INFLUENCE OF POST-AEROBIC EXERCISE NUTRITION ON PROTEIN TURNOVER AND MITOCHONDRIAL BIOGENESIS

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Abstract

The desire to age well is a common goal among the human population. How to do so is therefore, a popular question. One theory of ageing involves the accumulation of damage to mitochondrial protein and the subsequent loss of function the damage causes. Increasing the rate of mitochondrial protein synthesis, a variable that declines with advancing age, is one way to improve quality of life in the twilight years. A review of literature lead to a multi-level approach, with measurements of protein synthesis made at the whole body, muscle, and molecular levels. An acute bout of aerobic exercise, followed by feeding, two factors which have a positive effect on the rate of mitochondrial protein synthesis, was used. