240 minutes. The last 60 minutes (t = 420 - 480 min) of this period was regarded as steady state period 2. Measurements of blood glucose and insulin concentrations were performed every 10 minutes during the steady state period.

After this phase, insulin infusion was terminated and the clamp was stopped. Subjects were served a meal immediately in order to neutralize any potential carry over effect of the insulin infusion.

Glucose infusion rates required to keep glucose constant at the clamp level of 100mg.dl were recorded throughout the hyperinsulinemic euglycaemic clamp and especially during steady state periods 1 and 2 and were used for the calculation of insulin sensitivity parameters. Total volume of blood sampling per experiment did not exceed 36.3ml.

Insulin sensitivity was calculated over the last 60-mins of each stage by quantifying the total amount of glucose infused (mg) and expressed relative to the circulating insulin concentration (µU/ml) and fat free mass (kgFFM).

3.2.9 Skeletal Muscle Biopsies

Skeletal muscle specimens were obtained by muscle biopsy from the vastus lateralis at 9am while subjects with in the fasted state before and after the 21 day bed rest period (BDC-1 and HDT20). An area of skin was anaesthetised (2% w/v lidocaine HCl) and a small (0.5 cm) incision was made. A sterilised Bergstrom skeletal muscle biopsy needle was inserted into the muscle and approximately 200 mg of tissue were removed while applying suction. Pressure was maintained on the wound for 10 minutes and the incision was closed with steristrip tape and wrapped tightly in a crepe bandage.

3.3 Bed Rest Intervention

Each subject completed each trial over the course of three bed rest campaigns. Regardless of what study trial they were assigned all subjects remained in the -6° HDT position for the entire study duration. The exercise protocol was conducted in the HDT position also.

3.3.1 Resistive vibration exercise protocol

The training was performed two times per week with 3 to 4 days intervals (HDT 2, 5, 12, 16, 21). All training was performed on an integrated training device (Novotec Medical, Pforzheim, Germany). This device is a combination of two systems already used in bed rest studies. The vibration system (Vibration training device including monitoring function and data recording) combined with a system designed to exercise in the -6˚ orientation (cf. photo).

Figure 3.1 Integrated Training Device for Resistive Vibration Exercise

Subjects were told to wear flat soled, non-cushioned training shoes to protect their feet. Subjects underwent two familiarization sessions during the 7 day baseline data collection period with the second familiarization session being scheduled at least 5 days before the onset of bed rest. The first exercise session during the bed rest period was on the second day.

Each session comprised of the following components:

Warm up: Bilateral squats from 10° to 90° knee angle; timing : 8 seconds (4 down, 4 up) controlled by metronome; number of repetitions: 8; load: 50% of the one repetition maximum (1-RM); vibration parameters: 8 mm peak-to-peak amplitude and 24 Hz vibration frequency.

Squatting exercise: Bilateral squats from 10° to 90° knee angle; timing : 8 seconds (4 down, 4 up) controlled by metronome; number of repetitions: 10; load at study commencement: 75% of the 1-RM during study commencement; progression: 5% load increase when more than 10 repetitions are possible; 5% load decrease when 6 or fewer repetitions are possible; vibration parameters: 8 mm peak-to peak and 24 Hz.

Single leg heel raises: From maximal dorsiflexion to maximal plantar flexion; heel contact with footplate is avoided by a bar under the forefoot; timing: as fast as possible; number of repetitions: unto exhaustion; load at study commencement: 1.3 times body weight; progression: 5% load increase when more than 50 seconds are possible, 5% load

decrease when 30 seconds or less are possible; vibration parameters: 8 mm peak to-peak and 26 Hz.

Bilateral heel raises: From maximal dorsiflexion to maximal plantar flexion; heel contact with footplate is avoided by a bar under the forefoot; timing: as fast as possible; number of repetitions: unto exhaustion; load at study commencement: 1.8 times body weight; progression: 5% load increase when more than 55 seconds are possible, 5% load decrease when 40 seconds or less are possible; vibration parameters: 8 mm peak to-peak and 26 Hz.

Table 3.3 Overview: Vibration Exercise Settings MNX Study

Exercise	Range	Load	Cadence	Duration	Vibration	Pause
Bilateral squats (warm-up)	knee angle: 10° to 90°	50% 1-RM	4 sec. up, 4 sec. down	8 repetitions	8 mm*, 25 Hz	120 sec
Squatting exercise	knee angle: 10° to 90°	75% 1-RM	4 sec. up, 4 sec. down	8 repetitions	8 mm*, 25 Hz	300 sec
Single leg heel raises A	Max. dorsiflexion to max plantar flection	1.3 times body weight	As fast as possible (0.4-0.7 Hz)	Unto exhaustion	8 mm*, 25 Hz	90 sec
Single leg heel raises B	Max. dorsiflexion to max plantar flection	1.3 times body weight	As fast as possible	Unto exhaustion	8 mm*, 25 Hz	240 sec
Double leg heel raises	Max. dorsiflexion to max plantar flection	1.8 times body weight	As fast as possible	Unto exhaustion	8 mm*, 25 Hz	120 sec
Toe raises	Maximal dorsiflexion	1.5 times body weight	-	60 sec	8 mm*, 25 Hz	-

3.3.2 Nutritional Supplementation

For all ESA bed rest studies, the aim is to maintain fat mass while accepting the fat free mass is likely to decrease. Fat mass is clamped by ensuring the total energy intake matches energy expenditure. This is achieved by measuring resting metabolic rate and adjusting the diet accordingly. This way, we see no change in fat mass (kg) and a reduction in lean muscle mass (kg). This is important in order to isolate the effects of bed rest and the countermeasures. If fat mass increased, we could not then attribute any changes that we see to bed rest or countermeasures as increased fat mass would be a confounding variable.

Subjects in the exercise and nutrition group (NEX) were given a whey protein supplement in addition to partaking in the resistive exercise vibration protocol (section 3.3.1). An isocaloric supplementation of whey protein (0.6 g/kg body weight/day) was given to the volunteers in the NEX group bringing the total protein intake to 1.8 g/kg body weight/day. Protein supplementation was given every day. On days without exercise, the supplement was applied in equal amounts with main meals. On days with exercise, half of the daily amount was given in a timeframe of 30 minutes after exercise and the other halve equally distributed with main meals. The product was Diaprotein®, a powder supplied by Nephrologische Präparate Dr. Volker Steudle (Linden, Allemagne). The composition was the following:

- Diaprotein® 100 g Powder
- Calorie intake 1573 kJ (370 kcal)
- Proteins 90 g
- Fat 0.2 g
- Lactose 2.5 g
- Sodium < 300 mg
- Potassium < 650 mg
- Calcium < 400 mg
- Phosphorus < 250 mg
- Relation Phosphorus/protein < 3 mg/g.

As one disadvantage of high protein intake along with low dietary alkalinity (characterized by low potassium intake) is that this may exacerbate bone loss. For this reason and since whey protein adds a certain acid load to the diet, supplementation of 90 mmol $KHCO_3$ per day was applied in 6 portions (with main meals and snacks) and compensated for that. Effervescent tablets of potassium bicarbonate were provided by Krüger GmbH & Co (Bergisch Gladbach, Germany).

3.4 Sample Analysis

3.4.1 Muscle Analysis

The muscle biopsy samples were divided into three parts; one was frozen in liquid nitrogen in labelled, punctured eppendorfs about 20 seconds after the sample was obtained. The second part was mounted in tragnum gum and cooled to the temperature of liquid nitrogen in isopentane. Both of these parts were stored at -80 ˚C until analysis. The last part was put straight into a preservation medium, BIOPS solution and was prepared immediately for analysis of mitochondrial function using the High Resolution Respirometer (HRR) O2K Oroboros. Muscle biopsies were performed by Pr. Jacques MERCIER (MD, PhD) a well-trained medical doctor who has several years of experience performing muscle biopsies (and as back-up Dr Maurice HAYOT (MD)).

3.4.2 Fibre preparation

After the muscle biopsy, about 8mg of muscle tissue was immediately placed in a preservation solution, ice-cold BIOPS (in mM; 2.77CaK2 EGTA, 7.23 K2 EGTA, 20 immidazole, 20 taurine, 6.65 MgCl2, 5.77 ATP, 3.95 phosphocreatine, 0.5 dithiothreitol, 50 K-MES, pH 7.1 at 0˚C). After rapid manual separation of the muscle fibres with two very sharp forceps on a petri dish in BIOPS under a microscope, the fibres were quickly placed in a BIOPS solution supplemented with 50ul.ml saponin and gently agitated at 4˚C for 30 minutes. Saponin specifically removes the sarcolemma and leaves the intracellular structures intact (Veksler, Kuznetsov et al. 1987, Kuznetsov 2004). The fibres were then washed in respiration medium, MiR05 (Kuznetsov, Veksler et al. 2008, Pesta and Gnaiger 2012). Fibres were blotted, weighed and immediately placed in the

two chambers (OROBOROS, Oxygraph-2k, Innsbruck, Austria; Gnaiger 2008) for respirometric measurements.

3.4.3 Mitochondrial Respiration Experimental Protocols

Respiration was measured at 37 ˚C with approximately 3-5mg of permeabilized muscle fibres in each chamber containing 2 mls MiR05. The software DatLab (Oroboros, Innsbruck, Austria) was used for data acquisition at 2 second time intervals (Gnaiger 2004). Two substrate-uncoupler-inhibitor-titration (SUIT) protocols for high resolution respirometry were applied following stabilization, carbohydrate (CHO) SUIT and fatty acid (FA) SUIT:

In the CHO SUIT (Chamber A), substrate combinations were used for electron transport through complex (CI) and complex II (CII) of the electron transport chain. Following the stabilization of ROUTINE respiration, LEAK respiration was measured in the presence of pyruvate and malate (PM) and in the absence of adenosine diphosphate (ADP) or Cytochrome C (CytC). LEAK respiration refers to the proton flux through the inner mitochondrial membrane. It is mainly controlled by and compensating for the proton leak across the inner mitochondrial membrane. OXPHOS was then measured with the addition of ADP and refers to the oxidative phosphorylation capacity at saturating ADP concentrations and it is the measure of the oxygen consumption coupled to the phosphorylation of ADP to ATP. The addition of Cytochrome C confirmed the integrity of the outer mitochondrial membrane (Kuznetsov 2004). Glutamate was then added as an additional substrate to generate NADH (PMG). Uncoupler titrations were performed in the ADP activated state, to achieve Complex I linked electron transport system (ETS) capacity (non-coupled) (Pesta and Gnaiger 2012). Maximal ETS capacity with convergent electrons flow from complex I and II was reached after addition of succinate (PMGS) (Complex I & II linked ETS capacity). Then, CI was inhibited by the titration of rotenone (R) to measure respiration through CII only (S(Rot)). Malonic acid (Mna) and Antimycin A (Ama) were then titrated to inhibit complex II and III, respectively. Residual oxygen consumption (ROX) remains even after the inhibition of the ETS. All mitochondrial respiratory states were corrected for ROX which accounts for non-mitochondrial cellular oxygen consuming processes (Gnaiger 2008). In the fatty acid SUIT, LEAK respiration was measured by the addition of palmitate and malate (PalM) to the chamber A. This was

followed by ADP (OXPHOS state) and Octanoylcarnitine (PalMOct) all feeding electrons into the electron transport via CI and electron transferring flavoprotein (ETF). The experiment proceeded with sequential titrations of the carbohydrate protocol (Lemieux, Semsroth et al. 2011).

Table 3.4 Carbohydrate (CHO) and fatty acid (FA) substrate uncoupler inhibitor titration protocols for the measurement of high resolution respirometry.

FATTY ACID SUIT

PRODUCTS	*(stock solution) M*	*(chambre) mM*	*Syringe Volume (µL)*
Palmitoyl-carnitine	0,01	0,04	8
Malate	0,8	5	12,5
ADP	0,5	2,5	10
Cytochrome c	0,004	0,01	5
Octanoyl carnitine	0,1	0,2	4
Pyruvate	2	5	5
Glutamate	2	10	10
FCCP	0,001	0,001	2
Succinate	1	10	20
Rotenone	0,001	0,005	10
Malonate	2	5	5
Antimycine A	0,005	0,0025	1

CARBOHYDRATE SUIT

PRODUCTS	*(stock solution) M*	*(chambre) mM*	*Syringe Volume (µL)*
Pyruvate	2	5	5
Malate	0,8	5	12,5
ADP	0,5	2,5	10
Glutamate	2	10	10
FCCP	0,001	0,001	2
Succinate	1	10	20
Rotenone	0,001	0,005	10
Malonate	2	5	5
Antimycine A	0,005	0,0025	1
Ascorbate	0,8	2	5
TMPD	0,2	0,5	5

3.4.4 Protein Extraction

Approximately 25 – 30 mg of crude muscle was placed in a freeze dryer overnight (Alpha 1-2 LD plus, Fischer Bioblock Scientific) in order to remove excess moisture. The samples were then dissected using two forceps under a microscope to remove any connective tissue and blood. The remaining sample was weighed and homogenized in 100ul of ice cold homogenization buffer (Okadiac buffer, protease inhibitor, DTT, sodium orthovanadate, PMSF) per 1 mg of tissue for the determination of protein content. Using a hand held homogenizer, the sample was mixed for approximately 20 seconds. Samples were placed on ice for 15 minutes and centrifuged (14,000 g for 10 minutes at 4˚C) and protein concentration of the supernatant was determined using the Bradford method (Bradford 1976).

3.4.5 Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

An aliquot of muscle homogenate (40ug of protein) was mixed with Laemmli buffer containing β – mercaptoethanol and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 40 µg of protein for each sample was loaded onto the gel. Proteins were separated by SDS-PAGE 4-20% precast gels and a horizontal gel box by running for approximately 1 hour 20 minutes at 160Volts in running buffer. Molecular weight (MW) marker was used to define the size of the proteins. On completion of the separation of proteins using SDS-PAGE, proteins were then transferred to a solid support membrane made up of polyvinylidene difluoride (PVDF) on the Iblot (iBlot ^{TM} Device, Thermo Fisher Scientific). Blotting makes it possible to detect the proteins on the membrane using specific antibodies. The proteins transferred from the gel are immobilized at their respective relative migration positions at the time point when the electric current of the gel run was stopped. Membranes were soaked in ponceau, cut and blocked with Tris- buffered saline (TBS) (pH 7.6) with 0.1% Tween (TBS-t) containing 5% non-fat dried milk protein for 2 hours at room temperature. Membranes were incubated overnight at 4˚C with primary antibodies for the respective protein targets outlined in the table below. The following morning, membranes were washed in TBS-t and incubated at room temperature with the appropriate secondary antibody for 1.5 hours. Immunoreactive proteins were detected

in a dark room using chemiluminescence (ECL) and subsequently quantified by densitometry. Targeted antibodies are listed below.

Table 3.5 Antibodies directed against target proteins for immunoblot assay

Target	Supplier	Product	Concentration
Cox IV	Cell Signaling Technology	4844	1:1000
COX5a	Abcam	ab110262	1:1000
SDHa	Cell Signaling Technology	5839	1:1000
GapDH	Santa Cruz	Sc-25778	1:1000
OXPHOS Antibody Cocktail	Abcam	Ab110411	1:1000
FOX01	Cell Signaling Technology	9454	1:1000
Akt	Cell Signaling Technology	9272	1:1000

3.4.6 Citrate synthase

Sample Preparation

5mg wet weight muscle tissue was added to a cryovial with 100 µl cell lytic ™ M Cell Lysis Reagent and homogenized using a hand held homogenizer. After leaving the homogenized sample on ice for 10 minutes, the sample was centrifuged at 14000 g for 10 minutes. The supernatant was placed in a new eppendorf and protein assay was completed.

Citrate synthase assay

Citrate synthase is the initial enzyme of the Citric Acid Cycle. The enzyme catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid (Morgunov and Srere 1998). Citrate synthase activity was assayed by following the rate of change in absorbance (A_{412}/minute). The assay medium contained assay buffer, acetyl

CoA and 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB). Protein was added to the mastermix (2 mg/ml), vortexed and 190 µl of the solution was added to the plate. The plate was read on a plate reader with a kinetic function at 412 nm. The plate was read at baseline for 1.5 minutes. 10 µl of 10 mM oxaloacetate was quickly added in order to start the reaction. The absorbance of the reaction was read for 1.5 minutes in order to measure total activity.

The activity of the sample was calculated as follows:

$$Units\ (\mu mole.ml^{-1}.min^{-1}) = {(\Delta A_{412}).min^{-1} \times V.dil}\Big/{\varepsilon^{mM} \times L \times V_{enz}}$$

Equation 3.2

(ΔA_{412})/min = 90sec- 30sec plus 60sec-0sec.
V (ml) = for assay in 96 well plate = 0.2ml
V_{enz} (ml) = Vol of enzyme in sample
ε^{mM} (mM^{-1} cm^{-1}) - the extinction of coefficient of TNB at 412nm is 13.6
L (cm) = pathlength for absorbance = 0.552
dil = dilution factor of original sample

3.4.7 Statistical Analysis

All data are presented as mean ± standard error (SE). All measurements obtained for high resolution respirometry were normalized for the wet weight of the muscle fibres and for wet weight plus citrate synthase. All western blot analysis were normalized for a loading control, GAPDH. Two-way (trial x time) repeated measures ANOVA with pair-wise comparisons was used to determine differences between the three groups for variables with serial measurements. Where a main effect was found, the student Newman-Keuls post-hoc test was used to determine where the difference existed. Where a significant finding was reported but no group X time interaction, we reported an effect of time, combining all pre bed rest groups and all post bed rest groups.

Assuming a power of 80% and an α level of 5%, the sample size calculation revealed that n=7 subjects have to complete the study in order to obtain significant results. Assuming a certain dropout rate, a sample size of n=12 should suffice in order to obtain significant findings for primary outcomes.

3.5 Cell Culture Techniques

3.5.1 The Use of a Laminar Air Flow Cabinet in Cell Culture

All cell culture work was conducted in a class II Laminar Airflow Cabinet (Holen Laminar Air). Before and after use, the laminar airflow cabinet was cleaned with 70% industrial methylated spirits (IMS). All items introduced to the laminar air-flow cabinet were also swabbed with 70% IMS. Only one cell line was used in the laminar air-flow cabinet at a time and upon completion there was a 15 minute period to allow the laminar air-flow cabinet to clear. This was to eliminate any potential for cross contamination between cell lines. All laminar air flow cabinets were cleaned once a week with industrial disinfectant, Virkon (Antech International, P0550). All cells were incubated at 37˚C. Cells were fed fresh media or sub-cultured as necessary in order to maintain active cell growth. C2C12 mouse muscle myoblasts and myotubes (ATCC, Manassas, VA) were used in this body of work. C2C12 cells are all anchorage-dependent cell lines. Media was made up using Dulbecco's Modified Eagle's medium (DMEM) containing 25 mM glucose and with the addition of 50 ml foetal calf serum (10% v/v FCS) and L-glutamine under sterile conditions and stored at 4˚C.

3.5.2 Sub Culturing Of Cells

The cell culture medium was removed from the tissue culture flask and discarded in to sterile waste bottle. The flask was then rinsed with 1 ml Trypsin/EDTA solution (0.25% Trypsin (Gibco, 043-05090), 0.01% EDTA (Sigma E9884) solution in PBS (Oxoid, BRI4a)). This was to ensure any residual media was removed. Trypsin was then added to the flask and incubated at 37˚C for 5-10 minutes until all cells were detached from the surface of the flask. The volume of trypsin varied depending on the volume of flask i.e., 1 mL for T25, 2 mL for T75. The trypsin was deactivated by adding the same volume of complete media to the flask. The cell suspension was then removed and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 1000 rpm for 5 minutes. The supernatant was subsequently discarded and the pellet was suspended in complete medium. A cell count was performed and an aliquot of cells was used to re-seed a flask at the required density.

3.5.3 Assessment of Cell Number and Viability

Cells were trypsinized, pelleted and resuspended in media. A drop from the cell suspension was applied to a chamber of a plastic coverslip enclosed haemocytometer. Cells were counted in 16 squares four times. The average cell number per 16 squares was then multiplied by a factor of 10^4 and the relevant dilution factor to determine the number of cells per mL in the original cell suspension.

3.5.4 Cryopreservation of Cells

Cells for cryopreservation were pelleted and re-suspended in a suitable volume of foetal calf serum (FCS). An equal volume of 10% Dimethylsulphoxide (DMSO)/FCS solution was added dropwise to the cell suspension. The cell suspension was aliquoted into 1 mL volumes in cryovials (Greiner, 122278) and immediately placed in the vapour phase of a liquid nitrogen container (-80˚C). After a period of 3 hours, vials were removed from the vapour phase and transferred to the liquid phase (-196˚C) for long term storage.

3.5.5 Thawing of Cryopreserved Cells

5 mL of freshly warmed growth media was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen and thawed quickly at 37˚C. The cells were removed from the vial and placed in appropriately sized tissue culture flask with a suitable volume of growth media and allowed to attach overnight. The following day, flasks were fed with fresh media in order to remove any non-viable cells.

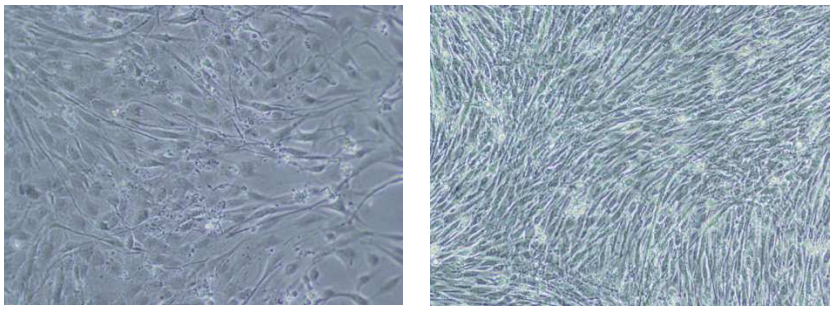
3.5.6 Monitoring of sterility of cell culture solutions

Sterility testing was performed for all cell culture media and all cell culture related solutions. Samples of prepared basal media were incubated at 37˚C for a period of seven days to ensure that there was no bacterial or fungal contamination present in the media.

3.6 Cell Culture Experimental Procedures

3.6.1 C2C12 cell differentiation

Mouse C2C12 myoblast were maintained in DMEM supplemented with 10% FCS. When cells reached confluence, the medium was changed to a differentiation medium containing 2% horse serum which was changed every 48 hours. After 4 additional days, the differentiated C2C12 cells had fused into myotubes. The myotubes were then subjected to either palmitate or high glucose and insulin treatment.



C2C12 Mouse Myoblasts C2C12 Mouse Myotubes

Figure 3.2 C2C12 Differentiation

3.6.2 High Insulin and Glucose Treatment

Following differentiation of C2C12 myoblasts into myotubes, cells were treated with 40 mM glucose and 100 nM insulin (high Glu/Ins) for 48 hours.

3.6.3 Fatty Acid Treatment

Following differentiation of C2C12 myoblasts into myotubes, cells were treated with 100nM palmitate (P0500, Sigma) for 24 hours.

Preparation of Palmitate – BSA complex:

100mM sodium palmitate (P0500, Sigma) was prepared by incubation of appropriate amount of palmitic acid (256.42 mg/mL) in 1M NaOH at 70°C for 30 minutes. Solution was then filtered through 0.45 µm synringe filter. Palmitic acid was bound to 5% (w/v) fatty-acid – free bovine serum albumin (BSA) to make a 5 mM FFA/5% BSA solution.

After cooling to room temperature, 4 ml of 5 mM FFA/5% BSA was added to 16 mL 2% FCS DMEM to make a 1 mM FFA/1% BSA solution. This was used to treat the cells.

3.6.4 Mitochondrial Respiration Experimental Protocols

To assess the function of the cells in the palmitate or high insulin/glucose treatments, two cell suspensions with a final concentration of 5 x 10^5 cells per mL of Mir05 were immediately placed in the two chambers (OROBOROS, Oxygraph-2k, Innsbruck, Austria; Gnaiger 2008) for respirometric measurements. Chamber A contained the negative control cells and chamber B contained the treated. Respiration was measured as described in section 3.4.3. One additional measurement that was applied was the estimation of reactive oxygen species (ROS) using the Oroboros Oxygraph O2K. This is described in section 3.6.6.

3.6.5 ROS Generation

Reactive oxygen species (ROS) were measured using two different protocols. ROS are chemically reactive oxygen containing molecules that are generated as a natural by-product of oxygen metabolism. ROS are continuously generated and eliminated under normal physiological conditions and have important function in cell signalling. Under stressful conditions, ROS levels can increase leading to oxidative stress. ROS was therefore measured using the O2k-Fluo LED2-Module which is a component of the O2k-Fluorometer. The DCF-DA (2', 7' – dichlorofluorescein diacetate) assay was also used for the measurement of ROS. In both assays, ROS is measured by hydrogen peroxide fluorescence.

3.6.6 ROS Production (O2K Fluorometry)

The O2k-Fluo LED2-Module is an amperometric add-on module to the O2k-Core, adding a new dimension to HRR. Optical sensors are inserted through the front windows of the O2k-glass chambers with light emitting diode (LED) for green and blue excitation. Hydrogen peroxide (H_2O_2) release (measured as fluorescences/min) was measured in the permeabilized cells with the titration of 10 µM Amplex Red (Molecular Probes) as a trapper of H_2O_2, catalysed by horseradish peroxidase (1 U/mL). H_2O_2 reacts with Amplex red generating a fluorescent compound resorufin which is stable once formed.

Fluorescence was continuously measured with a spectrofluorometer equipped with temperature control and stirring (wavelengths, excitation of 560 nm and emission 590 nm) (SAFAS Xenius, Monaco). Cells were added to the measurement buffer (MiR05) with Amplex Red and horseradish peroxidase and sequential substrate addition was performed as per section 3.6.4.

3.6.7 ROS production (DCF-DA)

Cells were washed twice with 500 µL/well using Hank's Balanced Salt Solution (HBSS) and incubated with DCF-DA [(2', 7' – dichlorofluorescein diacetate)(Invitrogen)] at a final concentration of 10 µM in 500 µL/well at 37 °C, 5% CO_2 incubator for 30 minutes. Following incubation, the dye solution was removed using a Gilson pipette. The cells were then washed twice with HBSS and one replica (untreated and treated) was treated with 100 µM amount H_2O_2. The fluorescent measurement of the oxidized DCFDA dye was measured after 10 minutes at the respective excitation and emission wavelengths of 490 nm and 545 nm in in a dual beam plate reader.

3.6.8 Protein Extraction and SDS PAGE

Cells were washed twice with ice cold PBS. All procedures following were completed on ice. 1 mL of NP-40 lysis buffer was prepared containing 10 µL 100 mM PMSF, 10 µL 100 mM DTT and 40 µL 50X protease inhibitors. 100 - 200 µL of lysis buffer was added to cells and cells were incubated on ice for 30 minutes. After incubation on ice, lysates were centrifuged at 14000 rpm for 15 minutes at 4˚C. Supernatant containing extracted protein was transferred to a fresh chilled eppendorf tube. Protein quantification was determined using the Bradford method (Bradford 1976). Western Blot analysis was conducted as described in section 3.4.5. Targeted antibodies are listed below.

Table 3.6 Antibodies directed against target proteins for immunoblot assay.

Target	Supplier	Product No.	Concentration
COX5a	Abcam	ab110262	1:1000
β Actin	Cell Signalling Technology	4967	1:1000
OXPHOS Antibody Cocktail	Abcam	ab110411	1:1000
UCP3	Abcam	ab10985	1:1000
Akt	Cell Signalling Technology	9272	1:1000
Phospho-Akt (Ser473)	Cell Signalling Technology	9271	1:1000

3.6.9 24 well plate assays – set up

ROS production, changes in membrane potential and mitochondrial content were all measured in cells exposed to high glucose/insulin load and palmitate treatment. For the purpose of these assays, cells were seeds and grown on 24 well plates. C2C12 cells were grown to confluence in 24 well plate in DMEM containing 10% FCS. When cells were confluent, the media was switched to differentiating media containing 2% horse serum and changed after 24hours. At 48 hours, the cells had differentiated into C2C12 myotubes and were ready to treat. Cells were treated with either 100 nM palmitate or 40mM glucose and 100nM as previously described. Determination of ROS production, mitochondrial mass and membrane potential was conducted using the protocols described below.

3.6.10 Determination of mitochondrial membrane potential (ΔΨ)

An effect of cell treatments on ΔΨ was estimated fluorimetrically with JC-1 dye (5,5',6,6' – tetrachloro - 1,1',3,3' – tetraethylbenzimodazolcarbocyanine iodide; Molecular Probes, Invitrogen). Myotubes grown in 24 well culture plates were washed twice with HBSS and

then stained with JC-1 dye at a concentration of 200 nM in DMSO and incubated for 30 minutes at 37°C in the dark. Following incubation, cells were washed again with 500ul HBSS using a Gilson pipette. To assess complete dissipation of ΔΨ, 5 µM FCCP was added to one replica of treated and untreated cells. Fluorescence was measured after 10 minutes at the respective excitation and emission wavelengths of 490 nm and 545nm in in a dual beam plate reader. The treatment layout for DCFDA and JC1 assays are outlined below.

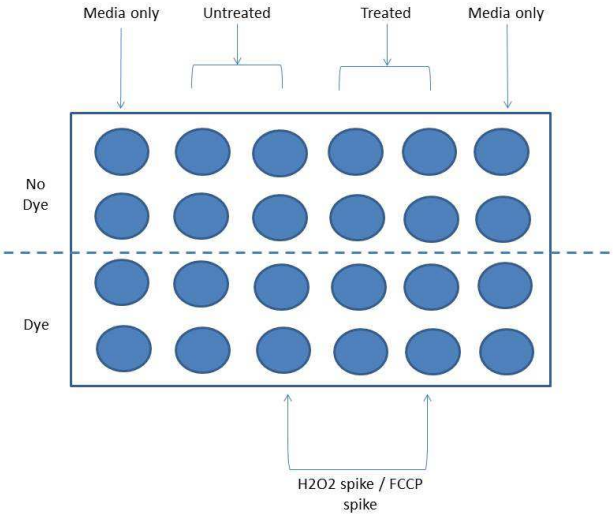


Figure 3.3 24 well plate set up for the determination of ROS production and membrane potential.

3.6.11 Mito Tracker Green

Mito Tracker FM™ (MTG) was obtained from Molecular Probes (Eugene, OR, USE) and dissolved in dimethylsulfoxide (DMSO) at 1mM stock concentration and stored at -20˚C in the dark. To label mitochondria, cells are incubated with MitoTracker probes which passively diffuse across the plasma membrane and accumulate in active mitochondria. Myotubes grown in 24 well culture plates were washed twice with HBSS and then stained with MTG dye at a concentration of 200 nM in DMSO and incubated for 30 minutes at 37°C in the dark. Following incubation, cells were washed again with 500ul HBSS using a Gilson pipette. Fluorescence was measured immediately at the respective

excitation and emission wavelengths of 490 nm and 545nm in in a dual beam plate reader. Images were also obtained using a fluorescent microscope (Leica).

3.7 SiRNA Transfection Experimental Procedure

3.7.1 RNA Interference (RNAi)

RNAi using short interfering RNAs (siRNAs) was carried out to silence specific genes that encode for a protein that may play a key functional role in the electron transport chain (ETC) in the miotochondrial matrix. COX5a is one of thirteen subunits of complex IV of the ETC and we were interested in its role in metabolic function. The siRNA used were chemically synthesized and purchased from Santa Cruz and were introduced to the cell via lyposomes.

3.7.2 Transfection Optimization

In order to determine the optimal conditions for siRNA transfection, an optimization with a siRNA for kinesin (Ambion Inc., 16704) was carried out using C2C12 cell line. Cell suspensions were prepared at 1 x 10^5, 2 x 10^5 and 3 x 10^5 cells per mL. Solutions of negative control and kinesin siRNAs at a final concentration of 30nM were prepared in OptiMEM (GibcoTM, 31985). Lipofectamine solutions were prepared in optiMEM at a range of concentrations and incubated at room temperature for 10 minutes. After incubation, Lipofectamine solutions were added to negative control or kinesin siRNA. The solutions were gently mixed and again incubated for 10 minutes at room temperature. Replicates of the 100ul of the siRNA/Lipofectamine solutions were added to a 6 well plate as follows:

Control →	2ul	3ul	4ul
Kinesin →	2ul	3ul	4ul

Figure 3.4 Transfection experiment plate layout

The cell suspensions were added to the plates at a final concentration of 1 x 10^5, 2 x 10^5 and 3 x 10^5 cells per well. The plates were gently mixed and sealed in parafilm and

incubated at 37˚C for 24 hours. After 24 hours, the transfection mixture was removed from cells and plates were fed with fresh medium. The plates were assayed for proliferation at 96 hours post transfection using the acid phosphatase assay. Optimal conditions for transfection were determined as the combinations of conditions that gave the greatest reduction in cell number after kinesin siRNA transfection. The results of this experiment indicated that the optimal conditions for the delivery of the siRNA transfection experiment were using a cell concentration of $1x10^5$ cells/ml and 2ul of lipofectamine reagent.

3.7.3 Transfection experiments

Using the optimal conditions COX5a siRNA was applied. Solutions of siRNA at a final concentration of 30 nM were prepared in optiMEM (GibcoTM, 31985). Lipofectamine was prepared in optiMEM and was incubated at room temperature for 10 minutes. After incubation, an equal volume of lipofectamine was added to siRNA. These solutions were gently mixed and incubated for another 10 minutes at room temperature. Replicates of 100 µl of siRNA/lipofectamine solution were placed in a 6 well plate. The cell suspension was then added to the plates. After 24 hours, the transfection was removed from the cells and plates were fed with fresh medium. The plates were assayed for changes in proliferation at 96 hours using the acid phosphatase assay. The cells that were subject to COX5a knock down were used for mitochondrial function analysis (section 3.6.4) DCFDA assay (section 3.6.7), JC 1 assay (section 3.6.10) and MTG (section 3.6.11). They also underwent protein extraction and were used for western blot to confirm the knock down of COX5a (section 3.4.5).

3.7.4 Acid Phosphatase assay

Acid phosphatase assay is performed in order to estimate the knock down efficiency of the siRNA. Following an incubation period of 4 days, media was removed from the plates. Each well was rinsed with PBS and 1 mL of freshly prepared phosphatase substrate (10mM p-nitrophenol phosphate (Sigma 104-0) in 0.1M sodium acetate (Sigma, S8625), 0.1% triton X-100 (BDH, 30632), pH 5.5) was added to each well. The plates were incubated for 1-2 hours at 37˚C in a dark room. Colour change was monitored throughout this period. The enzymatic reaction was stopped by the addition

of 1M NaOH. The plate was read in a dual beam plate reader at 405nm with reference wavelength of 620nm.

3.7.5 Statistical Analysis of Cell Culture Experiments

Data presented as mean ± SE. Analysis of the difference in the means was calculated using an unpaired student's t-test. Statistical significance was set at a level of 0.05. All statistical analysis was performed using SigmaPlot12.

Chapter IV

Physiological Response to Bed Rest

4.1 Physiological Measurements

4.1.1 Physical Characteristics of Subjects at Selection

In total, 12 subjects volunteered to partake in the bed rest study. All 12 subjects completed campaign 1. However, subject i withdrew from the study during campaign 2 after the baseline period. During campaign 3, 3 additional volunteers withdrew from the study (F, H and L). Only subjects that completed all three trials were included in the analysis. Therefore, 8 subjects completed the control (CONT), resistive vibration exercise (RVE) protocols and the nutrition and exercise protocol (NEX). The following anthropometric measurements were made at selection and were necessary to ensure that each volunteer satisfied the inclusion criteria.

Table 4.1 Physical Characteristics of Subjects.

Subject	Age	Height (M)	Weight (kg)	BMI (kg/M^2)	VO_2 peak (ml/min/Kg)
A	44	1.75	76.3	24.91	33
B	40	1.69	61.1	21.39	38
C	42	1.77	78.5	25.06	37
D	36	1.90	80.7	22.35	48
E	41	1.72	59.0	19.94	46
G	40	1.77	71.2	22.73	34
J	25	1.74	71.8	23.72	39
K	29	1.84	81.8	24.16	40
Mean ± SE	37.13 ± 2.37	1.77 ± 0.02	72.55 ± 3.04	23.03 ± 0.63	39.38 ± 1.87

Data are presented as mean ± SE.

4.1.2 Body Composition

There was a significant reduction weight and lean muscle mass after bed rest ($p<0.05$). Fat mass did not change with bed rest. Due to the reduction in lean muscle mass, there was a significant increase in percent fat after bed rest ($p<0.05$). An interesting observation was that there was no significant change in leg or arm lean mass suggesting that postural muscles and abdominal muscles may have been targeted by bed rest. Therefore, a reduction in whole body lean muscle mass could be attributed to the loss in muscle mass of abdominal and postural muscles.

Table 4.2 Body composition changes before and after 21 days BR.

	Control (n=8)		RVE (n=8)		NEX (n=8)		
	Pre	Post	Pre	Post	Pre	Post	
Weight (kg)	73.44±3.46	70.48±3.68	73.14±3.37	70.28±3.16	71.83±3.25	70.07±3.06	**
Body Fat (%)	21.01±2.00	21.46±2.19	19.96±1.82	21.09±1.92	19.95±1.73	20.64±1.65	**
WB FFM (kg)	57.79±2.5	55.12±2.69	58.48±2.87	55.36±2.59	57.43±2.70	55.47±2.47	**
WB FM (kg)	15.65±1.97	15.36±2.13	14.66±1.60	14.92±1.64	14.41±1.57	14.61±1.52	
Arm fat (kg)	1.72 ± 0.20	1.86± 0.31	1.66 ±0.19	1.78 ± 0.22	1.68 ± 0.20	1.70 ±0.20	
Arm Lean (kg)	7.16± 0.49	7.21 ± 0.57	7.54 ± 0.57	7.30± 0.58	7.29 ± 0.53	7.16 ± 0.53	
Leg Fat (kg)	5.81 ± 1.07	4.29 ± 0.42	5.53 ± 0.74	3.96 ± 0.33	3.85 ± 0.64	5.65 ± 0.59	
Leg lean (kg)	17.45 ± 0.50	17.50 ± 0.64	17.60 ± 0.87	18.29 ± 0.67	16.48 ± 0.55	19.10 ± 0.40	

Data are presented as mean± SE. WB FFM – whole body fat free mass, WB FM – whole body fat mass. *significantly different compared to pre bed rest for each trial. **significantly different to pre bed best as an effect of time.

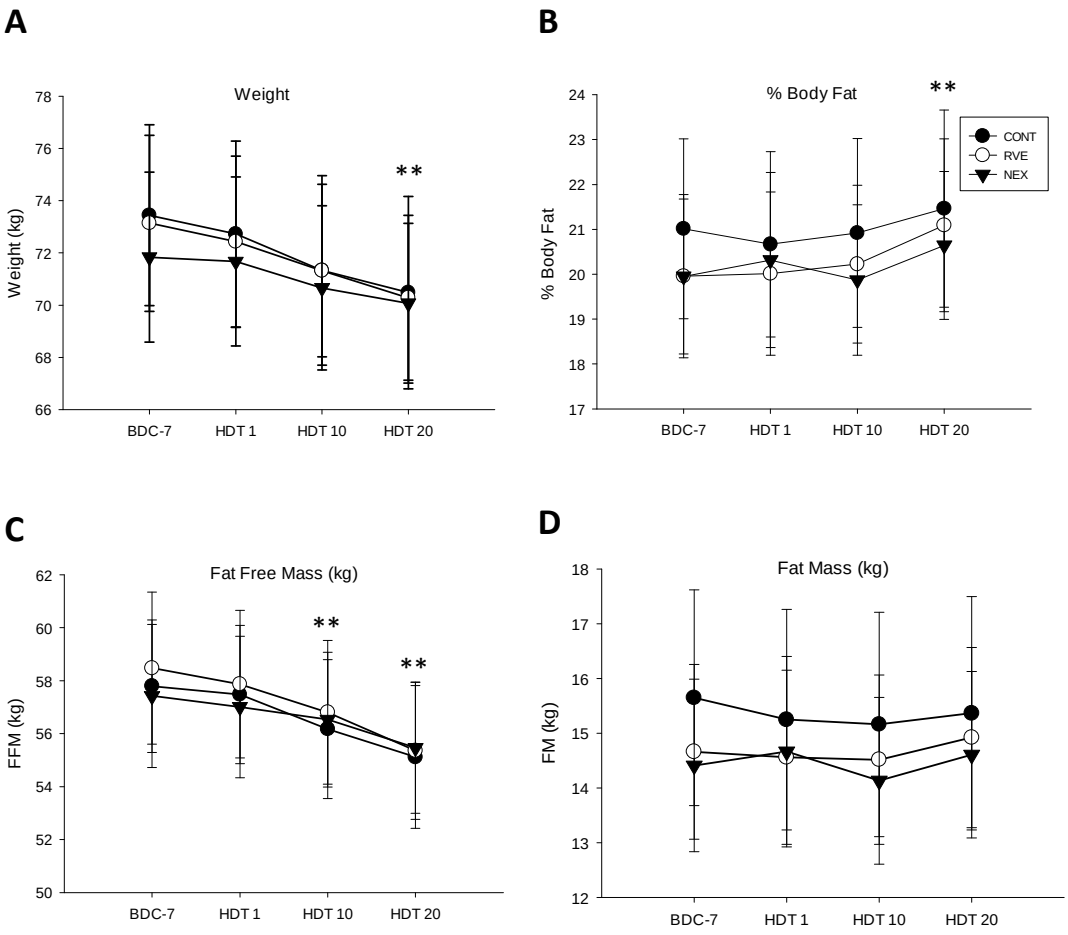


Figure 4.1 Body composition measurements during bed rest using dual-energy x-ray absorptiometry (DEXA). (A) Changes in weight (kg), (B) percent body fat, (C) fat free mass (FFM) (kg) and (D) fat mass (FM) (kg) throughout bed rest measured by DEXA. BDC-1 = baseline data collection at -7 days, HDT 1, 10 and 20 = head down tilt bed rest on day 1, 10 and 20. Data are presented as mean ± SE. **significantly different compared to pre (BDC-7) as an effect of time.

Body composition was also measured every second morning using bioelectrical impedance spectroscopy (BIS). Measured parameters included fat free mass (kg), fat mass (kg) and percent fat throughout bed rest. There was a significant reduction in fat free mass (kg) after bed rest ($p<0.05$). However, there was no difference in fat mass and percent fat mass after bed rest.

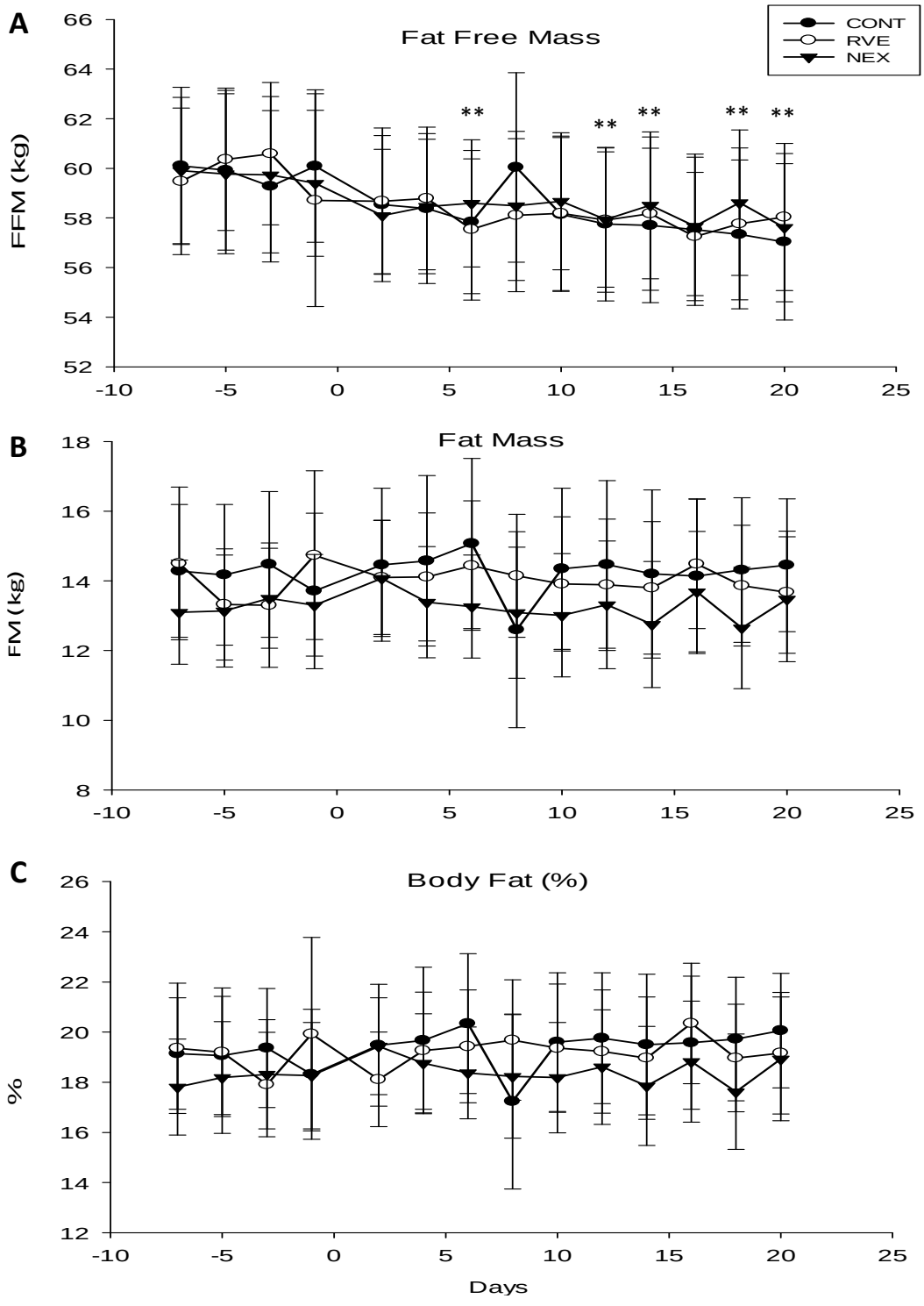


Figure 4.2 Bioelectrical Impedance Spectroscopy (BIS). BIS analysis every second morning for the measurement of (A) Fat Free Mass (FFM) (kg), (B) fat mass (FM) (kg), (C) percent body fat (%). Data are presented as mean ± SE. **significant changes compared to pre as an effect of time.

4.1.2 Resting Energy Metabolism

Resting energy expenditure was measured at six time-points throughout baseline and bed rest in only two groups (CONT and RVE). There was a significant difference between the baseline measurement and all other time-points, including a significant reduction in RMR post bed rest compared to pre ($p<0.05$). When normalized for FFM at four timepoints, there was no change in resting energy expenditure with bed rest. There were no changes noted in substrate utilization (RQ).

Table 4.3 Resting Metabolic Rate and Respiratory Quotient (RQ) before and after bed rest.

	Control		RVE	
	BDC-7	HDT 20	BDC-7	HDT 20
RMR (kcal//min)	6.64±0.30	6.14±0.32**	6.65±0.35	6.26±0.29**
RQ	0.87±0.03	0.86±0.004	0.86±0.01	0.87±0.02

Data are presented as mean ± SE. **significantly different to pre.

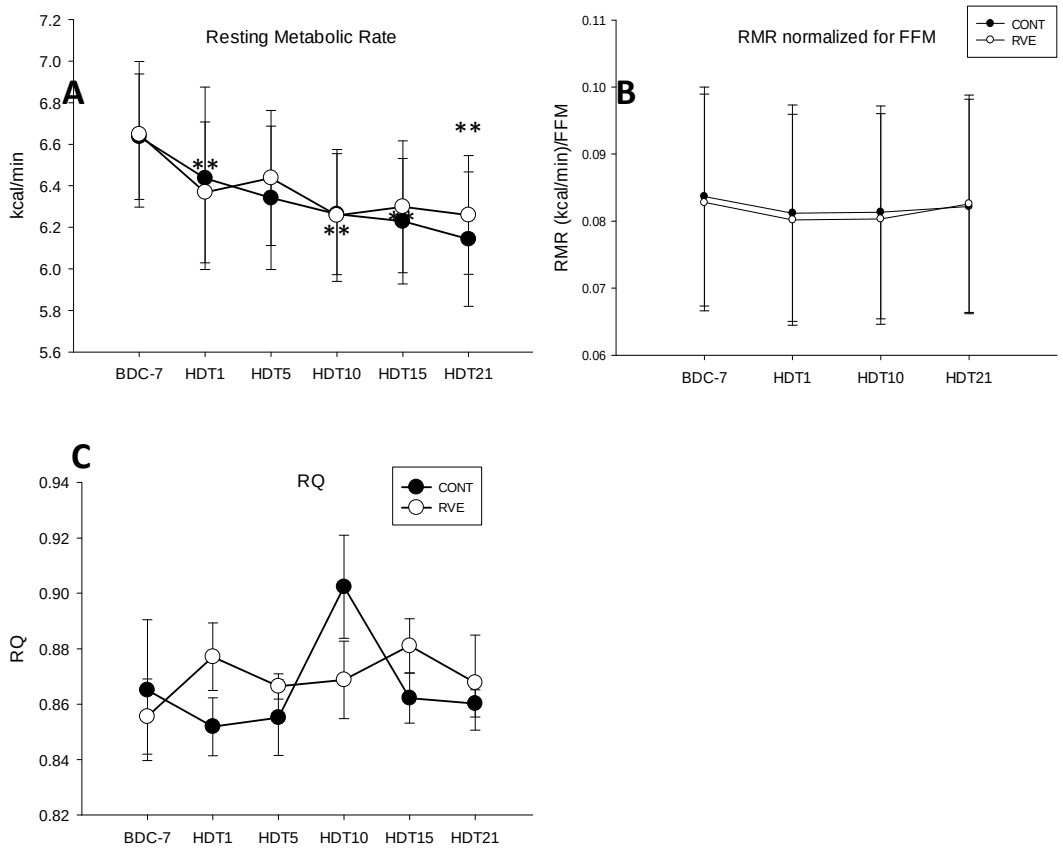


Figure 4.3 (A) Resting metabolic rate (RMR)(kcal/min), (B) RMR (kcal/min/FFM) and (C) substrate utilization (RQ) throughout bed rest. BDC = baseline data collection, HDT= head down tilt. Data are expressed as mean ± SE. **Significantly different to pre.

4.1.3 Aerobic Fitness

A reduction in absolute ($ml \cdot min^{-1}$) and relative ($ml \cdot kg^{-1} \cdot min^{-1}$) VO_2 max following bed rest was observed ($p<0.05$). This decrement was also noted when normalized for fat free mass ($p=0.053$). The deconditioning effect of bed rest resulted in an increase in peak RER ($p<0.05$) and Peak HR ($p<0.05$). At maximal intensity, peak power was lower than before bed rest ($p<0.05$).

Table 4.4 Maximal exercise test parameters pre and post bed rest.

	Control		RVE		NEX		P<0.05
	BDC-5	R+1	BDC-5	R+1	BDC-5	R+1	
Peak HR	181.75±3.45	184±3.27	177±4.51	185.63±3.84	179.75±3.54	186.75±3.35	**
VE (l/min)	123.74±10.27	97.92±7.15	122.67±13.59	115.85±12.03	92.29±11.1	93.88±10.34	
RER	1.29±0.02	1.31±0.02	1.26±0.02	1.26±0.02	1.26±0.02	1.32±0.03	**
VCO2 max (ml/min)	3686±278.18	2931.40±195.45	3631.30±307.03	3363.60±250.74	3419.63±244.59	3323.50±253.99	**
VO2max (ml/min)	2817.10±211.3	2238.10±132.84	2793.80±209.27	2613±185.56	2687.25±193.86	2474.13±208.27	**
VO2 max (ml/min/kg)	38.13±2.17	31.38±1.91	37.88±1.73	36.25±1.60	37.25±2.32	34.38±2.15	**
Power (Watt)	266.25±17.16	205.63±13.11	272.50±25.64	244.38±16.35	247.50±16.93	228.75±16.76	**

Data are presented as mean ± SE. **significantly different to pre bed rest values as an effect of time.

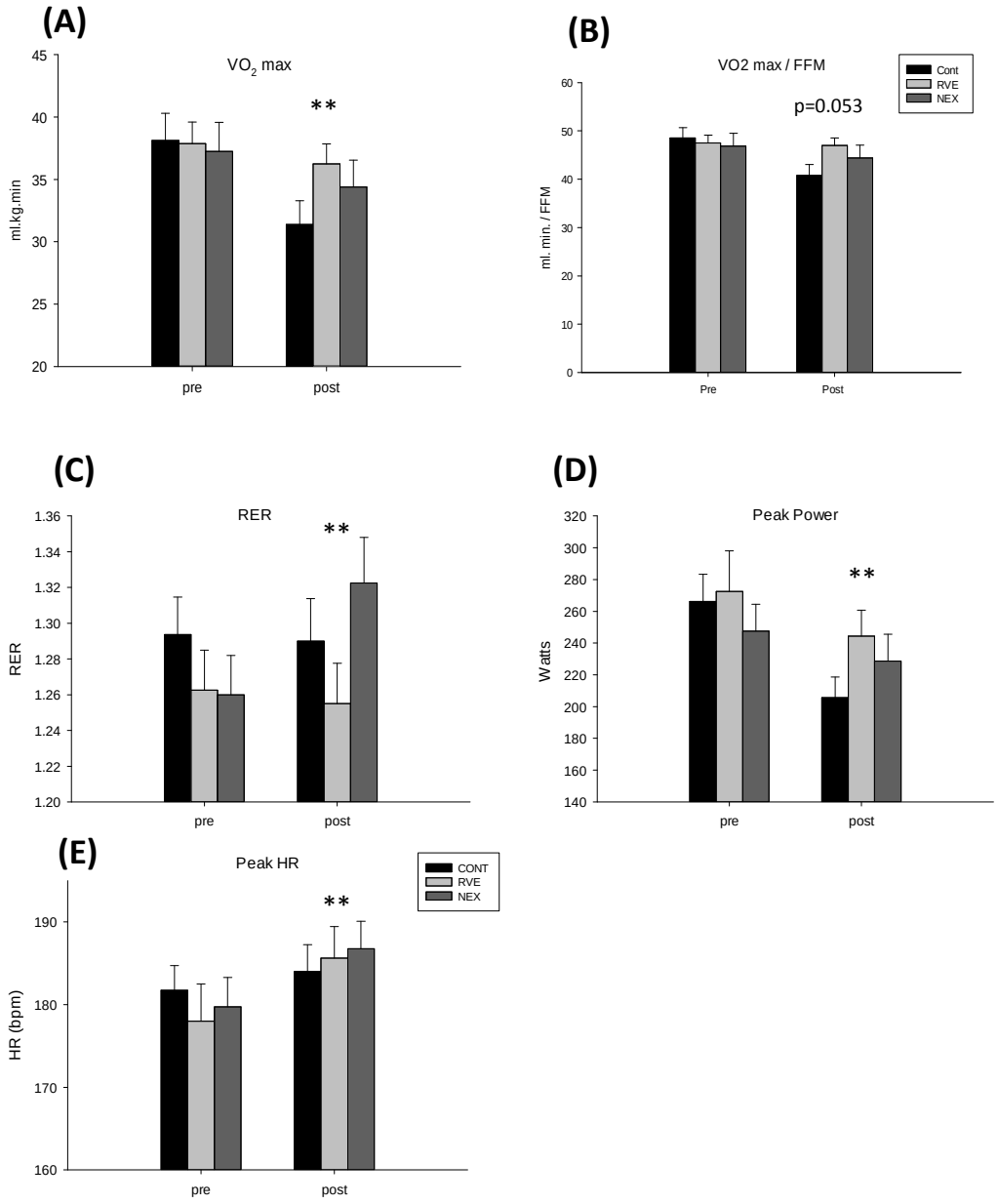


Figure 4.4 Maximal exercise response before and after bed rest. Measured parameters include (A) relative VO_2 max, (B) VO_2 max (relative to fat free mass) (C) respiratory exchange ratio (RER) and (D) peak power (Watts) and (E) peak heart rate (HR). Data are presented as mean ± SE. **significantly different to pre as an effect of time.

4.1.4 Hyperinsulinemic Euglycemic Insulin Clamp

The hyperinsuliInemic euglycemic insulin clamp was completed in two stages. Each stage lasted 4 hours. The last hour of each stage was regarded as steady state. During the first stage, the insulin infusion was 0.25µU/kg/min from t=0 minutes to 240 minutes. During stage one, lower glucose infusion rate was noted in all groups when normalized for fat free mass and insulin ($p<0.05$). In stage two, (241 to 480 minutes) insulin infusion was

increased to 1.0 µU/kg/min. In stage two, reduction in glucose infusion rate was noted in the control trial compared to baseline when normalised for fat free mass and insulin ($p<0.05$). No changes were noted for the RVE or NEX trials in stage two.

Table 4.5 Hyperinsulinemic Euglycemic Insulin Clamp. Stage 1 and 2 glucose infusion per unit of insulin relative to fat free mass.

	M(mg/kgFFM/µU/min)			
	Stage 1		Stage 2	
	Pre	Post	Pre	Post
Control	0.39 ± 0.04	0.25 ± 0.03**	0.27 ± 0.02	0.22 ± 0.01**
RVE	0.39 ± 0.04	0.30 ± 0.04**	0.27 ± 0.02	0.27 ± 0.02
NEX	0.39 ± 0.04	0.33 ± 0.04**	0.27 ± 0.02	0.25 ± 0.02

Data are expressed as mean ± SE. FFM = fat free mass. **significantly different to pre.

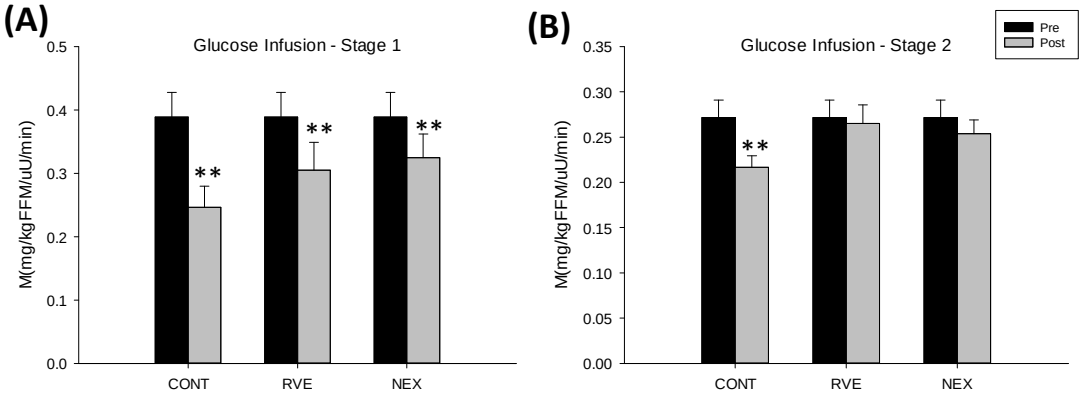


Figure 4.5 Glucose infusion per unit of insulin. (A) stage 1 of the hyperinsulinemic euglycemic insulin clamp relative to fat free mass (FFM) and (B) stage 2 relative to FFM. Data are presented as mean ± SE. **Significantly different to pre as an effect of time.

4.1.5 Isometric Maximum Isometric Voluntary Contraction

Isometric extension and flexion contractions were performed on 4 muscle groups: knee extension and flexion, ankle extension and flexion. There was a significant reduction in knee extension and flexion after bed rest compared to pre bed rest ($p<0.05$). A reduction in ankle extension and flexion post bed rest was also noted ($p<0.05$). When we normalized all values for FFM, no changes were noted, suggesting that the reduction in whole body fat free mass is responsible for the reduction in strength.

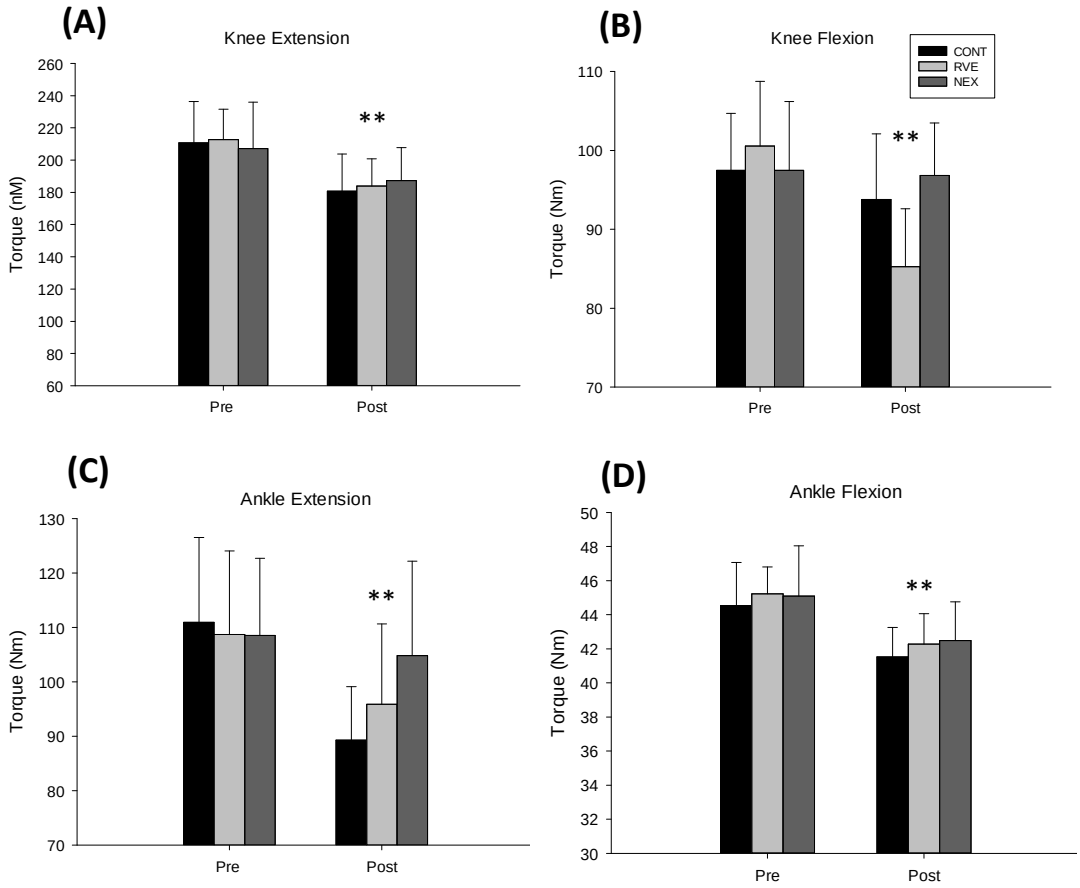


Figure 4.6 Isometric muscle voluntary contraction. (A) knee extension, (B) knee flexion, (C) ankle extension and (D) ankle flexion. Data are expressed as mean ± SE. **significantly different to pre values as an effect of time.

CHAPTER V

Cellular Response

5.1 High Resolution Respirometry

Muscle biopsies were obtained before and after bed rest. Analysis of muscle mitochondrial oxygen consumption was measured immediately in saponin permeabilized muscle fibres (2-4 mgs) by high resolution respirometry at an oxygen concentration of 200 nmol O_2/ml. Samples were analyzed using two different substrate uncoupler inhibitor titration (SUIT) protocols, a carbohydrate (CHO) SUIT and fatty acid (FA) SUIT as described in section 3.4.3. Residual oxygen consumption was measured at the end of each experiment with the addition of malonate and antimycin A, inhibiting complex II and III of the electron transport chain. The oxygen consumption ascribed to the auto oxidation of these substrates was subtracted, hence normalizing for ROX.

Subsequent quantification of citrate synthase (CS) activity was conducted and as a measure of mitochondrial content was used, in addition to ROX to normalize mitochondrial respiration for mitochondrial content. Therefore, absolute respiratory rates (oxygen flux) are expressed as picomoles of oxygen per milligram of wet weight per second (pmol/(s*ml) normalized for ROX and subsequently normalized for CS.

5.1.1 Membrane Integrity

The integrity of the outer mitochondrial membrane was assessed with the addition of 10 mM cytochrome C in the FA SUIT. There was no change in the level of respiration after the addition of cytochrome C (p=0.48 – Pre and p=0.74 - Post) ensuring the permeabilization did not compromise the integrity of the outer mitochondrial membrane.

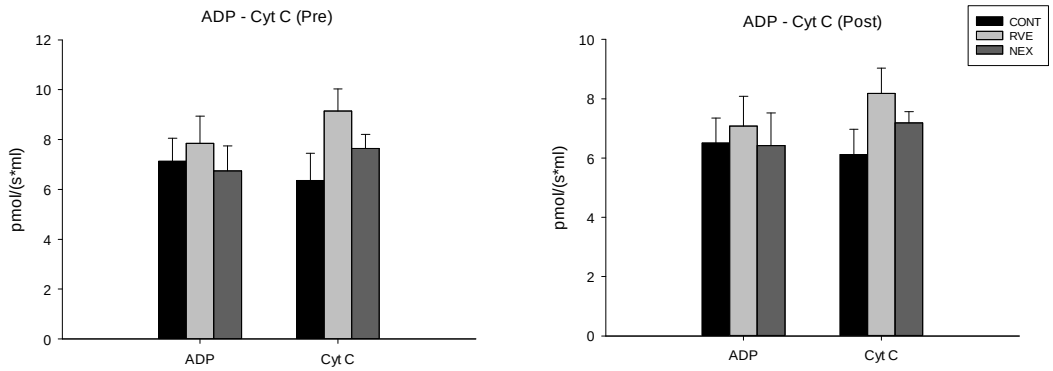


Figure 5.1 Addition of Cytochrome C to ensure mitochondrial membrane integrity. Cytochrome C was added to assess membrane integrity in the FA SUIT. There was no change in the level of respiration after the addition of cytochrome C (p=0.48 – Pre and p=0.74 - Post). Data are presented as mean ± SE

5.1.2 Citrate Synthase Activity

Citrate synthase (CS) is the first enzyme of the tricarboxylic acid (TCA) cycle. It catalyzes the reaction between acetyle Coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid. The activity of CS is an indication of mitochondrial enzyme activity and has been commonly used as a marker of mitochondrial content. There was a significant effect of time on CS after bed rest ($p<0.05$).

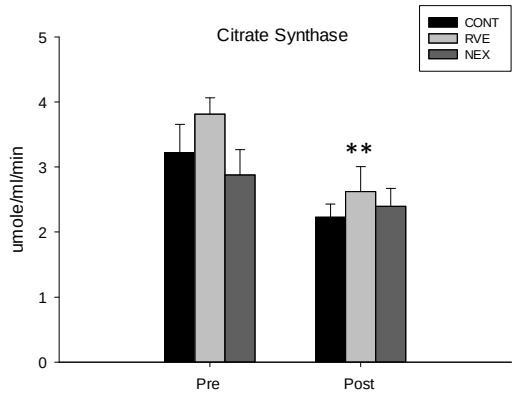


Figure 5.2 Citrate synthase measured before and after bed rest. Data are presented as mean ± SE. ** Significant effect of time.

5.1.3 Leak Respiration

Leak respiration, as measured by the addition of pyruvate and malate in the CHO SUIT and palmitate and malate in the FA SUIT was significantly reduced when normalized for wet weight in both the CHO and FA SUIT experiments (p=<0.05). This response was shown as an effect of time with no effect of trial. No change was reported when normalized for CS.

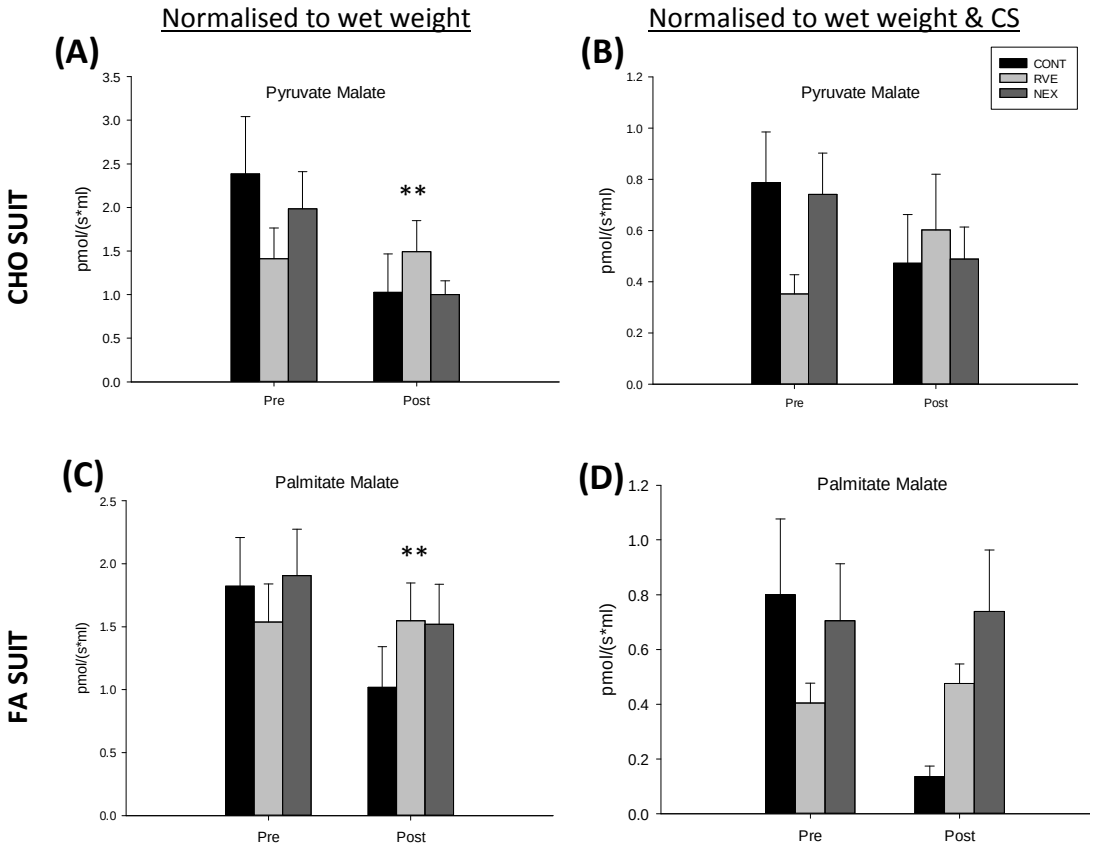


Figure 5.3 LEAK respiration in (A) carbohydrate SUIT with addition of pyruvate and malate normalized to wet weight, (B) normalized to wet weight and CS, (C) fatty acid SUIT with titration of palmitate and malate normalized for wet weight and (D) wet weight and CS. Data are expressed as mean ± SE. ** significant effect of time where no effect of trial was noted.

5.1.4 ADP Stimulated Respiration (OXPHOS)

ADP stimulated respiration (OXPHOS) displayed no change after bed rest in any group when normalized for wet weight in the CHO or FA SUIT. However, when OXPHOS was normalized for wet weight and CS, a significant increase in respiration existed in both the CHO and FA SUIT ($p<0.05$). Furthermore, RVE and NEX interventions showed a significant difference to the control group in the FA SUIT post bed rest which indicates that there is a different response between the CHO and FA SUIT protocols especially in the control group. ($p<0.05$).

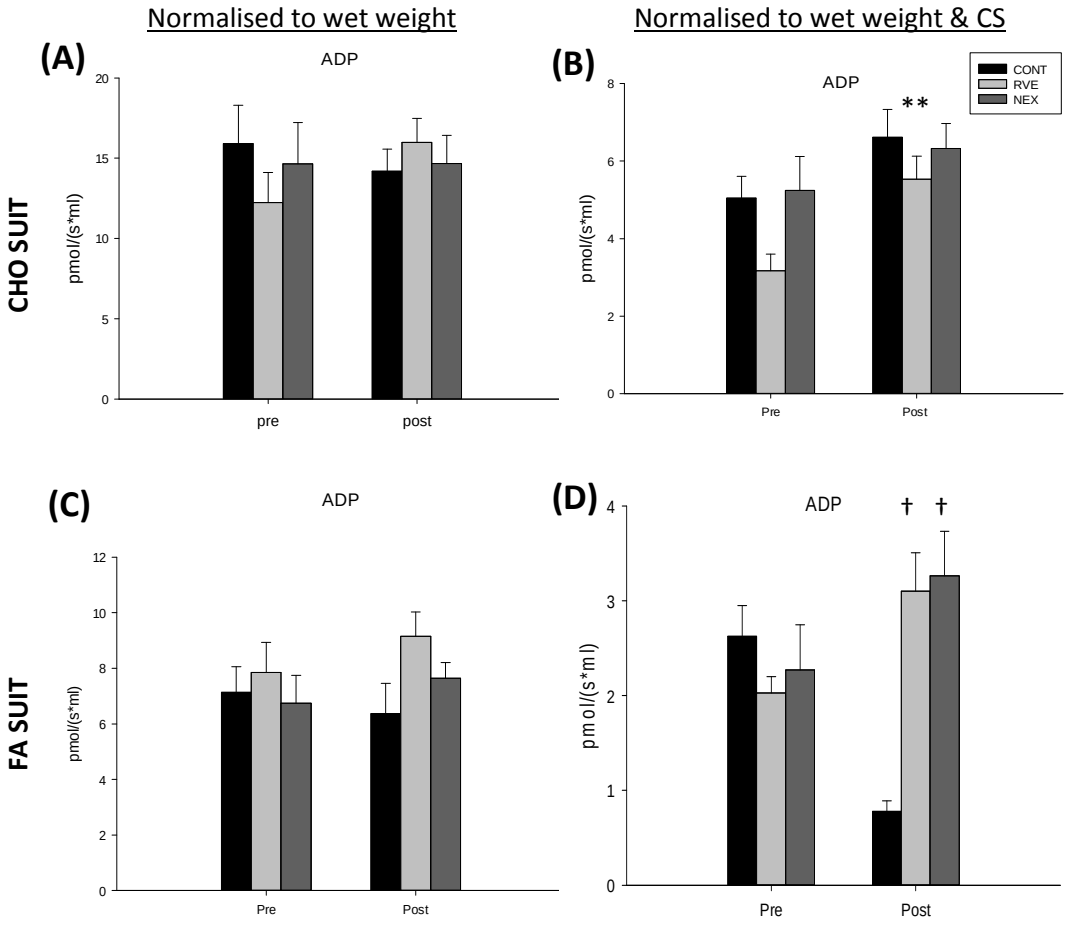


Figure 5.4 OXPHOS capacity. (A) CHO SUIT normalized for wet weight (B) CHO SUIT normalized for wet weight and CS, (C) FA SUIT in the presence of saturating ADP concentrations, normalized for wet weight, (D) wet weight and CS. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted. † significantly different to control trial in the post bed rest period.

5.1.5 Uncoupling of the ETC (FCCP)

With the addition of the uncoupler, FCCP, we measured complex I linked electron transport system (ETS) capacity. There was no change in complex I linked ETS capacity when normalized with wet weight. However, when normalized for wet weight and CS, a significant increase post bed rest was noted in the CHO SUIT. In the FA SUIT, within trial differences existed in the post bed rest period, respiration in RVE was reduced compared to control and increased in NEX compared to control ($p<0.05$). Furthermore, post bed rest control was significantly lower than pre ($p<0.05$).

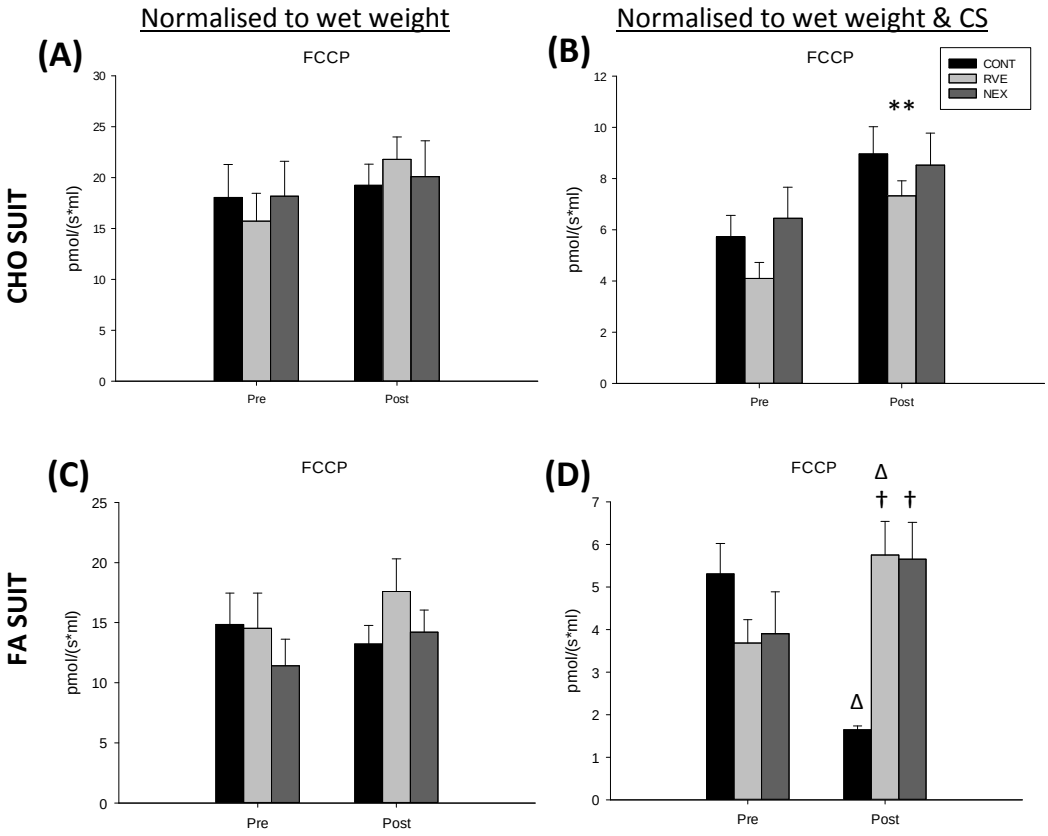


Figure 5.5 Complex I linked ETS capacity. (A) CHO SUIT normalized for wet weight (B) wet weight and CS, (C) FA SUIT normalized for wet weight and (D) wet weight and CS. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted. † significantly different to control trial post bed rest. Δ significantly different to pre bed rest as an effect of trial.

5.1.6 Succinate Driven Respiration (ETS)

In the CHO SUIT, respiration driven by succinate for complex II immediately following the FCCP titration gives us a measure of complex I & II linked ETS (maximal ETS).

Maximal ETS showed no change when normalized for wet weight post bed rest. Normalization for wet weight and CS produced a significant increase in respiration as an effect of time ($p<0.05$). Further analysis expressed a difference between trials with a difference between the NEX and RVE trial ($p<0.05$) and the control trial and RVE ($p<0.05$). In the FA SUIT, a similar trend exists where no change was identified when respiration was normalized for wet weight. However, when normalized for wet weight and CS, an effect of time is noted showing an increase in respiration for both the RVE and NEX trials ($p<0.05$). A reduction in the control group post bed rest is noted ($p<0.05$). A further comparison of the trials in the post bed rest period shows a significant increase between both the RVE and NEX trial and the control trial ($p<0.05$).

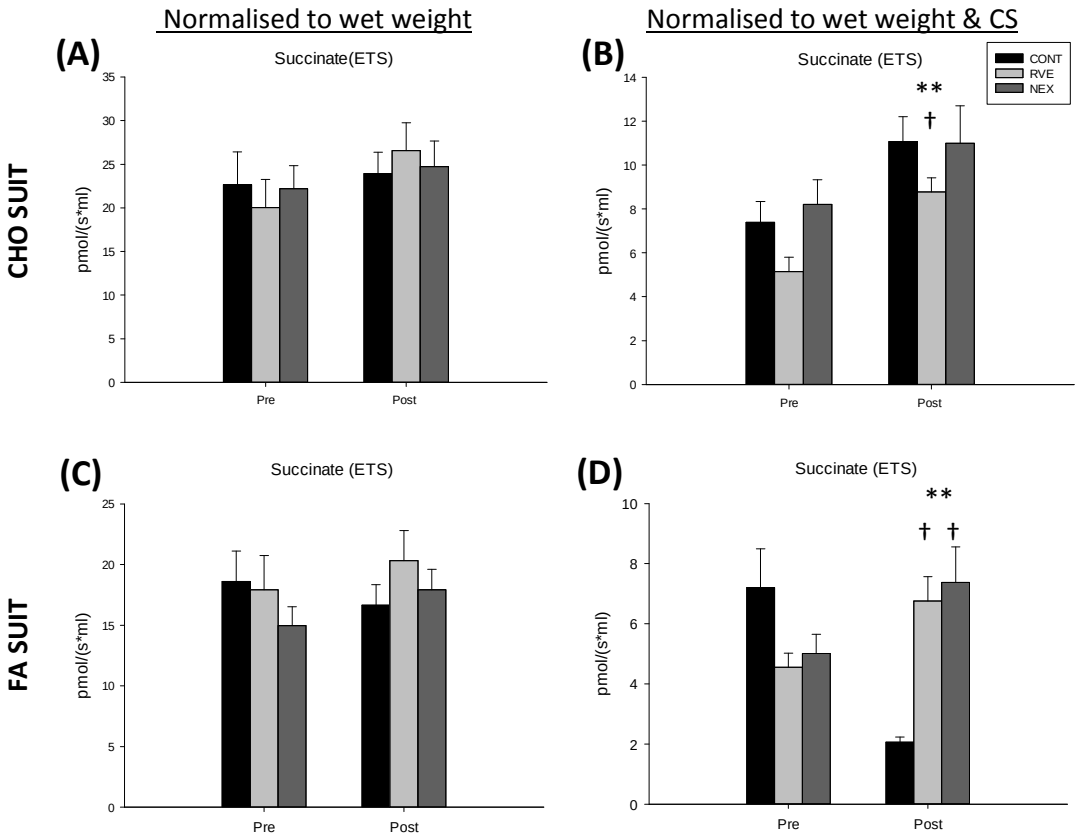


Figure 5.6 Complex I & II linked ETS capacity (maximal capacity). (A) CHO SUIT normalized for wet weight (B) wet weight and CS, (C) FA SUIT normalized for wet weight and (D) wet weight and CS. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted. † significantly different to control trial in the post bed rest period.

5.1.7 Rotenone Complex I Inhibitor

Inhibition of Complex I with the addition of rotenone allowed us to assess respiratory flux through complex II. No change was noted in either CHO or FA SUIT when normalized with wet weight. In the CHO SUIT, there was an increase in respiration when normalized for wet weight and CS as an effect of time post bed rest. In the FA SUIT, within trial differences existed in the post bed rest period, respiration in RVE and NEX were different compared to control ($p<0.05$).

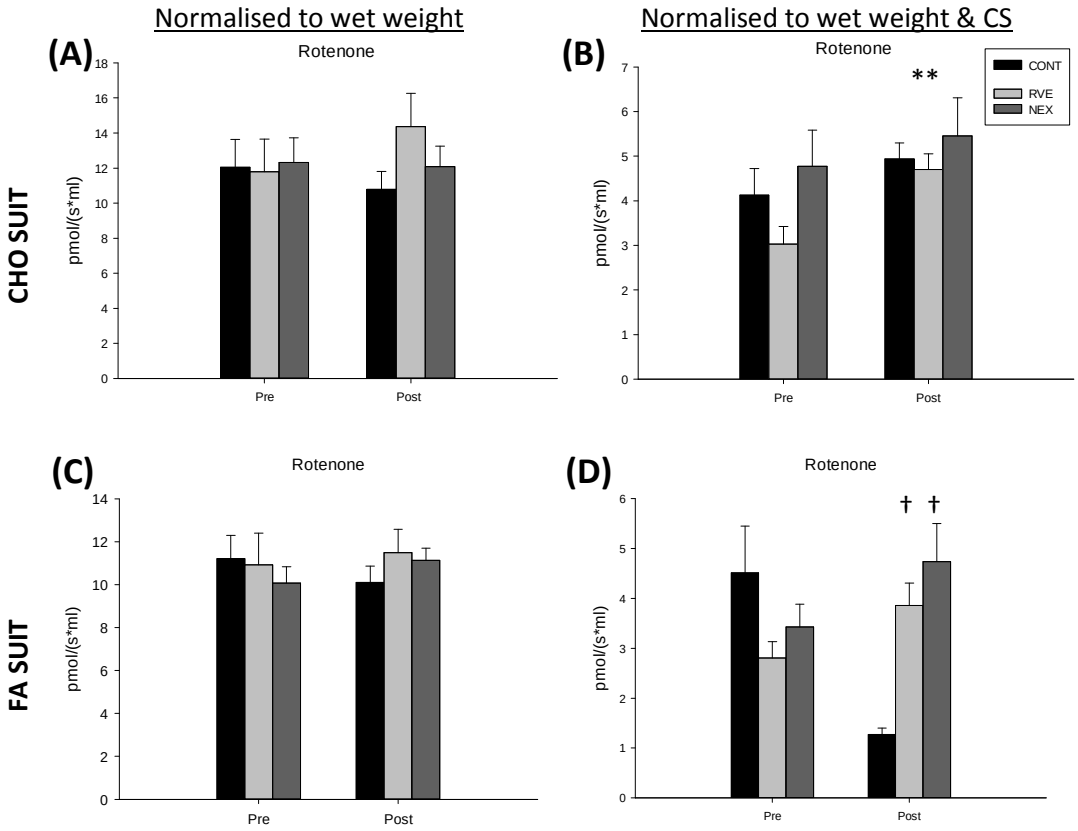


Figure 5.7 Complex II capacity in (A) CHO SUIT and (B) FA SUIT with the addition of complex I inhibitor, rotenone, normalized for wet weight, wet weight and CS, wet weight and porin. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted. † significantly different to control trial post bed rest.

5.1.8 Ascorbate and TMPD

In the CHO SUIT only, there was no change in respiratory capacity of complex IV when normalized with wet weight. However, when normalized with wet weight and CS, an effect of time was noted showing an increase in respiration post bed rest ($p<0.05$).

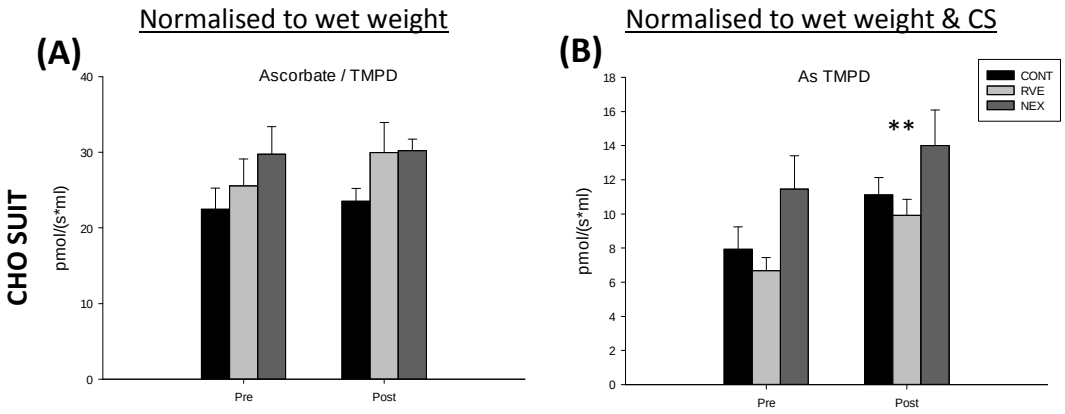


Figure 5.8 Respiratory flux through complex IV with the addition of complex II and III inhibitors, ascorbate and TMPD in (A) CHO SUIT, normalized for wet weight and (B) wet weight and CS. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted.

5.1.9 Octanoylcarnitine

No difference in respiration existed with the addition of medium chain fatty acid, octanoylcarnitine, when respiration was normalized for wet weight. An effect of time was reported with wet weight and CS normalization ($p<0.05$). Further analysis showed a difference between the two intervention trials and the control trial in the post bed rest period ($p<0.05$).

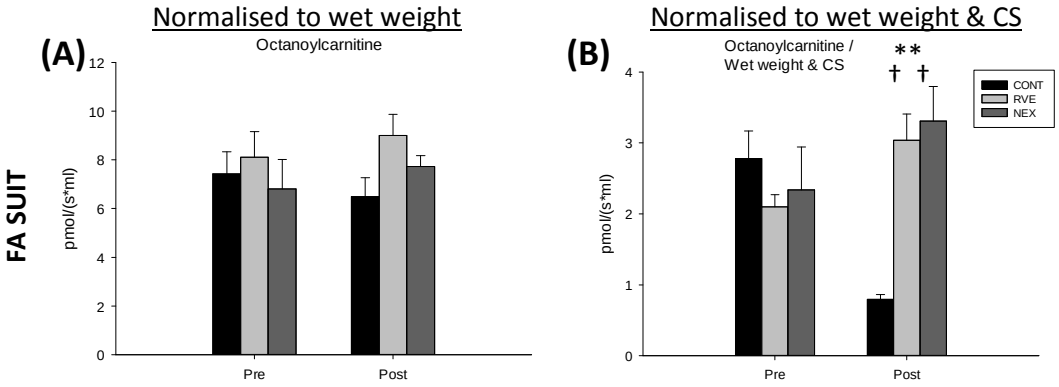


Figure 5.9 Respiration with the addition of octanoylcarnitine in the FA SUIT. normalized for wet weight, wet weight and CS, wet weight and porin. Data are expressed as mean ± SE. ** significant effect of time and no effect of trial was noted. † significantly different to control trial post bed rest.

5.1.10 Correlation Analysis

There was a significant correlation between the change in insulin sensitivity (section 4.1.4) and mitochondrial respiration with FCCP (complex I linked ETS capacity) for the control group only. It was a positive correlation whether normalised to WW (r=0.854, p=0.00691) or CS (r=0.891, p=0.00299). The greater the decrease in insulin sensitivity, the greater the decrease in electron transport system capacity. There were no correlations with changes in fitness nor were there any significant correlations with the intervention data suggesting that the countermeasures may have been effective in counteracting the reduction in insulin sensitivity.

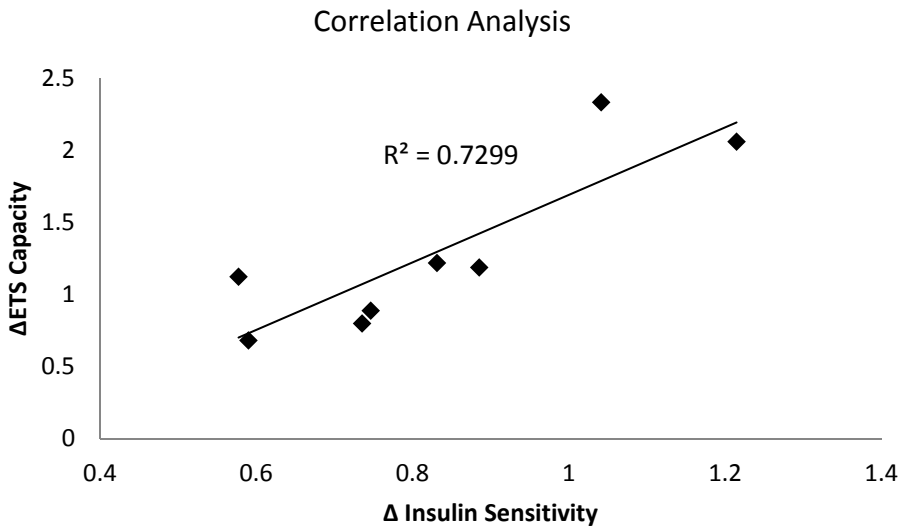


Figure 5.10 Correlation Analysis between changes in ETS capacity and change in insulin sensitivity.

5.10 Western Blot Analysis

Subsequent Western Blot analysis of all samples was conducted. Blots were probed for proteins involved in mitochondrial function and cellular metabolism.

5.10.1 OXPHOS Cocktail

No change existed for complex I (NDUFB8), complex IV (MTC01) or complex V (ATP5a). Complex III (UQCRC2) showed a significant decline with an effect of time ($p<0.05$). A reduction in complex II SDHB was also noted ($p<0.052$).

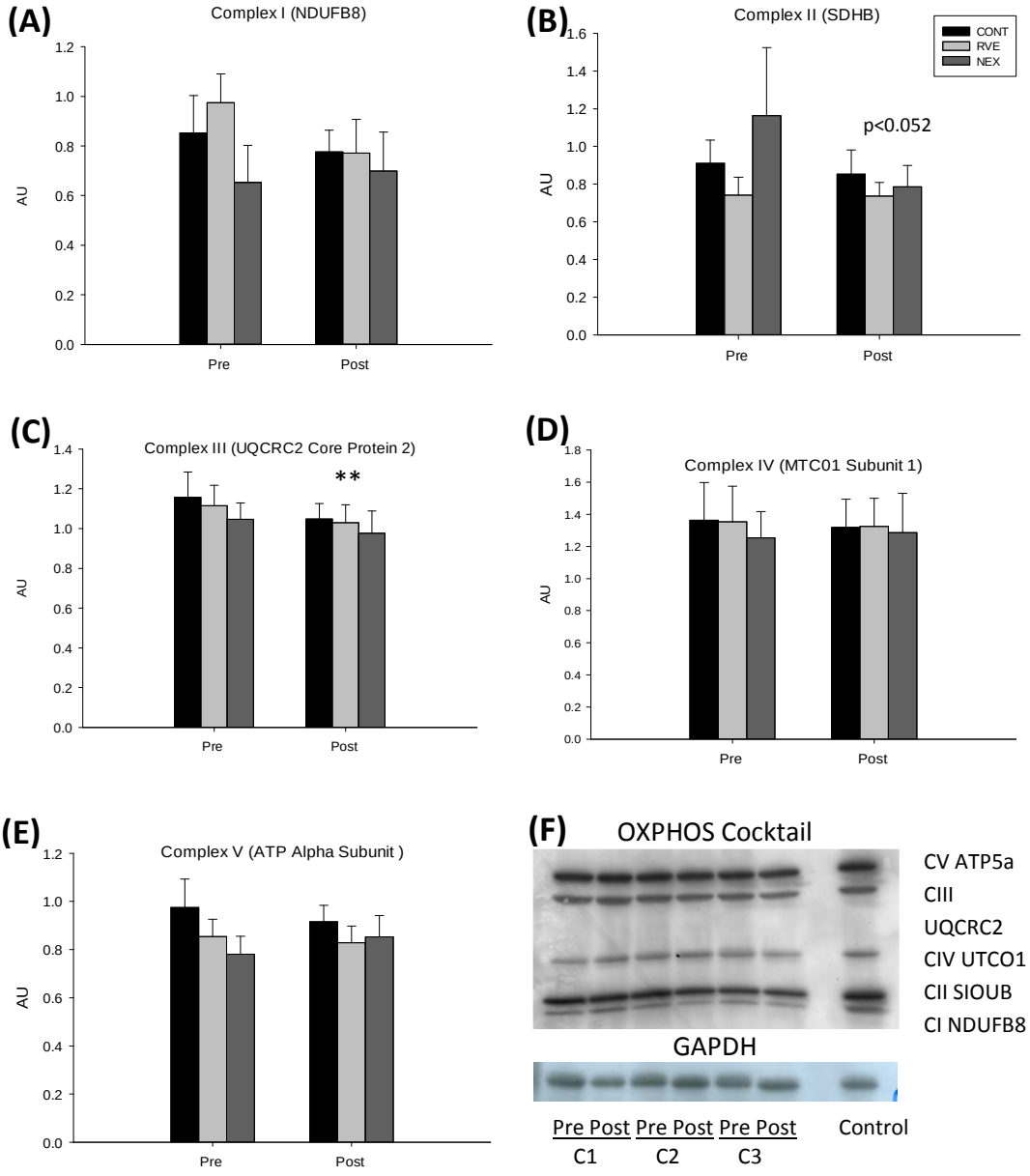


Figure 5.11 Total OXPHOS Western Blot Antibody Cocktail. (A) NDUFB8, Complex I NADH dehydrogenase 1 beta subcomplex subunit 8, an accessory subunit of NADH dehydrogenase (complex I) located on the inner mitochondrial membrane. (B) SDHB Complex II, succinate dehydrogenase iron-sulfur subunit complex II. (C) UQCRC2, cytochrome b-c1 complex subunit 2, complex III. (D) MTCO1 mitochondria encoded cytochrome C oxidase also known as cytochrome C oxidase (COX I) subuit of complex IV. (E) CV ATP5a, alpha subunit of complex V. Data are expressed as mean ± SE. ** significant effect of time for all groups combined.

5.10.2 Other Mitochondrial Proteins

There was no change in the expression of COX IV, SDHa or mitochondrial membrane protein, porin. A significant difference was noted for COX5a as an effect of time ($p<0.05$).

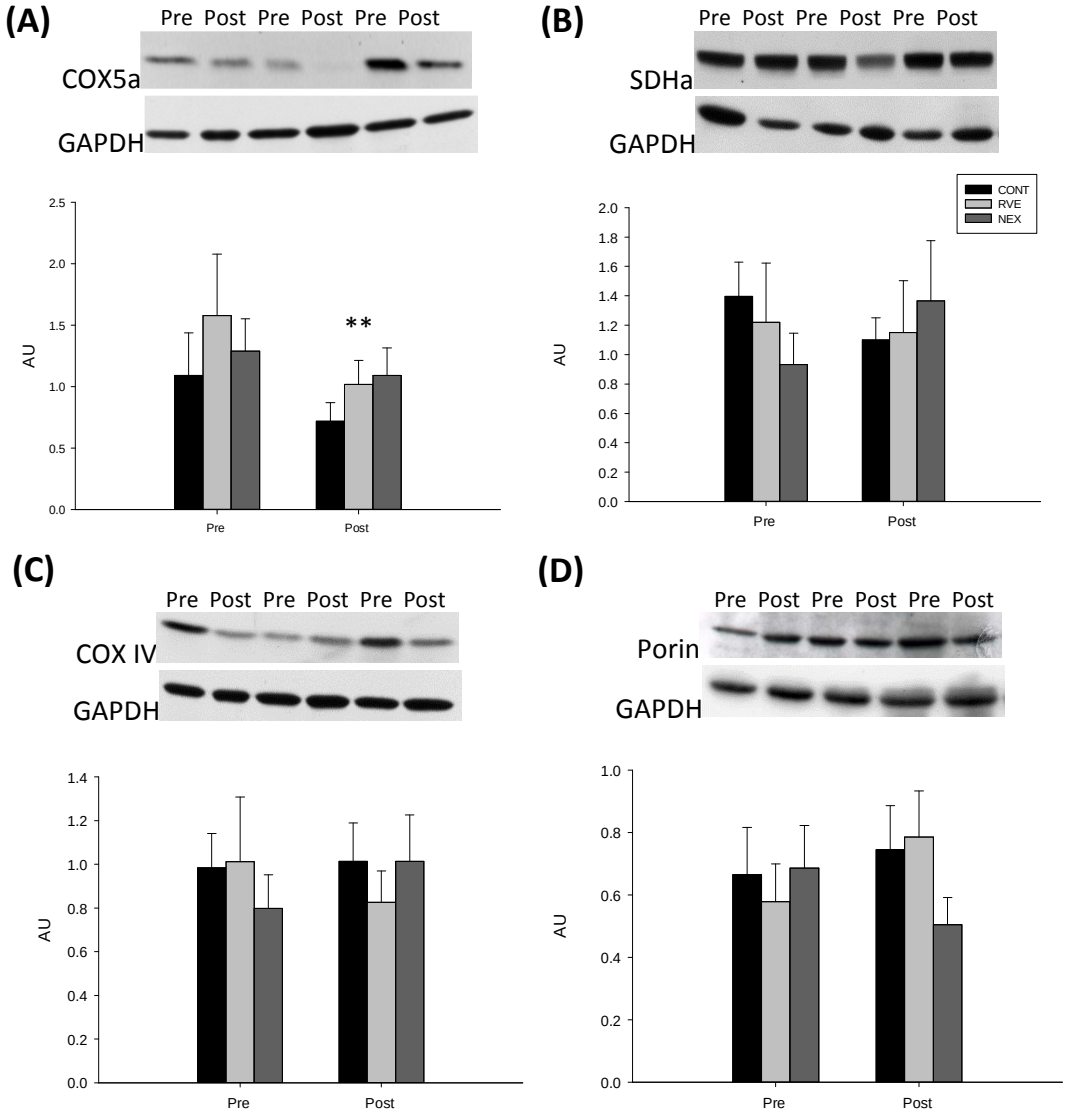


Figure 5.12 Mitochodrial Proteins quantified by Western Blot. (A) Cox5a, cytochrome C oxidase subunit 5a, subunit of complex IV. (B) SDHa participates in both the TCA cycle and the electron transport chain. It is one of 4 subunits of complex II. (C) COX IV is a subunit of complex IV and is responsible for initiating assembly of the complex. (D) Porin, otherwise known as voltage-gated ion channels (VDAC) are structural proteins that are responsible for outer membrane mitochondrial permeability. Data are expressed as mean ± SE. ** significant effect of time for all groups combined.

5.10.3 Proteins related to Cellular Metabolism

There was no change in the expression of Akt after bed rest. There was a significant increase in FOX01 as an effect of time ($p<0.05$).

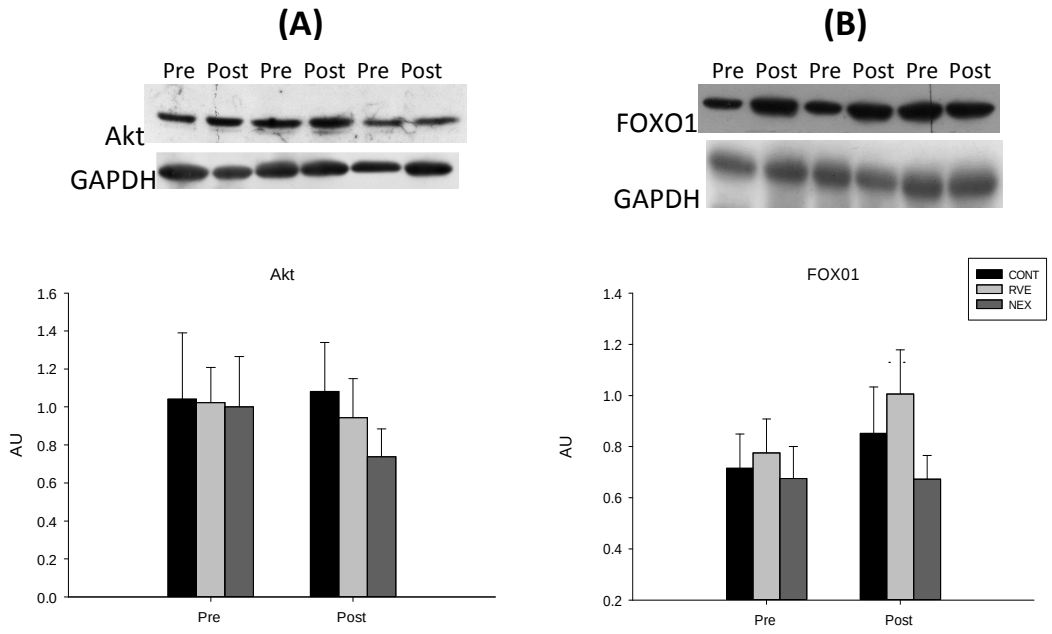


Figure 5.13Proteins relating to cellular metabolism. (A) Akt (also known as protein kinase (B) and (B) Forkhead box protein 01 (FOX01). Data are expressed as mean ± SE. ** significant effect of time for all groups combined.

Chapter VI

Cell Culture

Developing an insulin resistant model in C2C12 mouse muscle cells became of interest following the findings of the bed rest study. With a reduction in insulin sensitivity and changes in mitochondrial function and mitochondrial proteins, particularly, a decrease in COX5a expression in the human samples, we developed this in vitro model of metabolic stress in order to further explore the cell response. We wanted to further investigate the role of COX5a in insulin resistance and mitochondrial function. We used two models of metabolic stress, high glucose and insulin for 48 hours and palmitate treatment for 24 hours. We wanted to assess the function of the mitochondria, ROS production, membrane potential and proteins involved in mitochondrial function. All of this work was conducted in C2C12 myotubes. Subsequently, we transfected the C2C12 myoblasts for COX5a knock down and assessed all of the same parameters in order to identify the effect a reduction of this protein could have on cell metabolism and function. We acknowledge that this is not a replica of the model of bed rest; however, it does provide us with information regarding what happens to the cell in stressful metabolic environment.

Control cells from each treatment condition should not be compared as the high insulin/glucose controls were cultured in 10% FCS DMEM low glucose (5mM) glucose media and the control cells for the palmitate treatment was in 10% FCS DMEM 25mM glucose. Additionally, the incubation times was 24 hours and 48 hours for the high glucose/ insulin and palmitate treatments and all experiments were conducted on different days. Taking this into consideration, we suggest that control experiments from the two treatments should not be compared.

6.1 C2C12 Differentiation

When C2C12 cells are placed in serum poor media (2% horse serum), differentiation is induced. The C2C12 cells withdraw from the cell cycle and fuse to form multinucleated myotubes. Myoblast differentiation in vitro into myotubes was thought to more closely represent skeletal muscle myofibres.

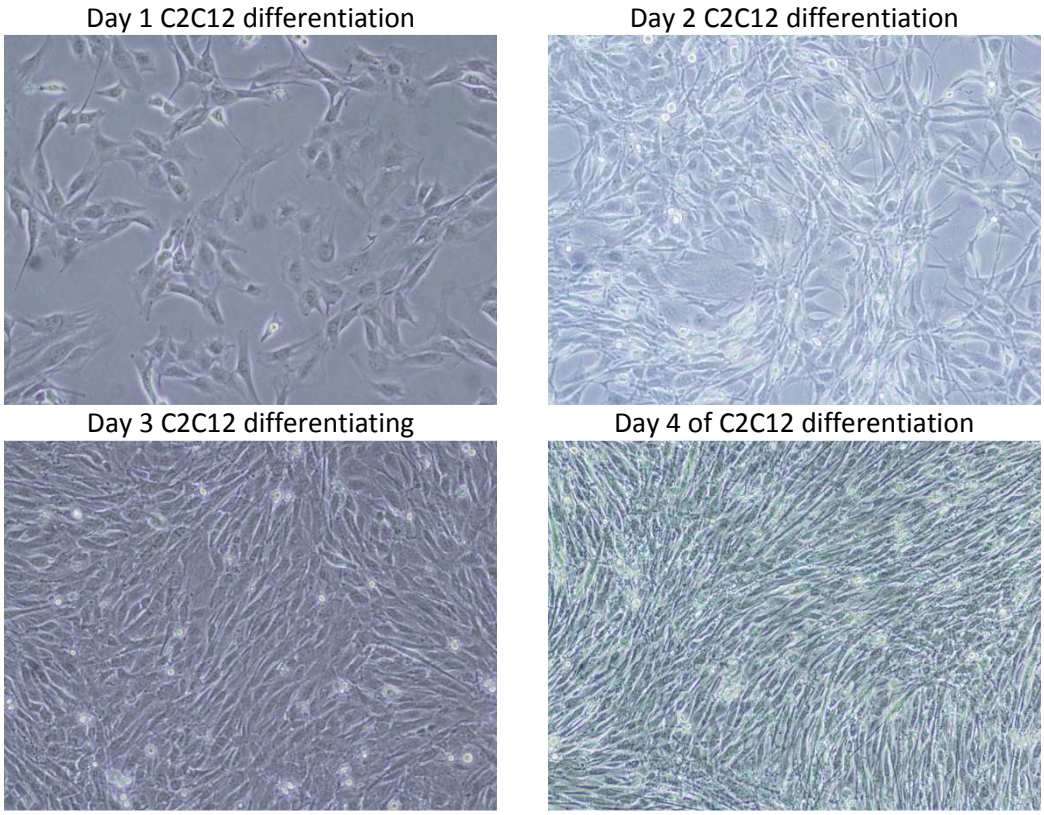


Figure 6.1 C2C12 cell differentiation protocol over 96 hours.

6.2 Citrate Synthase

Citrate synthase was measured in all control and treated cells and used as a marker of mitochondrial enzyme activity. No significant change in citrate synthase activity was noted.

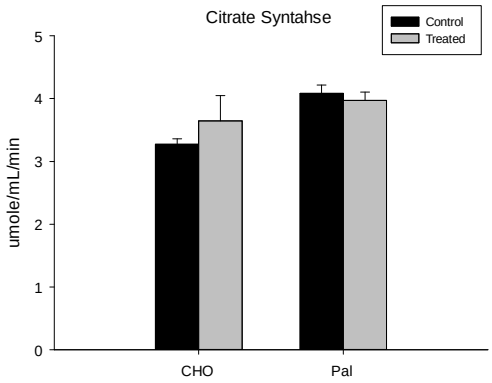


Figure 6.2 Citrate synthase (CS) measured in C2C12 cells with high glucose/insulin (CHO) treatment and high lipid treatments (Pal). Data are presented as mean ± SE.

6.3 Mito Tracker (MTG)

To label mitochondria, cells were incubated with MitoTracker probes which passively diffuse across the plasma membrane and accumulate in active mitochondria. MitoTracker green fluorescence was measured at the respective excitation and emission wavelengths of 490 nm and 545nm in in a dual beam plate reader. There was no change in mitochondrial content in the high glucose/insulin treated cells. There appeared to be an increase in mitochondrial content in the high lipid treated cells (p=0.054). Fluorescent images were subsequently obtained using a fluorescent microscope. We did not normalize fluorescence with cell number. If we were to repeat these experiments, we would employ normalization steps.

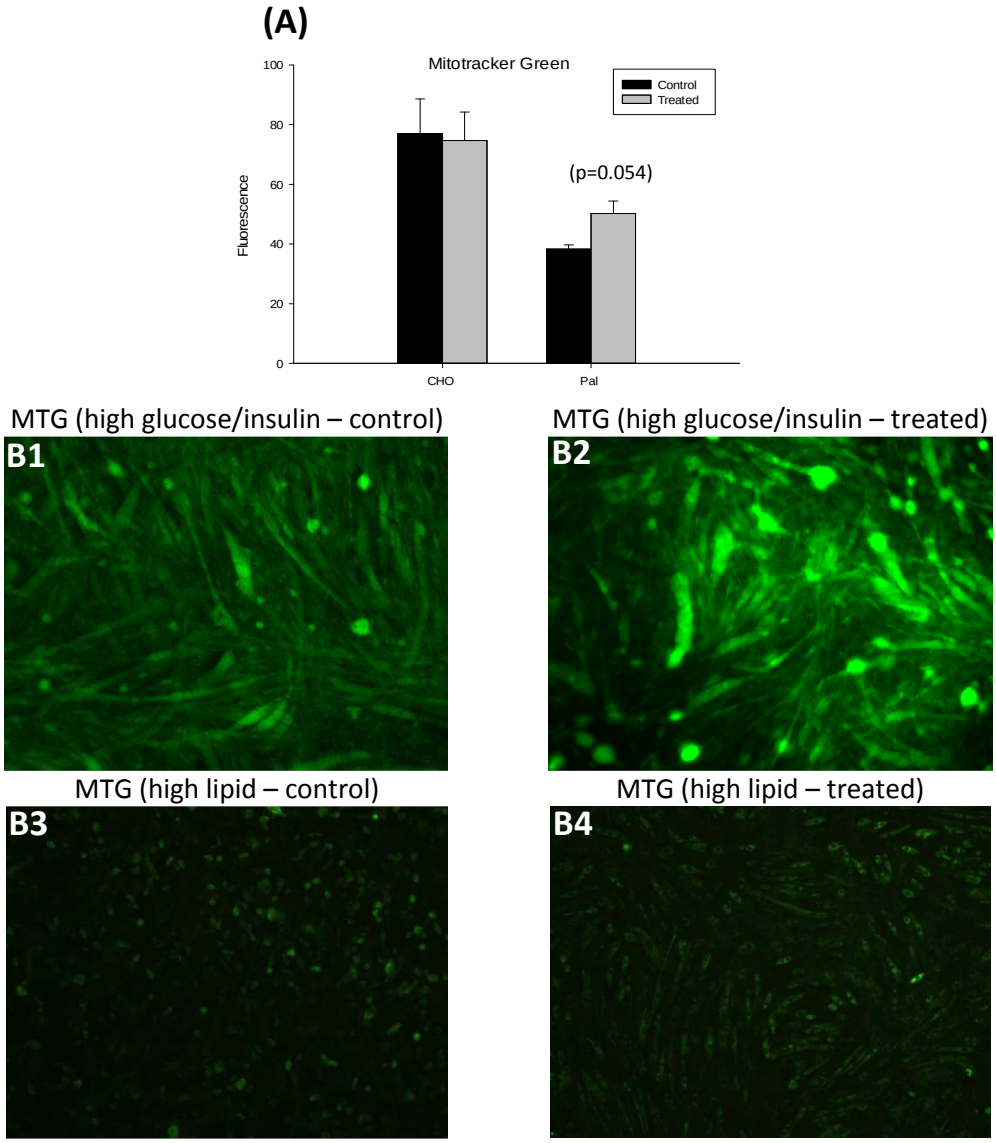


Figure 6.3 MitoTracker Green assay in C2C12 myotubes (A) mitochondrial content in high glucose/insulin (CHO) and high lipid (Pal) treated cells. Data are expressed as mean ± SE. (B) Fluorescent images of MTG (B1) high glucose/insulin control, (B2) high glucose/insulin treated (B3) high lipid control and (B4) high lipid treated. Images obtained by fluorescent microscope.

6.4 High Resolution Respiration

6.4.1 LEAK Respiration

The following analysis was completed using an average of 3 experiments for both high glucose/insulin treated cells and high lipid treated cells. All cell experiments are automatically normalized for cell number during the experiment. LEAK respiration was normalized to ROX and ETS. LEAK respiration as measured by the addition of glutamate and malate was significantly increased in the high glucose/insulin treatments ($p<0.05$). No change was noted in the high lipid treatment.

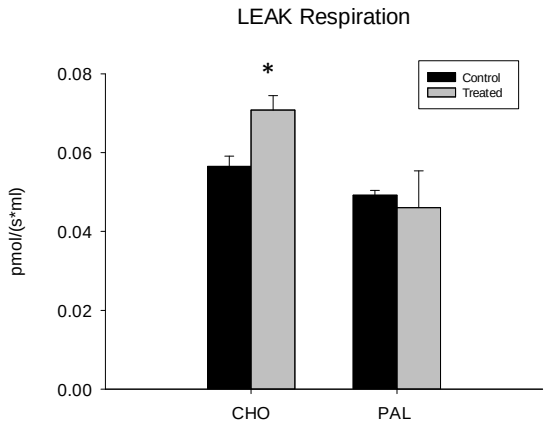


Figure 6.4 Leak Respiration with the addition of glutamate and malate in CHO (high glucose/insulin) and PAL (high lipid) treated C2C12 myotubes. Data are expressed as mean ± SE. * Significantly different to control.

6.4.2 ADP stimulated Respiration

There was no change in OXPHOS capacity in either treatment condition in C2C12 myotubes when normalized for ROX and ETS.

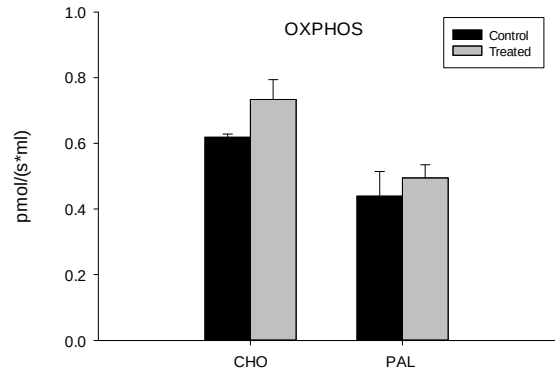


Figure 6.5 ADP stimulated respiration (OXPHOS) with the addition of ADP. Data are expressed as mean ± SE.

6.4.3 Succinate Driven Respiration

No changes were noted in either treatment condition with the addition of complex II driven substrate, succinate, when normalized for ROX and ETS.

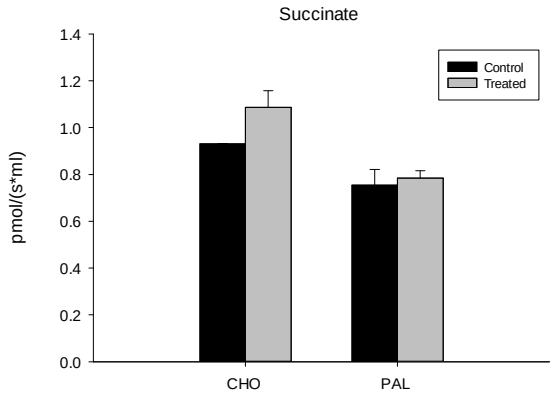


Figure 6.6 Respiration with the addition of succinate. Data are expressed as mean ± SE.

6.4.4 Uncoupled Respiration (FCCP)

No change was present in either condition with the titration of FCCP, when normalized to ROX.

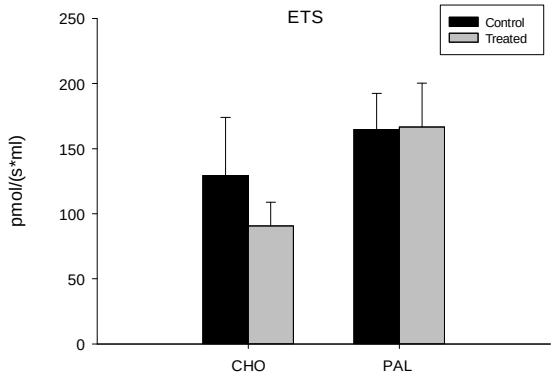


Figure 6.7 Maximal ETS respiration with the addition of FCCP. Electron transport chain capacity (ETS) in CHO (high glucose/insulin) and PAL (high lipid) treatments in C2C12 myotubes. Data are expressed as mean ± SE.

6.4.5 Complex I inhibitor (Rotenone)

Complex II capacity was assessed by the addition of complex I inhibitor, rotenone. No significant changes were noted in either treatment condition when normalized for ROX and ETS.

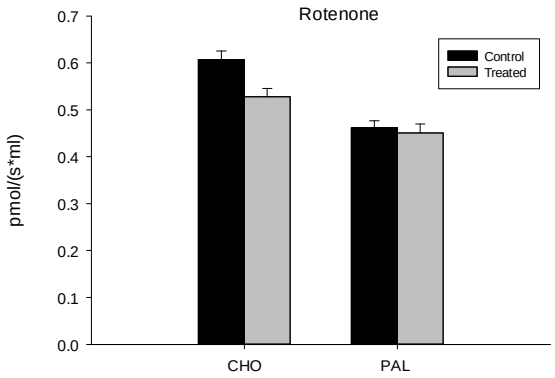


Figure 6.8 Respiration with the addition of Rotenone. Complex II capacity with the addition of complex I inhibitor, rotenone in CHO (high glucose/insulin) and PAL (high lipid) treatments in C2C12 myotubes. Data are expressed as mean ± SE.

6.5 ROS production

Reactive oxygen species (ROS) were measured using two methods. The O2k-Fluo LED2-Module is a component of the O2k-Fluorometer. ROS was measured using this module on the Oroboros oxygraph O2k in parallel to HRR measurements and normalized to ETS capacity. This measurement was performed on high glucose/high insulin and high lipid treated cells. There was no change in ROS production in treated cells compared to control using the O2k-Fluo LED2-Module to measure it. The DCF-DA assay was also used to quantify ROS. There was significant increase in ROS production in the high glucose/insulin treated cells compared to control ($p<0.05$).

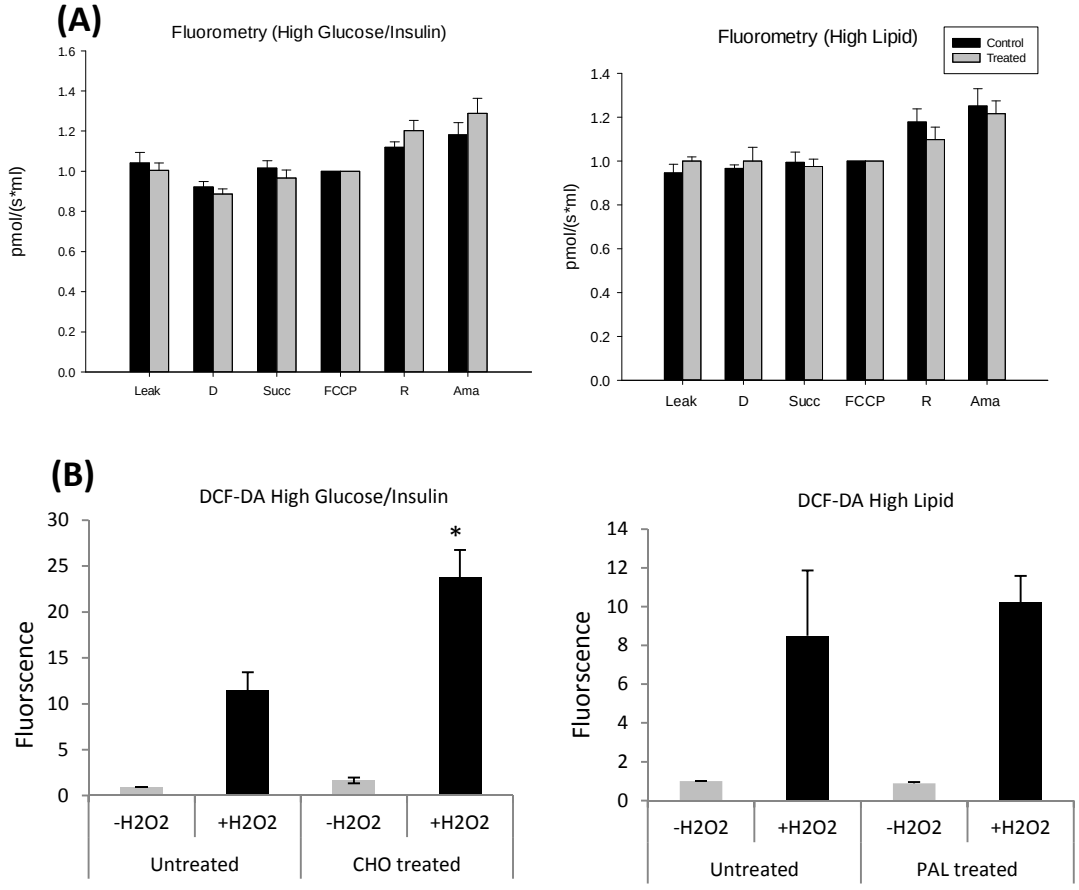


Figure 6.9 ROS production as measured by (A) O2k-Fluo LED2-Module and (B) DCF-DA assay. Data are presented as mean ± SE for O2k Fluorometry and fold change ± SE for DCF-DA.
*** Significantly different to untreated.**

6.6 Membrane Potential (JC1)

$\Delta\Psi_M$ is an important parameter of mitochondrial function and has been used as an indicator for cell health in C2C12 cells treated with high glucose/insulin and high lipid. JC-1 enters the mitochondria and changes its fluorescent properties based on the aggregation of the probe. In healthier cells, a higher $\Delta\Psi_M$ exists and JC-1 forms complexes known as J-aggregates with intense red fluorescence. In less healthy cells, a lower $\Delta\Psi_M$ exists and JC-1 remains in the monomeric form and this exhibits a green fluorescence. Therefore, in both treatment conditions compared to control, the higher ratio of red to green fluorescence, the higher the polarization of the inner mitochondrial membrane. An interesting reduction in mitochondrial membrane potential was noted in the palmitate treated cells (p=0.09).

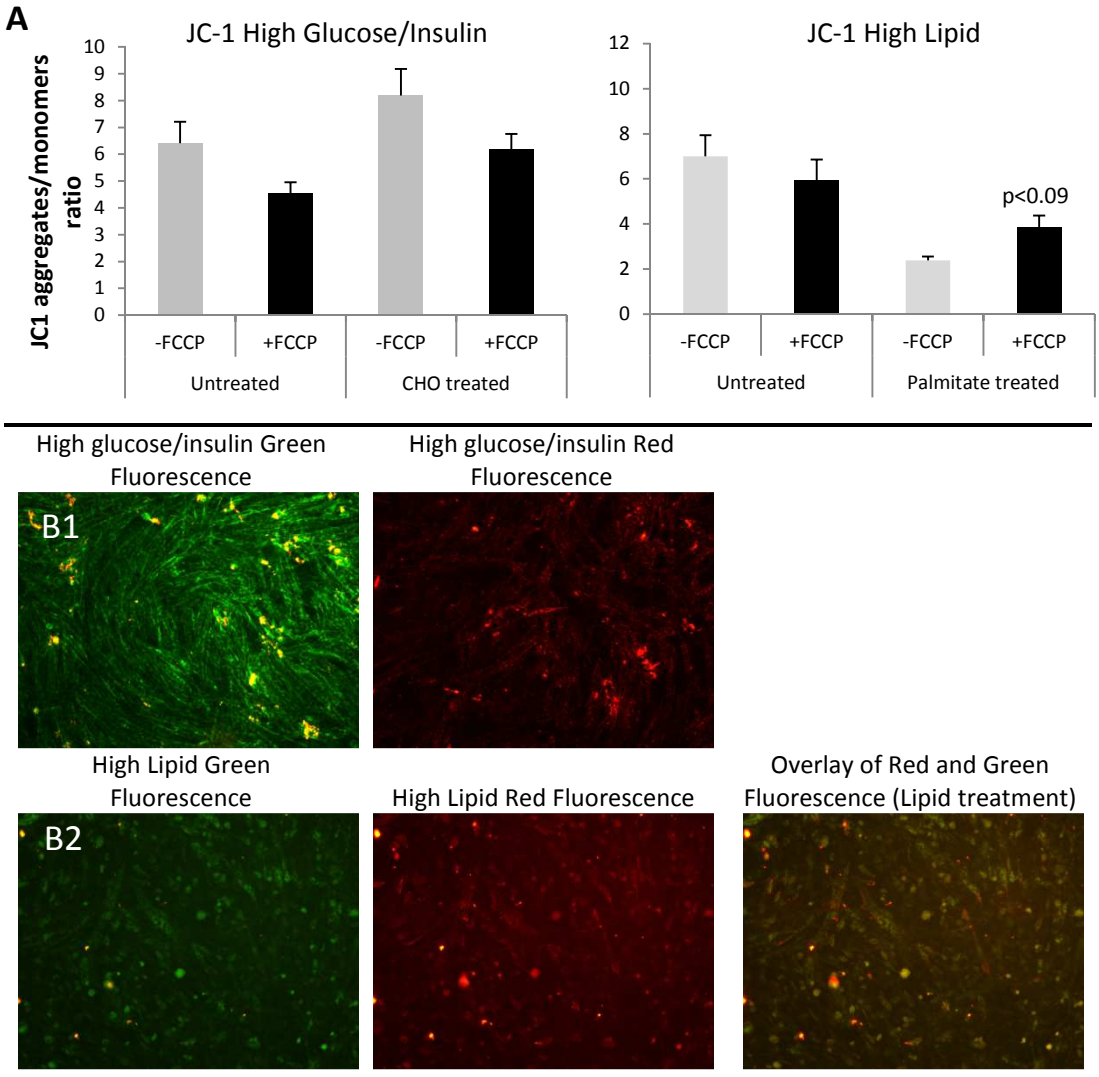


Figure 6.10 Mitochondrial membrane potential. (A) Mitochondrial membrane potential using the JC-1 assay (B) Fluorescent images display green fluorescence red fluorescence (J-aggregates) and green fluorescence (monomers) in (B1) high glucose/insulin and (B2) high lipid treated cells. An overlay image of the ratio of red to green fluorescence is also shown for the lipid treatment condition.

6.7 Western Blot Analysis

6.7.1 Mitochondrial Proteins

Mitochondrial proteins were quantified using Western Blot analysis. No change was noted for any of the mitochondrial proteins in either treatment condition.

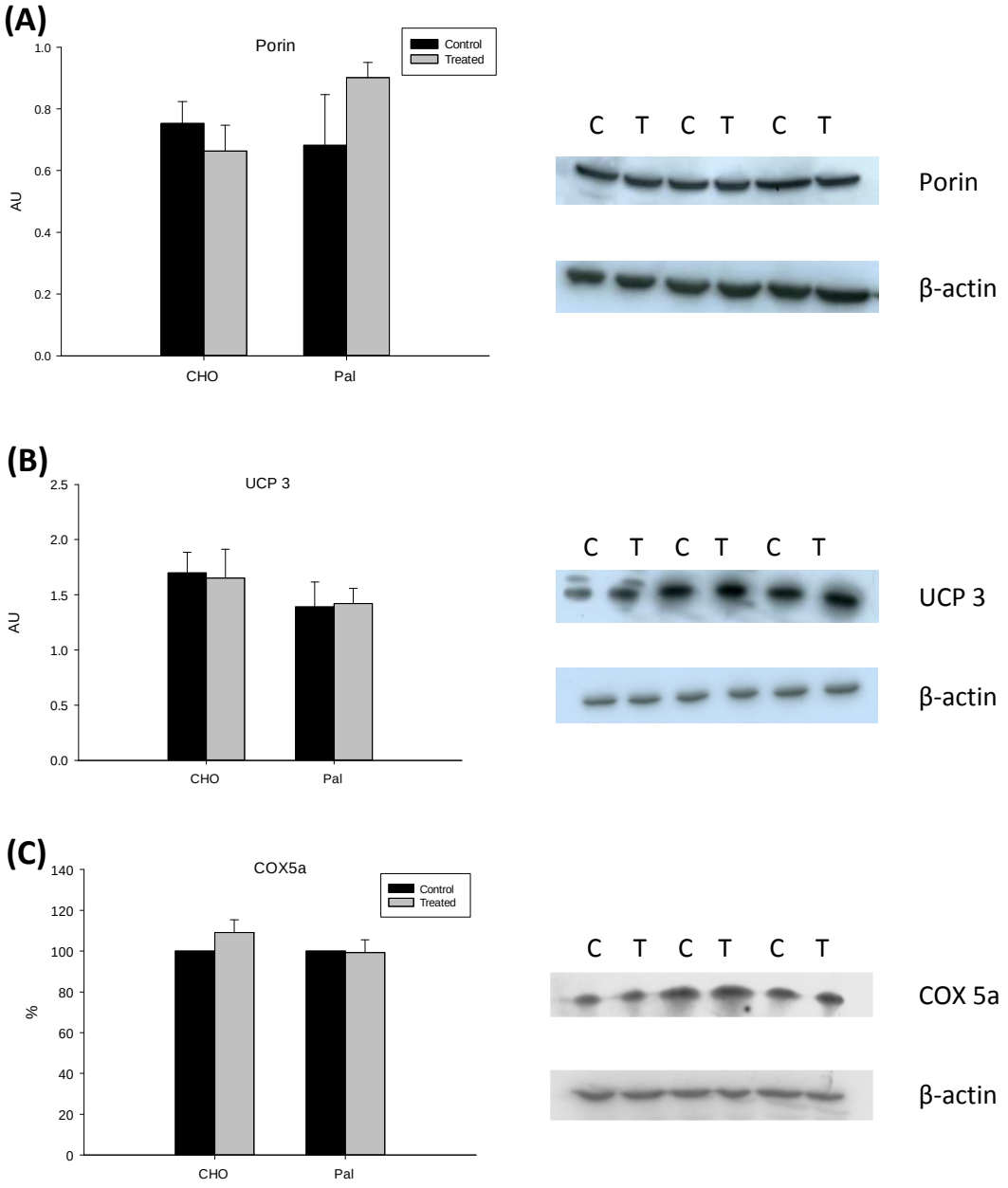


Figure 6.11 Mitochondrial proteins. (A) Porin, (B) UCP3, (C) COX5a. Data are expressed as mean ± SE. COX5a is presented as percent change.

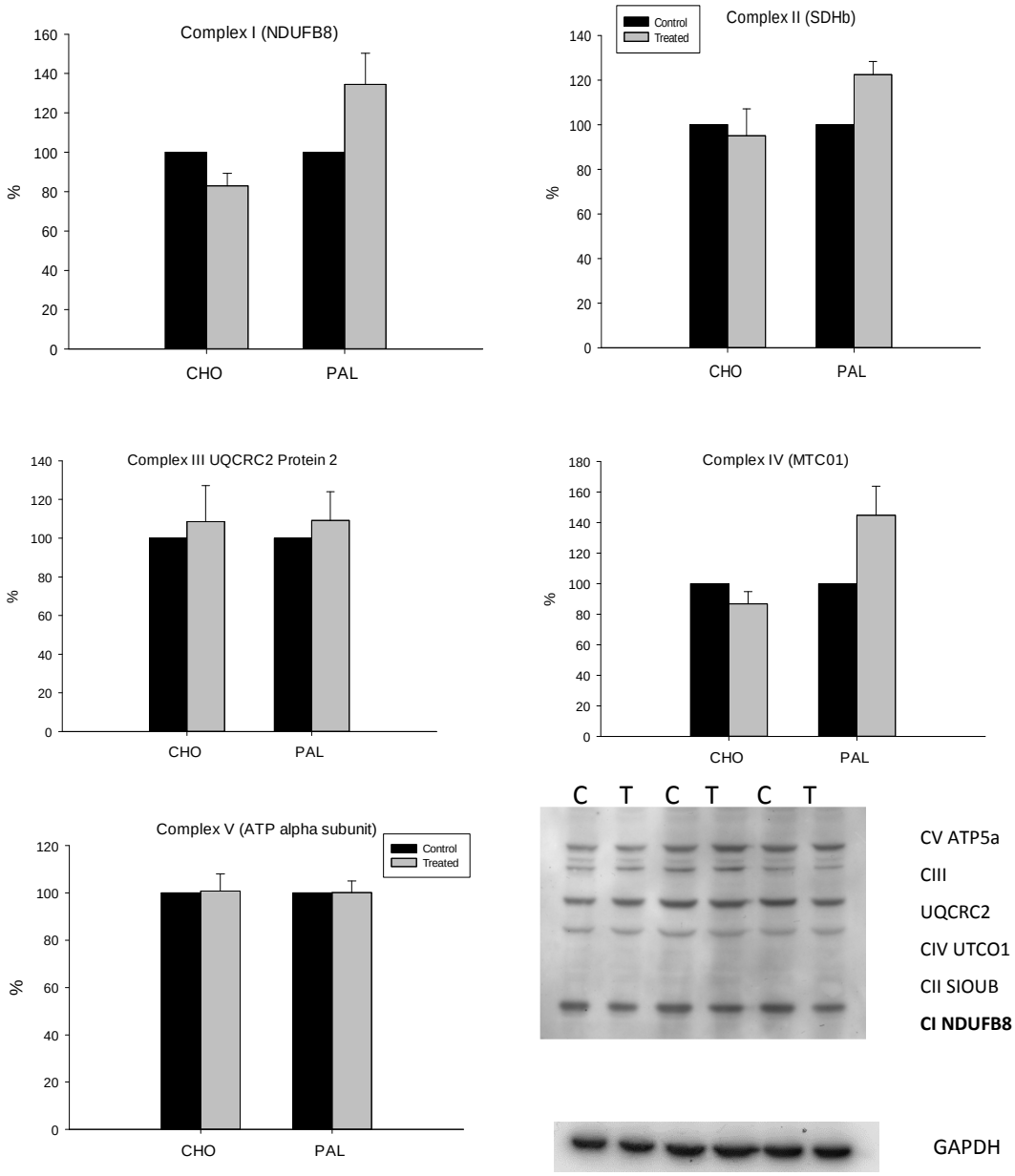


Figure 6.12 Total OXPHOS Western Blot Antibody Cocktail. Complex I (NDUFB8), Complex II (SDHB), Complex III (UQCRC2), Complex IV (MTCO1) and CV (ATP5a). Data are expressed as mean ± SE. * Significantly different to pre.

6.7.3 Akt Phosphorylation

There were no changes noted when Akt phosphorylation (Ser473) was normalized for Akt activity. There was no change in Akt expression. There was a reduction in Akt phosphorylation following the high lipid treatment compared to control (p=0.057).

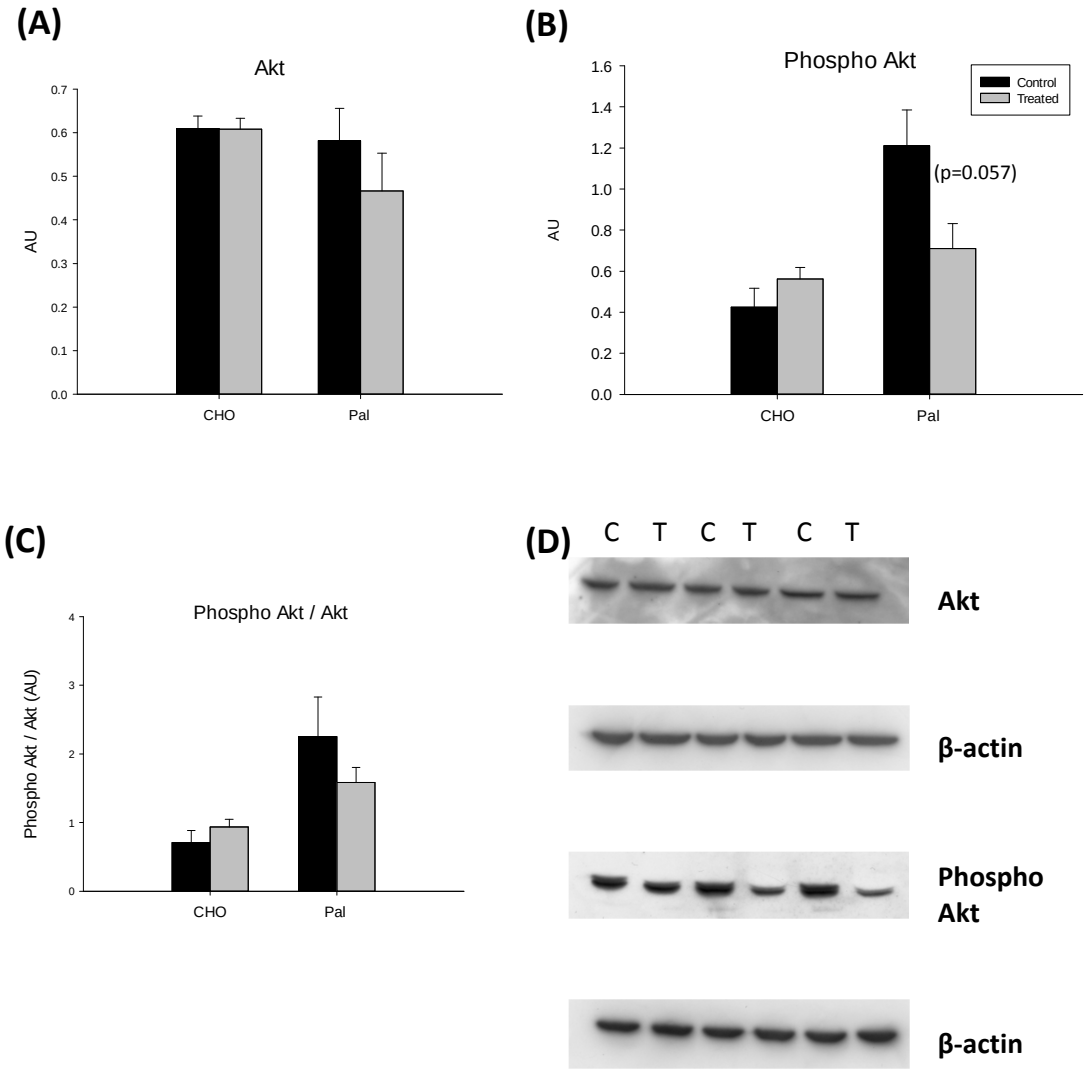


Figure 6.13 Phospho Akt/Akt. (A) Quantification of Akt and phosphorylation of Akt (Ser473) with high glucose/insulin and high lipid treatments. (B) Phosphorylation of Akt normalized for Akt. (D) Representative blots for Akt/ β -actin and phosphor Akt/ β –actin. Data are expressed as mean ± SE.

6.8 SiRNA

6.8.1 Mitochondrial Respiration

All transfection experiments were performed on C2C12 myoblasts. RNAi using short interfering RNAs (siRNAs) was carried out to silence specific genes that encode for COX5a. COX5a has become of interest due to it being down regulated in the human muscle samples from the bed rest study. COX5a is one of thirteen subunits of complex IV of the ETC and we were interested in its role in metabolic function. Mitochondrial function was assessed using the oroboros. There were no significant differences noted with any substrate addition between control and transfected cells. However, a clear reduction existed for ADP stimulated respiration (p=0.076) and maximal ETC (p=0.083). Experimental results are based on n=3.

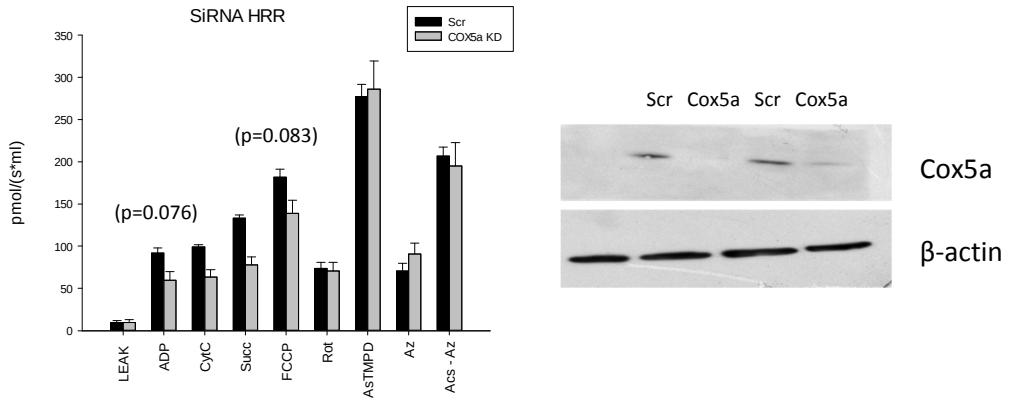


Figure 6.14 COX5a SiRNA. Mitochondrial respiration in SiRNA transfected C2C12 myoblasts (n=3). Knockdown efficiency of 80% for COX5a as measured by acid phosphatase colorimetric assay. Representative blots for COX5a knockdown/ β-actin. Data are expressed as mean ± SE.

6.8.2 MitoTracker Green (MTG)

MitoTracker green fluorescence was measured at the respective excitation and emission wavelengths of 490 nm and 545nm in in a dual beam plate reader. There was no change in mitochondrial content in SiRNA transfected cells compared to scrambled negative control cells.

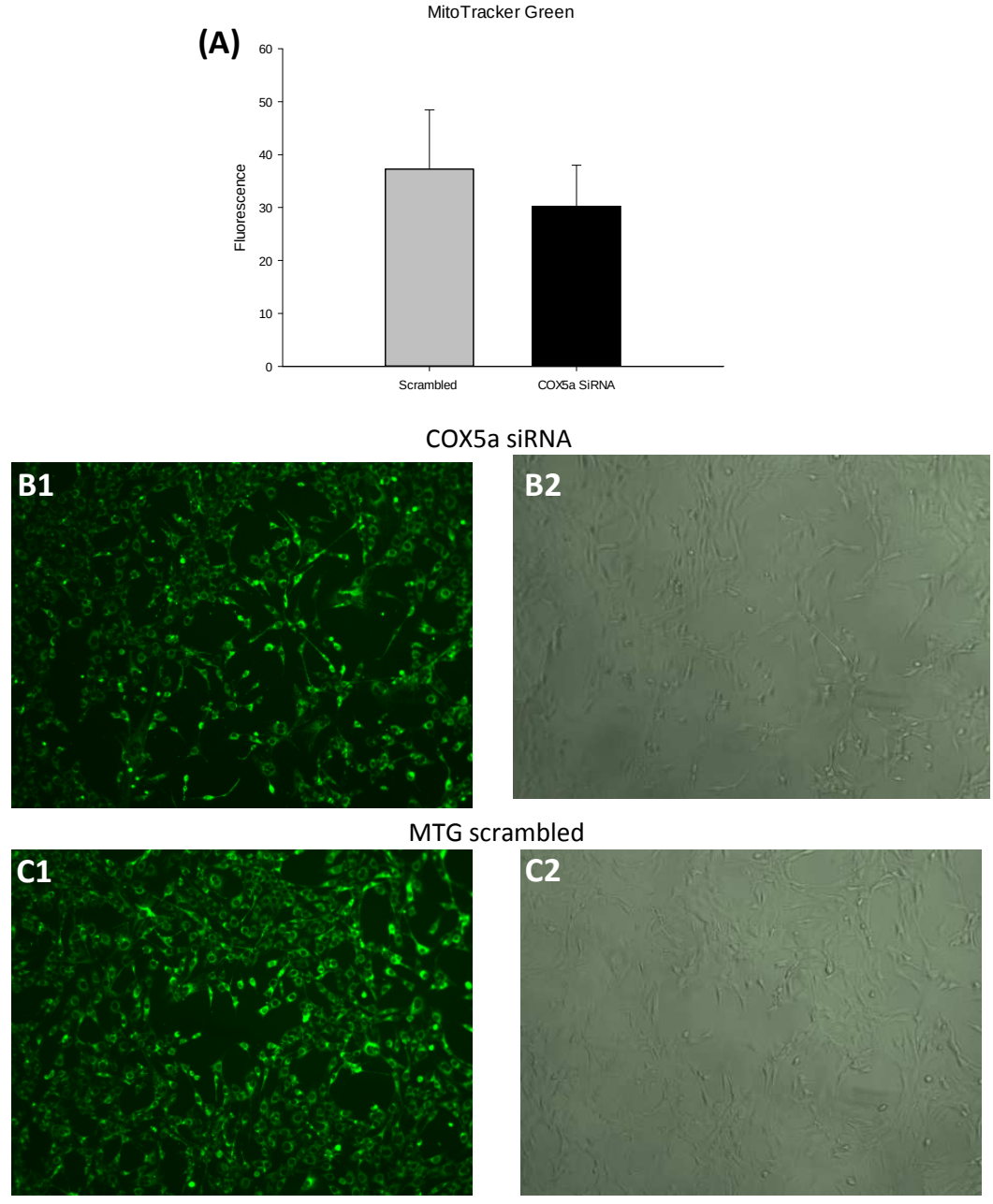


Figure 6.15 MitoTracker Green assay in C2C12 myotubes (A) mitochondrial content SiRNA transfected cells (COX5a) and scrambled (Scr) negative control cells. Data are expressed as fold change ± SE. (B) Fluorescent images of MTG (B1) fluorescent image with COX5a transfection (B2) COX5a transfection with normal image (C1) scrambled (Scr) negative control fluorescent image and (C2) scrambled (Scr) negative control normal image.

6.8.3 ROS Production (DCF-DA)

The DCF-DA assay was also used to quantify ROS. No change in ROS was noted with either treatment condition.

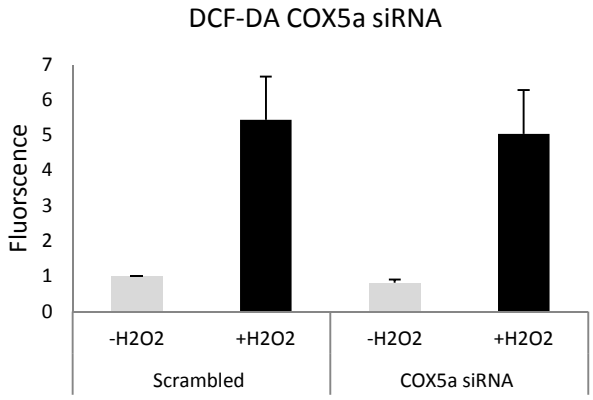


Figure 6.16 Mitochondrial ROS production. Negative control (Scr) and SiRNA transfected cells (COX5a). Data are expressed as fold change ± SE.

6.8.4 Membrane Potential (JC1)

$\Delta\Psi_M$ was assessed in COX5a transfected cells compared to control. No significant difference was noted in COX5a transfected cells compared to controls.

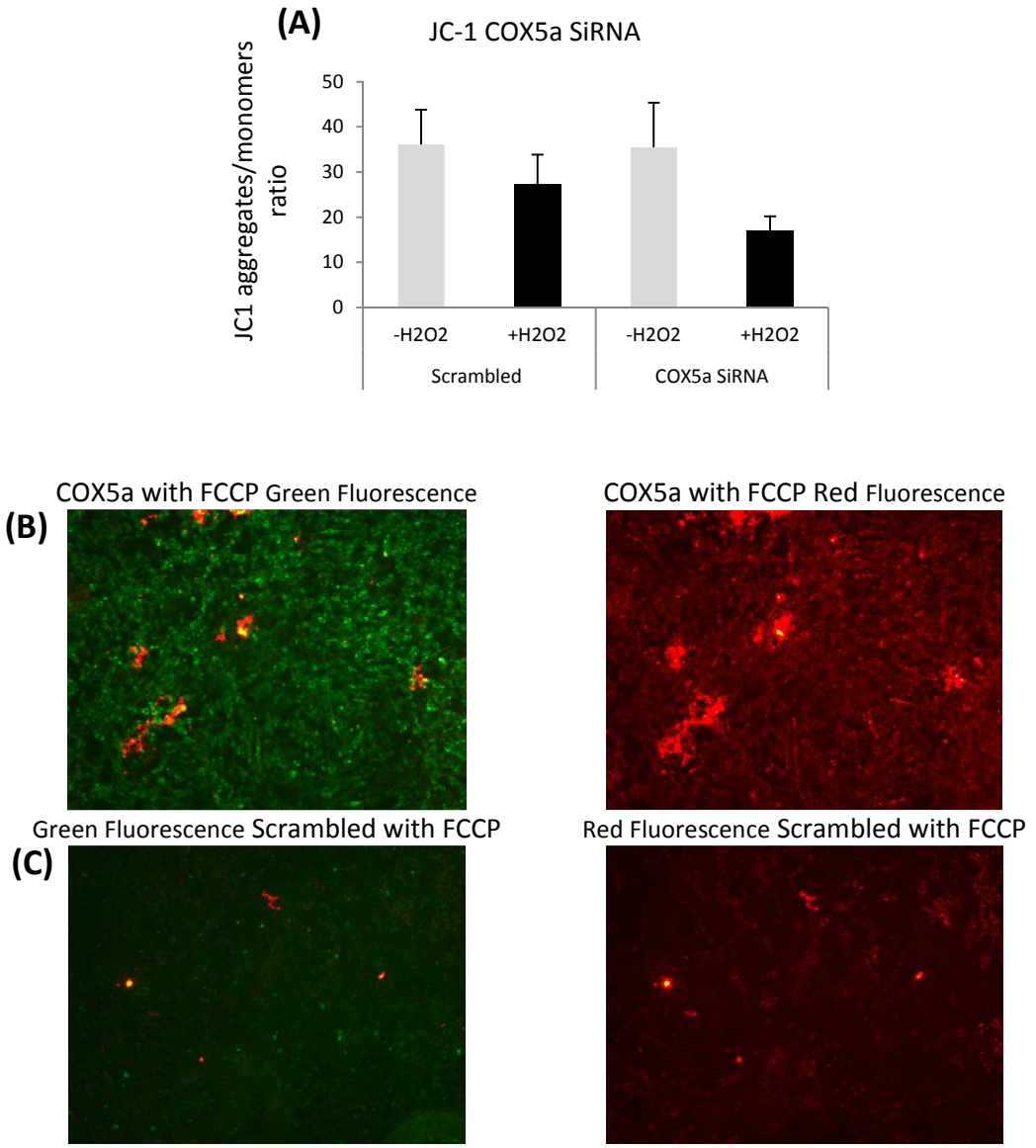


Figure 6.17 Mitochondrial Membrane Potential. (A) Mitochondrial membrane potential measured using the JC-1 assay. (B) Fluorescent images display green fluorescence red fluorescence (J-aggregates) and green fluorescence in (B) COX5a transfected cells with and without FCCP and (C) negative control cells (Scr) with and without FCCP. Data are expressed as fold change ± SE.

Chapter VII

Discussion

7.1 Overview

The main finding of this thesis was that the physiological changes following 21-days of bed rest were accompanied by subtle changes in mitochondrial function. The decrease in LEAK respiration may be linked to increased ROS production and the correlation between insulin sensitivity and maximal uncoupling suggests a general decrease in the efficiency of mitochondria. In support of this finding, a decrease in citrate synthase activity was observed and this may reflect a decrease in mitochondrial mass and/or tricarboxylic acid cycle activity. While mitochondrial function may have been maintained, as indicated by similar oxygen consumption relative to tissue mass, the decrease in citrate synthase activity adds support to a decrease mitochondrial efficiency.

In cell culture models that have been previously shown to induce insulin resistance there was an increase in LEAK respiration when exposed to high glucose/insulin for 48-hrs and an increased sensitivity to ROS. The increased LEAK is likely to represent an adaptive response to excess nutrient availability but demonstrates early changes in complex I following metabolic dysregulation. In contrast, when myotubes were exposed to palmitate for 24-hrs there was a change in the membrane potential indicative of decreased mitochondrial stability. Therefore, the early changes in mitochondrial efficiency may depend on whether there is an excess of carbohydrate or palmitate.

Finally, we made the novel observation that the expression of cytochrome oxidase subunit 5a (COX5a) was decreased following bed rest. When the expression of this protein was knocked down in cultured cells there was a reduction in mitochondrial function in complexes I-III. Therefore, the early changes in complex I may be linked to altered expression of proteins in other ETS complexes.

7.2 Energy Regulation

The present study was designed to maintain the fat mass of each subject during 21-days of bed rest. This was achieved by monitoring body composition every second morning using bioelectrical impedance spectroscopy (BIS) which quantified fat mass and fat free mass. Body weight was also measured every morning. In addition, to BIS, a DEXA scan was conducted at 8 time points throughout each bed rest campaign. Any changes that were noted in body composition allowed us to alter dietary intake to ensure the subjects

remained in energy balance. Our results suggest that the subjects remained in energy balance during the study as there was no change in fat mass. However, there was a ~3kg reduction in lean muscle mass that was similar to the decrease in body weight. Interestingly, leg and arm fat free mass, as measured by DEXA, did not show any change which suggests that the loss of lean muscle mass could be in postural and abdominal muscle primarily. This is in contrast to other studies that have reported a decrease in leg muscle mass but the reliance on DEXA scanning to provide the necessary precision may be a limiting factor in this case. Finally, resting energy expenditure was also measured at 6 time points throughout bed rest which allowed the dietician to adjust energy intake to match energy expenditure, taking into account energy expenditure from upper body movement also.

In previous bed rest studies, diet has been adjusted in order to clamp body mass to pre bed rest values (Gretebeck, Schoeller et al. 1995). In other studies, this approach was chosen with the idea that the outcome measurements of the bed rest would be independent of any changes in energy balance or under nutrition. Since bed rest reduces physical activity energy expenditure by 40% and muscle atrophy causes a further reduction in resting energy expenditure (Blanc, Normand et al. 1998), maintaining body mass results in a positive energy balance and an increase in fat mass (Gretebeck, Schoeller et al. 1995). In order to standardize bed rest studies in a way that controls energy balance, real time energy intake should be monitored and altered based on energy expenditure.

The loss of fat-free mass during bed rest is due to muscle disuse independent of energy balance (Stein 2000). In fact muscle atrophy is even more pronounced when there is a positive energy balance. In the present study, fat mass did not vary significantly in any group confirming that they did maintain a stable energy balance during bed rest. This is subject to confirmation as doubly labelled water was administered to a subgroup of participants to measure total daily energy expenditure and is pending analysis. Interestingly, similar results were obtained in a 42 day bed rest study when healthy men were provided food at meals in an *ad libitum* manner (Blanc, Normand et al. 1998). Total energy expenditure was measured using doubly labelled water (DLW) for 15 days before and 15 days during bed rest. During bed rest, total energy expenditure and total energy intake were significantly reduced and resting energy expenditure was maintained. The reduction in total energy expenditure was due to the 39% reduction in physical activity

energy expenditure and not to lean body mass loss. This study also suggests that during long term bed rest energy intake is intrinsically adjusted to match total energy expenditure and maintain energy balance. In the present study, a gradual reduction in resting metabolism rate could be attributed to the reduction in lean muscle mass.

While the decrease in RMR was expected, we did not find a change in resting RQ, unlike other studies (Blanc, Normand et al. 2000, Bergouignan, Schoeller et al. 2006, Bergouignan, Trudel et al. 2008). Our results suggest there was no change in substrate oxidation while previous studies have shown that physical inactivity induces a shift in resting substrate use from fat to carbohydrate oxidation (Bergouignan, Trudel et al. 2009). This shift is independent of energy balance (Stein and Wade 2005). The maintenance of steady state body weight requires suggests that the daily oxidation of fuel matches the amount and composition of nutrients in the diet (Jéquier and Tappy 1999). It may be expected that body composition changes are linked to changes in substrate oxidation balance. Previous studies have shown that RQ increases from 4–14%, depending on the duration of bed rest (Blanc, Normand et al. 2000, Bergouignan, Schoeller et al. 2006, Bergouignan, Trudel et al. 2008). The change in fuel use is independent of muscle atrophy but may be linked to an increase in glycolytic muscle fibres and a reduction in oxidative muscle fibres (Trappe, Trappe et al. 2004). This interpretation is not supported in animal studies that showed the shift in substrate use preceded the change in muscle fibre type (Grichko, Heywood-Cooksey et al. 2000). Therefore, the mechanisms involved in these changes in substrate use need further clarification.

Another consideration for the interpretation of results from this study is that while we are reporting no change in fat mass, it is possible that there was a redistribution of fat into the cells. For clarification of this we are conducting a lipidomic analysis with Matej Oresic at the Steno Diabetes Research Centre but the results were not available in time for submission of this thesis. The reduction in fat oxidation coincides with ectopic fat storage during bed rest (Bergouignan, Trudel et al. 2008). A significant increase in intramuscular lipid after 2 months of bed rest in women was correlated with a reduction in dietary palmitate oxidation (Bergouignan, Trudel et al. 2008) suggesting dietary fat is diverted from oxidation in the muscle towards muscle lipid incorporation. In another bed rest study, Cree et al. (2010) reported a negative correlation with intramuscular lipids and decreased glucose uptake. Lipid accumulation in skeletal muscle is associated

impaired insulin stimulated glucose uptake (Krssak, Petersen et al. 1999). Excess lipid accumulation can induce lipotoxicity as reflected by the accumulation of ceramides and diacylglycerides (DAG) (Moro, Bajpeyi et al. 2008). These lipid intermediates impair insulin signalling through increased serine phosphorylation of insulin receptor and IRS1 and reduced serine phosphorylation of Akt (Morino, Petersen et al. 2006).

In conclusion, maintaining energy balance during bed rest appears to be the best approach to prevent an increase in fat mass. This approach accepts that muscle mass loss is inevitable and further work is necessary to identify the optimal countermeasure. This may require a more rigorous exercise programme but the benefit has to be carefully considered as increased energy expenditure will increase the risk of a negative energy balance.

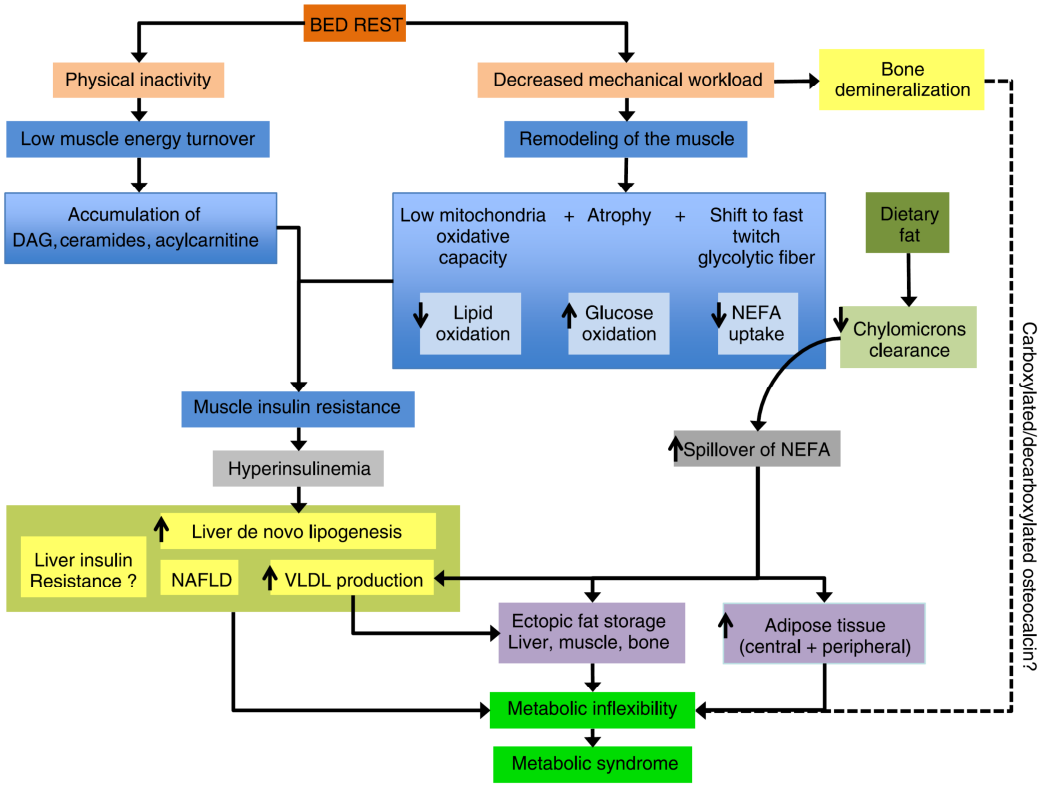


Figure 7.1 Hypothetical metabolic alterations cascade induced by bed rest that can explain how physical inactivity induced metabolic flexibility. Adapted from Bergouignan et al. (2011).

7.3 Oxygen consumption

The current bed rest study and many others have reported a reduction in VO_2 max following bed rest. There was also a reduction in peak power, peak ventilation, an increase in peak HR in addition to RER. In this study, aerobic capacity was assessed before and after 21 days bed rest but previous studies reported a rapid decline in the first few days of bed rest and a more gradual decrease thereafter (Greenleaf, Bernauer et al. 1989, Convertino 1997, Capelli, Antonutto et al. 2006). Interestingly, the rate of decline in aerobic capacity becomes progressively smaller as bed rest duration increases, up to 42 days (Capelli, Antonutto et al. 2006), demonstrating the body's ability to adapt to its new environment.

VO_2 max data obtained on four subjects by Greenleaf et al (1989) at 7, 14, 21 and 29 days of bed rest show the greatest average daily rate of VO_2 max decline occurred in the first week. Early changes in VO_2 max (2 weeks) with bed rest are associated with decreased blood volume (Convertino 1995) and lower maximal cardiac output (Capelli, Antonutto et al. 2006). While the present work does not present data on cardiac output and oxygen delivery, it is well documented (Capelli, Antonutto et al. 2006) that both parameters are decreased in parallel with a reduction in aerobic capacity following bed rest. These findings suggest that the early decrement in VO_2 max is associated with reduced cardiac output (Lee, Moore et al. 2010). The rate of decrease with longer duration bed rest may be associated with peripheral and central factors. Today, it is universally accepted that there is a physiological upper limit to the body's ability to consume oxygen. The prevailing view is that in the exercising human, VO_2 max is limited primarily by the cardiorespiratory system (heart, lungs and blood) and not the ability of the muscle to take up oxygen. However, the pathway of oxygen from the air to the mitochondria contains a series of steps, each of which could pose as a limitation for maximal O_2 flux.

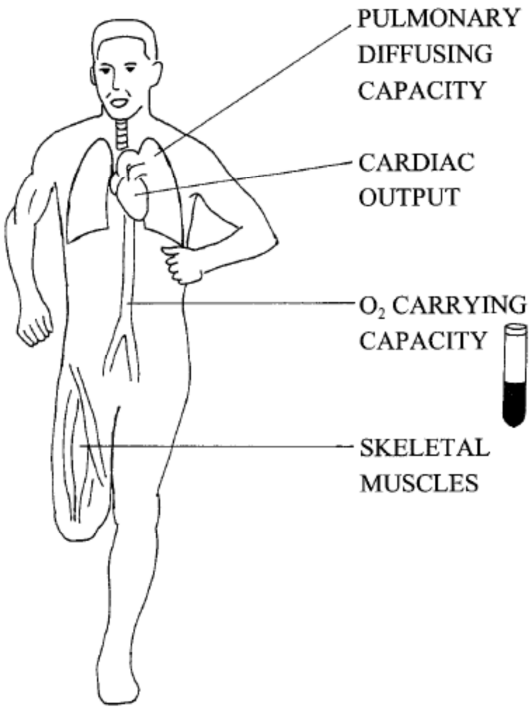


Figure 7.2 Physiological factors that potentially limit maximum oxygen uptake (VO2 max) in exercising humans. Adapted from Bassett & Howley (1999).

While max ventilation ($l \cdot min^{-1}$) was significantly reduced in the present study after bed rest, the literature suggests that pulmonary limitation to VO2 max is unlikely because at sea level, the lungs perform their job of saturating the arterial blood with oxygen successfully. Even during maximal intensity exercise, O2 saturation (SaO2) remain above 95% (Powers, Lawler et al. 1989). Additionally, elite athletes are more likely to have arterial O2 desaturation at maximal exercise due to the decreased transit time of red blood cells in pulmonary capillaries as they have a much high maximal cardiac output compared to untrained individuals (40 vs 25 $L \cdot min^{-1}$). Therefore, it is unlikely that ventilatory max posed a limitation to VO_2 max in this study. A likely limiting factor is maximal cardiac output, the product of stroke volume and heart rate. In the present study, maximal heart rate increased after bed rest suggesting an attempt to increase cardiac output to achieve the same workload. This also indicates that heart rate has the ability to increase and the inability to increase stroke volume is the main cause of the reduction in cardiac output. This is supported by Ferretti et al (1997) who demonstrated that the reduction in maximal cardiac output was solely due to a reduction in stroke volume. This could be a result of a reduction in plasma volume, diminished cardiac function or impaired vascular function. In bed rest, maximal cardiac output declines rapidly in the first weeks and does not change much thereafter (Capelli, Antonutto et al.

2006), suggesting that peripheral factors possibly at the level of the muscle may be responsible for any further decrement in VO_2 max.

7.4 Skeletal Muscle Adaptation

In this study we report a significant reduction in lean muscle mass, peak oxygen consumption and maximal force production, determined by decrements in knee and ankle extensor and flexor muscles. These results suggest that skeletal muscle function is impaired following 21-day bed rest. The gradual decrease in actively respiring lean muscle may impact maximal aerobic capacity as local tissue changes may contribute more with longer duration bed rest. While we did not have MRI data, other studies support our findings and show that muscle size is reduced 3% after 7 days bed rest (Ferrando, Stuart et al. 1995, Akima, Kubo et al. 2001), with 4-5% strength loss per week (Berg, Larsson et al. 1997, Funato, Matsuo et al. 1997, Bamman, Clarke et al. 1998).

Another aspect of muscle morphology that would affect maximal oxygen consumption is muscle fibre type. A shift from slow to fast myosin isoform composition has been reported with bed rest (Trappe, Trappe et al. 2004). Type I slow fibres are characterized by a more oxidative phenotype that contain a higher number of mitochondria and a greater oxygen consuming capacity. Therefore, the reduction in type I fibres could contribute to the reduction in skeletal muscle oxygen uptake. When we consider these physiological changes at the level of the muscle, it is imperative that we consider peripheral alterations in the muscle as potential reasons for reduced maximal oxygen consumption.

7.5 Mitochondrial function: Leak respiration

Within the muscle cells, O_2 is consumed in the final step of the mitochondrial electron transport chain. Both the content and the intrinsic capacity of mitochondria play an important role in the capacity of the skeletal muscle to oxidize fuel. The present study is the first study to assess mitochondrial function with bed rest. We hypothesized that physical inactivity, through the model of bed rest, would decrease mitochondrial function in healthy young men. We have reported a reduction in LEAK respiration (State 4) with the addition of pyruvate and malate and palmitoylcarnitine and malate and in

the absence of ADP in permeabilized skeletal muscle fibres after bed rest. LEAK respiration referes to the process whereby protons 'leak' back into the matrix of the mitochondria through alternative conductance pathways, uncoupling substrate oxidation from ATP synthesis. While these pathways may appear to be futile, there are a number of reputed beneficial roles including thermogenesis and body mass regulation due to the high energy cost (Rolfe and Brand 1996), an improved ability to regulate energy metabolism, a safety valve to avoid excessive membrane potentials and attenuation of free radical production (Rolfe and Brand 1997). According to the free radical theory of aging (Harman 1955), the process of oxidative metabolism in aerobic cells is accompanied by the production of oxygen to superoxide and other ROS such as hydrogen peroxide and hydroxyl radicals. ROS can be potentially damaging to cell and while cells have a powerful antioxidant defence system to protect against ROS damage, a lot of attention has been focused on antioxidants and not much given to the regulation of ROS. It has been suggested that an increase in LEAK respiration could reduce ROS production by causing the oxidation of ubiquinone (Brand 2000). The reduction in LEAK respiration in this study suggests that the cells more likely to produce ROS. However, to date there is no direct evidence that a reduction in LEAK could increase ROS production.

The *in vitro* work in this thesis shows an increase in LEAK respiration in myotubes, when normalized to ETS, treated with high glucose and insulin. This indicated that mitochondrial coupling has been affected by the metabolic stress. This could reflect the nutrient overload used in the model, which is different to the human bed rest study. It is not possible to replicate the in vivo response using a cell culture model but the addition of high glucose/insulin and palmitate have been used in previous experiments to induce insulin resistance and were deemed suitable. However, a careful and balanced interpretation of these findings is required.

There was no significant increase in real time ROS production, as measured by fluorometry but there was an increased sensitivity to hydrogen peroxide measured with the traditional DCFDA assay. There is a link between changes in leak respiration and increased ROS. When proton leak is increased there are more electron-rich intermediates present (e.g. ubisemiquinone) which are available for ROS generation. In the palmitate treated cells, there was no change in LEAK respiration or ROS production but there was an interesting 3-fold decrease in the mitochondrial membrane potential as measured using the JC-1 assay. While this does not appear to contribute to ROS

production or mitochondrial dysfunction, it may make the mitochondria more unstable which could potentially lead to mitophagy. It may also be possible that the increase in mitochondrial mass in the palmitate treated cells could be the reason that mitochondrial respiration is maintained. This early adaptive response in mitochondrial biogenesis has previously been reported as a means of compensating for excess nutrient availability. Alternatively, the 24-hr incubation may not have been long enough for ROS production to increase and longer duration experiments would help clarify the link between mitochondrial function and ROS production.

Mitochondrial superoxide production as well as ROS production is extremely sensitive to the electrochemical potential across the inner mitochondrial membrane which becomes apparent under state 4 respiration / LEAK respiration (resting, non-ADP stimulated respiration). Superoxide production has been reported to be increased during fatty acid oxidation (St-Pierre, Buckingham et al. 2002) which could suggest that ROS induced activation of uncoupling protein 3 (UCP3) may serve as a feedback mechanism to attenuate mitochondrial ROS production, thereby limiting ROS-induced cellular damage. UCP protein content was unchanged in our experiments though an increase in FA oxidation has been linked to an increase in UCP3 expression in other studies (Hoeks, Hesselink et al. 2006, Anderson, Yamazaki et al. 2007). Therefore, despite no change in the expression UCP3, this could explain the difference in H_2O_2 production in the high glucose / insulin and palmitate treated cells where the palmitate treated cell produced less ROS.

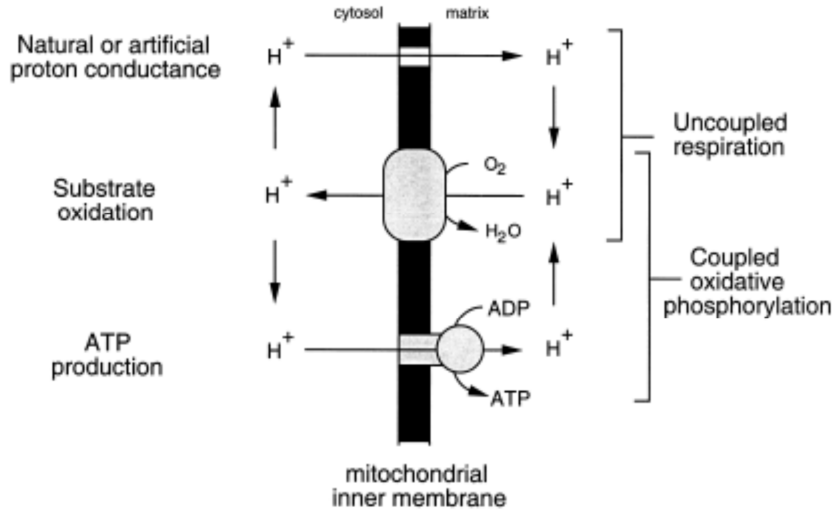


Figure 7.3 The chemiosmotic proton circuit in the mitochondrial inner membrane. The lower circuit showing coupled respiration and the upper circuit showing uncoupled respiration. Adapted from Brand 2000.

7.6 Normalizing for Mitochondrial Content

The interpretation of mitochondrial respiration data is dependent on normalising the results for the content of mitochondrial. In some cases, especially steady state conditions, this may not be difficult but in states of metabolic flux it can be challenging to accurately quantify mitochondrial content. There is a lot of variability in the literature and since the conclusions are influenced by the normalisation this issue needs to be discussed in detail. Mitochondrial content or volume is an important quantitative indicator of oxidative capacity and is commonly used to normalize respiratory capacity. The gold standard for measuring mitochondrial fractional area is two-dimensional imaging using transmission electron microscropy (TEM). Other biochemical markers of mitochondrial proteins, lipids, enzymes and DNA are used as biomarkers of mitochondrial content. In the bed rest study we normalized mitochondrial respiration to citrate synthase activity. The role of citrate synthase in the cell is to catalyse the initial reaction of the TCA cycle between acetyl CoA and oxaloacetate to form a six-carbon citrate. We reported a significant reduction in citrate synthase activity following bed rest. However, one concern is that citrate synthase is an enzyme of the TCA cycle and could fluctuate with TCA cycle activity. Therefore, it may be used as an indicator of TCA cycle activity or mitochondrial content. While citrate synthase activity may be a strong marker of content in lean or obese or insulin resistant phenotypes, another concern is that citrate synthase could respond to the dynamic nature of the bed rest intervention, independent of mitochondrial content as shown with exercise (Tonkonogi, Harris et al. 1997).

However, a study undertaken by Larsen et al (2012) examined a number of biomarkers of mitochondrial content and showed the strongest correlation with TEM. Cardiolipin, a phospholipid located on the inner mitochondrial membrane, showed the strongest concordance, then citrate synthase followed by complex I activity. In contrast mtDNA was not significantly correlated with mitochondrial content which was surprising as previous studies have shown a significant positive correlation between citrate synthase and mtDNA content in human vastus lateralis (Wang, Hiatt et al. 1999). An important consideration is that mitochondrial nucleotide contains a variable number of DNA copies making mtDNA a semi quantitative indicator of mitochondrial mass. Previous studies have validated the use of citrate synthease as a marker of mitochondrial content

showing similar change in activity of citrate synthase and morphological change in mitochondrial content (Reichmann, Hoppeler et al. 1985). If cardiolipin and citrate synthase were used to normalize mitochondrial function, Larsen et al (2012) suggest cardiolipin has advantages. Citrate synthase can contribute to the oxidation of substrates whereas cardiolipin is not directly involved in substrate oxidation. However, citrate synthase is easy to measure. We were unable to measure a greater number of biomarkers of mitochondrial content. However, whether citrate synthase is an indicator of mitochondrial content or TCA cycle activity the results indicate a significant decrease. The interpretation of our mitochondrial respiration data is based on both scenarios.

7.7 Mitochondrial Function

We hypothesized that there would be a reduction in mitochondrial function after 21 days bed rest. When we normalized mitochondrial respiration for wet weight we did not find any change in respiratory capacity but there was an increase in respiration relative to citrate synthase activity. This suggests that the muscle was able to maintain normal respiratory capacity but more oxygen was consumed per unit of citrate synthase. There was an increase in respiratory flux with ADP saturation, ETS capacity, complex I and IV capacities.

In type 2 diabetic skeletal muscle there was a decrease in respiration when normalized for wet weight but no change in respiration when normalized for mtDNA or citrate synthase (Boushel, Gnaiger et al. 2007). In healthy skeletal muscle our results indicate that the mitochondria have to work harder to maintain normal function. It could be that the increase in work rate is an early adaptive/protective response to physical inactivity and an attempt to prevent any further damage e.g. oxidative stress. It is also interesting that other research groups have shown a compensatory increase in mitochondrial oxidative capacity in the presence of impaired insulin sensitivity and glucose tolerance by feeding mice a high fat diet over 8-12 weeks (Garcia-Roves, Huss et al. 2007, Turner, Bruce et al. 2007, de Wilde, Mohren et al. 2008). This suggests that there is a compensatory response to increase the rate of oxidation in order to protect the cell from damage. However, this compensatory response may only be transient and is lost as insulin resistance and obesity progress (Sreekumar, Unnikrishnan et al. 2002). Our in vitro experiments support the data showing a compensatory increase in mitochondrial

content and/or function when cells are exposed to excessive nutrients. Longer duration experiments or different models may be required to investigate mitochondrial dysfunction.

We measured a number of mitochondrial proteins and found a decrease in COX5a expression but no change in COX IV, both subunits of complex IV of the ETC. This suggests there is not a uniform change in ETS subunit protein content during metabolic stress. We did not find a change in mitochondrial proteins when using a cocktail of antibodies. This cocktail is often reported as an indicator of subunit content but this interpretation is too general. The cocktail only quantifies one protein in each complex and may not reflect the rate of change in other subunits and therefore the function of the entire complex. This was also evident when we found a decrease in SDHB protein content. This subunit of complex II is responsible for the transfer of electrons from succinate to Co enzyme Q / ubiquinone. Interestingly, we did not find any change in SDHa content, as measured by the mitochondrial cocktail.

While the measurement of mitochondrial proteins and enzymes inform us about the oxidative capacity of the mitochondria, *ex vivo* measurement of O_2 consumption provide greater insight into mitochondrial function. The terms mitochondrial 'function' and 'dysfunction' are widely employed in bioenergetics, however precise definitions are difficult as different studies employ different methods to measure 'function'. However, the predominant physiological function of the mitochondria is the generation of ATP by oxidative phosphorylation (Brand and Nicholls 2011).

7.8 Muscle Atrophy and Insulin Resistance

We found a significant reduction in insulin sensitivity when normalized for FFM following bed rest. There was a significant correlation between the change in insulin sensitivity and change in Complex I linked ETS in the control group. While the changes in mitochondrial function contrast with reports in skeletal muscle from type 2 diabetics we also reported a significant reduction in mitochondrial content/TCA activity, as measured by CS. Therefore the relationship between insulin sensitivity and mitochondrial dysfunction is important. As skeletal muscle accounts for 80% of insulin-mediated glucose disposal, the tissue is considered an important player in the development of insulin resistance. Earlier reports suggest that the activity of oxidative enzymes in

skeletal muscle was reduced in type 2 diabetic patients (Simoneau and Kelley 1997, Simoneau, Veerkamp et al. 1999). Interest in the role of mitochondria in insulin resistance intensified when Kelley et al. (2002) described dysfunctional mitochondria in type 2 diabetes. Other influential work reported the reduction in expression of PGC-1α, an important regulator of mitochondrial biogenesis, in type 2 diabetics (Patti, Butte et al. 2003). Many other studies have identified reduced mitochondrial content in insulin resistant skeletal muscle (Kelley, He et al. 2002, Ritov, Menshikova et al. 2005, Boushel, Gnaiger et al. 2007, Chomentowski, Coen et al. 2010). All of these studies strongly implicate mitochondrial dysfunction in insulin resistance. To further support that mitochondrial dysfunction is associated with insulin resistance, intrinsic mitochondrial function and mitochondrial density was studied extensively. In this context, Ritov et al. (2005) showed a reduction in activity of the ETC in type 2 diabetics versus controls, a difference that could not be explained fully by the observed reduction in mtDNA which further suggests an intrinsic defect. Support of intrinsic mitochondrial defect in type 2 diabetics was reported by Mogensen (2007) who reported a reduction in mitochondrial respiration isolated mitochondria. Taken together, we can deduct that there is a relationship between the presence of mitochondrial dysfunction (intrinsic defect or reduced content) and insulin resistance.

Physical inactivity is a well-established risk factor for the development of insulin resistance and type 2 diabetes (Booth, Laye et al. 2007) and is shown to reduce insulin sensitivity in the present study. Physical inactivity causes a reduction in VO_2 max which is supported by others (Convertino, Bloomfield et al. 1997, Capelli, Antonutto et al. 2006), reduced skeletal muscle oxidative enzyme (CS), proteins and decreased lipoprotein lipase (Bey and Hamilton 2003). The participants in our study were healthy young males and were insulin sensitive. It is important that the results are interpreted as a decrease in insulin sensitivity and not as the development of insulin resistance, per se. It is reasonable to conclude that decreased insulin sensitivity following bed rest is associated with subtle changes in mitochondrial function or efficiency. Together, the findings of the present study and findings reported in the literature support the view that physical inactivity-mediated reductions in oxidative metabolic capacity may limit skeletal muscle metabolism of circulating nutrients. While physical inactivity may contribute to mitochondrial down-regulation and reduced insulin sensitivity we do not have sufficient

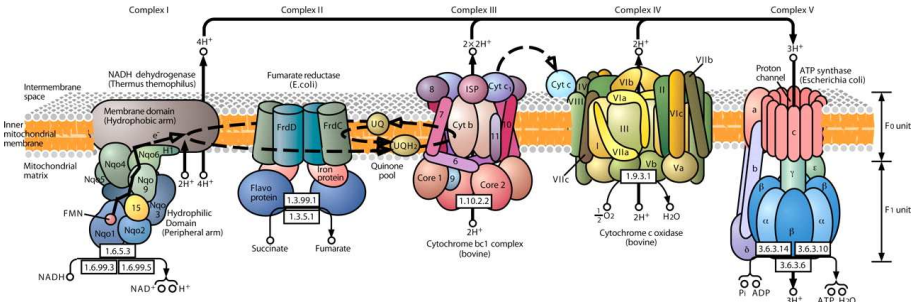
data at this time to confer a causal relationship between mitochondrial dysfunction and insulin resistance.

Physiological systems are often studied in isolation but it is challenging to develop an integrated approach. There is a high degree of overlap between the cellular events that regulate glucose uptake, protein balance and cell metabolism in skeletal muscle. A small change in one of the cascades could have an impact on the other processes. A reduction in insulin sensitivity is associated with decreased insulin stimulated PI3K activity, an upstream regulator of Akt activity and E3 ubiquitin-conjugating enzymes, Atrogin-1/MafBX and MuRF1 (Sandri, Sandri et al. 2004). We did not find a change in Akt protein content but did see an increase in FOXO1. Under normal conditions, Akt phosphorylates FOXO1 and causes its translocation to the cytoplasm, allowing for the expression of metabolic proteins such as PGC-1α and suppressing the expression of Atrogin-1 and MuRF1. We have only measured the protein content and there may have been changes in phosphorylation that would provide greater insight into the functional consequences. We were only able to obtain fasting biopsies and future studies should investigate the insulin stimulated response also. The measurement of other proteins in the insulin (IRS-1, GLUT-4) and atrophy (MuRF1, Atrogin-1) signalling cascades was also undertaken. Given the amount of biopsies and difficulties with some of the antibodies it was not possible to have all of this data completed.

7.9 COX5a

In the bed rest study we made the novel observation of a decrease in COX5a protein content. This protein has been of interest to our lab since a proteomic analysis of a mitochondrial-enriched fraction showed decreased expression following exercise training (Egan, Dowling et al. 2011). COX5a is one of 10 nuclear encoded subunits of Complex IV of the electron transport chain that also contained 3 mitochondrial encoded proteins. Complex IV is known to couple the transfer of electrons from cytochrome c to molecular oxygen and contributes to the inter-membrane proton gradient that forms the basis of ATP synthesis in the mitochondria. The function of nuclear-encoded subunits is unknown but, up to now, they have been associated with the regulation and assembly of the complex.

(a)



(b)

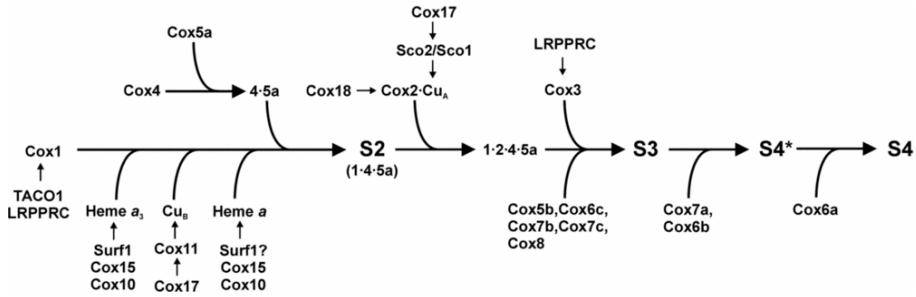


Figure 7.4 (a) Pathway of oxidative phosphorylation (Adapted from Kegg analysis www.genome.jp/kegg), (b) Proposed model for the assembly of Complex IV of the electron transport chain.

COX5a is located on the matrix side of the inner mitochondrial membrane, adjacent to the ATP binding domain COXIV, and is one of only three subunits not to have a transmembrane domain (Tsukihara, Aoyama et al. 1996). It is involved in the early formation of Complex IV, binding with COXIV prior to binding COX I (Fig. 7.4), and appears to be evolutionarily conserved with only 5 amino acid differences between human, mouse, rat, cattle and boar (Schmidt, Goodman et al. 2002).

There are very few studies reporting COX5a data in mammalian tissue, most of them are descriptive in nature and none have investigated a possible mechanistic role for the protein. In a mouse model of diabetes COX5a protein expression, but not mRNA, was lower in islet cells (Lu, Yang et al. 2008). COX5a mRNA has been reported to be lower in kidney cells following LPS treatment in mice (Feingold, Wang et al. 2008) and increased following cardiac myocyte electrical stimulation (Kia) and in skeletal muscle from aerobically trained humans (Parikh, Nilsson et al. 2008). In C2C12 myotubes COX5a mRNA expression was regulated by PGC-1α, a metabolic co-activator that induces mitochondrial biogenesis (Gerhart-Hines, Rodgers et al. 2007).

Our initial findings in a mitochondrial-enriched fraction from human skeletal muscle did not support the positive association between COX5a expression and mitochondrial function as reported in these studies. We found a marked increase in the mRNA and protein expression of mitochondrial gene transcription factors (PGC-1α, ERRα, NRF-1) and mitochondrial proteins (COXIV, CPT1α, malate dehydrogenase, ATP synthase β) to accompany the increase in whole body oxygen consumption following 14-days of exercise training (Egan, Dowling et al. 2011, Egan, O'Connor et al. 2013). These positive adaptations were accompanied by a 49% decrease in mitochondrial COX5a protein. We formulated a working hypothesis that COX5a was a negative regulator of Complex IV activity and protects against excessive electron transport chain activity. This was supported by one functional study in the literature that showed thyroid hormone binding to COX5a, regulating the ATP-binding domain on COXIV, and abolishing the allosteric inhibition of respiration by ATP (Arnold, Goglia et al. 1998). We believed the mitochondrial re-modelling found in the exercise training study from our lab would increase the capacity for ATP production but this would only be required during exercise. Therefore, more sensitive regulation of COX activity was required and a decrease in COX5a would facilitate this process.

The decrease in COX5a protein expression following bed rest appears to contradict the exercise training data and, despite many attempts, we have been unable to replicate the impact of thyroid hormone on mitochondrial function in mammalian cells. These findings suggest a re-formulation of the working hypothesis is required. However, the analysis of our exercise study was performed in a mitochondrial-enriched fraction and opens the possibility of an exciting hypothesis that COX5a is not just a structural protein but may leave the mitochondria and act elsewhere in the cell. This is supported by the fact that COX5a is one of only 3 Complex IV subunits not to have a trans-membrane domain (Tsukihara, Aoyama et al. 1996). This is untested and will require further investigation.

In order to determine if COX5a was having an impact on mitochondrial function we silenced the protein expression in C2C12 myoblasts. Our preliminary results show that we were able to successfully reduce COX5a expression by 80% and this was associated with a trend toward decreased oxidative phosphorylation and maximal uncoupling. However, there was no change in oxygen consumption by Complex IV. These data suggest that COX5a is necessary to maintain normal mitochondrial respiration and

supports the notion that it is not just a structural protein in the assembly of Complex IV. The role it may play in mitochondrial function is not known. In a previous study, nematodes treated with RNAi for COX5a took longer to reach adulthood and had a significantly reduced lifespan. They report that COX5a deficient nematodes had decreased intrinsic complex I enzymatic activity despite no change in complex I content (Suthammarak, Yang et al. 2009). Unlike our data in myoblasts, they found a decrease in Complex IV dependent respiration. While the metabolic regulation of mammals is more complex than nematodes these data support the notion that a decrease in COX5a protein can negatively impact the function of other complexes in the electron transport chain. One possibility is that COX5a is translocated to these complexes to regulate function but another, more likely, possibility is that the formation of super-complexes (I:III:IV) in the mitochondrial membrane means that the reduction of one protein may affect a number of complexes.

In conclusion, these data suggest a possible role for COX5a in the regulation of cellular metabolism. The protein content is decreased in whole muscle homogenates following 21-days of bed rest and our in vitro experiments demonstrate a decrease in mitochondrial function in C2C12 myoblasts. The mechanism of action requires further investigation.

7.10 Countermeasures

The present study implemented resistive vibration exercise as a countermeasure alone and in combination with whey protein supplementation. The benefits of exercise have been widely reported and, as a result, some form of exercise is a mandatory component of all bed rest studies. The subjects in the present study completed the exercise twice a week for less than 30 minutes, in the head down tilt position at all times. While heel raises were completed to exhaustion, squatting exercises were completed for 8 repetitions and a moderate intensity of 50 and 75% 1RM. The vibration parameters were 8 mm vibration amplitude and 25 Hz. The Berlin Bed rest study aimed to test the effectiveness of resistive vibration exercise in healthy males over 56 days bed rest. However, they completed 2 exercise sessions per day on 5 days/week. They reported that the high load resistance/vibration exercise was effective in reducing and delaying atrophy in the soleus and medial gartrocnemius and vasti muscles during bed rest. The

load and frequency of exercise in the present study may not have been sufficient to protect from deconditioning effects of bed rest.

While the present study shows an increase in mitochondrial respiration when normalized for citrate synthase, in the Fatty Acid SUIT, there was a clear increase in respiration for the two countermeasure groups and a reduction in respiration for the control group, again suggesting that exercise and protein supplementation did have a beneficial effect. We used a 2-way repeated measures ANOVA to test for statistical significance in this study. While this is appropriate given the trial and time components of the experimental design there may be other tests to discriminate more sensitively between trials. In our design we had one control and two intervention trials. While a visual inspection of the results suggest that the decrease in many variables during the control trial were partially mitigated in at least one of the intervention trials, there were no between trial differences. Instead we found an effect of time in most cases. While our interpretation is based on the statistical tests used in the study they may be overly stringent.

The physiological rationale of including exercise is well established. In brief, high intensity exercise improves VO_2 max, O_2 pulse and peak power output in healthy men and women (Astorino, Allen et al. 2012). Insulin sensitivity is improved following exercise training and there is a positive correlation between glucose disposal and PI3K activity in healthy males (Kirwan, del Aguila et al. 2000). Protein synthesis is promoted by exercise and protein degradation is reduced The increase in protein synthesis following resistance exercise correlates with the activation of PI3K, mTOR S6K and Akt (Baar and Esser 1999, Bolster, Kubica et al. 2003, Nader 2005). In addition, Akt has the additional effect of phosphorylating FOXO1 making it unable to enter the nucleus and drive transcription of Atrogin-1 and MuRF1 (Manning and Cantley 2007).

The best countermeasure developed so far has been the combination of resistance exercise and vibration exercise (Armbrecht, Belavý et al. 2010). The Berlin bed rest studies demonstrated that vibration had particular benefit for bone (Belavý, Beller et al. 2011). In the 1st Berlin bed rest study, a very demanding vibration exercise regime was applied (frequency = 19 – 25 Hz and amplitude was variable) 11 times per week. Muscle atrophy in the plantar flexors and knee extensors was attenuated by vibration exercise protocol (Blottner, Salanova et al. 2006, Mulder, Stegeman et al. 2006). In a 60 day bed

rest study, an aerobic exercise and resistance exercise countermeasure was shown to protect against loss of strength, however, no protective effect of protein supplementation without exercise was reported (Lee, Schneider et al. 2014). A study by Tabata (1999) demonstrated a remarkable increase in GLUT-4 expression (30% above pre rest values) after 19 bed rest and isometric resistance exercise, while there was a 15% decrease in GLUT-4 concentration in the control group (Tabata, Suzuki et al. 1999). No protective effects of muscle strength or mass were noted in the present study. However, while we saw a reduction in VO_2 max and an decrease in peak power, the countermeasures appear to show a lower decrement when compared to the control group. A similar trend was noted for insulin sensitivity in stage 2 of the clamp where a significant reduction in insulin sensitivity was only noted in the control group and no change was seen in either countermeasure group.

The optimum countermeasure is likely to be a combination of exercise and nutritional supplementation. In this study exercise and whey protein supplementation was used to minimize the loss of skeletal muscle mass. A 50% reduction in protein synthesis has been reported in 14 days of bed rest (Ferrando, Lane et al. 1996). Essential amino acids have been shown to stimulate muscle protein synthesis and prevent loss of lower body mass with bed rest (Paddon-Jones 2006). Whey protein in particular is known for its therapeutic effects. In fact, whey protein is a popular dietary protein supplement purported to induce antimicrobial activity, immune modulation, increased muscle strength and body composition and prevent cardiovascular disease and osteoporosis (Keri Marshall 2004). In the present study, exercise was applied in isolation and in combination with protein supplementation in two distinct groups. The results suggest that while the negative effects of bed rest were not statistically different to the control trial the changes were not so great. Additionally, whey protein supplementation has been shown to have an insulinotropic effect by stimulating insulin release and reducing postprandial glucose (Frid, Nilsson et al. 2005, Pal, Ellis et al. 2010). However, further work is needed to clarify the mechanisms involved and longer term effects.

7.11 Limitations

Some limitations became apparent while conducting the current studies. Bed rest studies provide us with a very controlled environment in order to assess physiological

changes. Such an environment can minimize limitations but there were a few that could impact the measurement outcomes. The current bed rest study used a cross-over study design and while this design is beneficial as subjects are their own control, we found that baseline measurements varied. Between each bed rest campaign, there was a 126 day washout period during which subjects were not under supervision and were permitted to live life as they wished. Upon return to the bed rest for campaigns two and three, it became apparent that some subjects had prepared for bedrest during the washout period. Another limitation in this study and one that is common to other bed rest studies was that subject measurements during the baseline period were obtained while subjects were ambulatory. However, they were confined to the clinic and so these physical activity measurements may not reflect free living conditions. There were 9 research teams working on the same study and this meant that volunteers had to follow a strict and perhaps stressful regime in terms of test measurements. While a lot of time and effort goes into the timing of each test and the order they are in, it may be seen as a limitation also.

The euglycemic hyperinsulinemic clamp measurement was only measured at baseline before the first campaign and after each of the three campaigns. Ideally, a clamp should have been conducted at each baseline time-point however, due to logistical issues, this was not feasible.

A final limitation that was noted in this study was that 4 subjects dropped out of the study thus decreasing the statistical power.

We had additional measurements that were not possible to complete before submission of this thesis. Doubly labelled water was administered to measure changes in whole body energy expenditure during bed rest but the lab in Strasbourg performing the analysis was shut for 1-yr because of asbestos in the roof. We have collaborated with a group to conduct label-free proteomic analysis but their initial sample preparation was not adequate and the analysis is currently being repeated. Finally we have lipidomic analysis being performed but our collaborative moved during the study and is only now performing the analysis.

Finally, using cell culture as a model to investigate physiological changes that we see in human models is a limitation in itself. While it does provide us with a useful model to

investigate specific pathways, isolated cells in culture do not reflect the complex physiological environment of the human body. For the SiRNA transfection experiment, we had hoped to optimize the transfection protocol in C2C12 myotubes, however, we were unsuccessful and ran out of time. This may be an experimental protocol that we will revisit.

7.12 Future Directions

Bed rest studies allow us to study human physiology in a very unique and controlled environment. The European Space Agency has highlighted their standardization protocol so that all bed rest studies in the future are comparable (Belavý, Bock et al. 2010). While a lot of work has been completed to support the use of resistive vibration exercise in bed rest, further work is needed to identify the optimal volume of exercise in order to counteract the effects of bed rest. Additionally, an aerobic component using high intensity interval exercise training may be necessary to avoid any major decrements in oxygen consumption because ultimately, energy metabolism depends on the body's ability to deliver oxygen sufficiently. Ideally, a combined countermeasure protocol that would target all or most body systems would be optimal. While bed rest is a great model to study physiology and identify countermeasures to further human exploration in space, when we look at bed rest as a model of physical inactivity and an initiator of metabolic dysfunction in skeletal muscle, it may be interesting to use less extreme models such as reduced ambulatory activity in a healthy population. This would more closely identify with sedentary behaviour and the role it plays in the onset of chronic disease.

The primary cause of insulin resistance remains elusive. Particular attention has been given to the role of mitochondria in insulin resistance (Lowell and Shulman 2005, Koves, Ussher et al. 2008, Anderson, Lustig et al. 2009),but the mechanistic evidence is not conclusive (Holloszy 2013). Other suggested contributors to insulin resistance are lipotoxicity, impaired fatty acid oxidation and intramyocellular lipid accumulation (Coen and Goodpaster 2012, Goodpaster 2013). The link between fuel metabolism in the mitochondria and fatty acid accumulation in the cell and the role they play in insulin resistance needs further clarification. The role of UCP3 in mitochondrial uncoupling has gained interest. However, many studies have focused on changes in proton conductance

using isolated mitochondria *in vitro*. However, the regulation of mitochondria is much more complex *in vivo* due to the dynamic nature of the mitochondria in intact cells (Chan 2006). Further work investigating the role of UCP3 in reducing the production of ROS in vivo is suggested. Many studies have suggested that insulin resistance is linked to muscle protein degradation due to the high level of interaction in their signalling pathways. Further identification of the main players in these pathways that are upregulated with physical inactivity is necessary in order to tailor specific countermeasures and therapeutic approaches to counteract muscle atrophy.

7.13 Conclusion

Being confined to bed rest has negative physiological consequences. Whole body physilogical changes are widely reported. Less is known about the cellular response to physical inactivity. A reduction in CS in the present study suggests that there are fewer actively respiring mitochondria. This is interesting as no change in mitochondrial respiration was noted, infact, when normalized to CS, an increase in mitochondrial respiration was reported. This could mean that the healthy mitochondrial that are there are working harder to maintain the same level of respiration or that CS is not a great indicator of mitochondrial content. As an enzyme, it may fluctuate in response to the dynamic nature of bed rest or is simply reflective of TCA cycle activity. However, a significant positive correlation between the reduction in maximal ETS and insulin sensitivity suggests a relationship between the two. One aim of the bed rest was to further optimize countermeasures that could mitigate the negative consequences of physical inactivity. Resistive vibration exercise alone and in combination with whey protein supplementation showed somewhat of a protective effect, however not significant. Further work will need to be done to identify appropriate countermeasures. Of particular interest was the reduction in COX5a protein. It has been generall regarded as a complex IV structural protein. However, when it is knocked down in an in vitro model, mitochondrial respiration is negatively affected, suggesting that it may have a regulatory function in mitochondrial function. The mechanisms involved in disuse muscle atrophy require further clarification, as do the mechanisms linking insulin sensitivity, mitochondrial function and muscle atrophy.

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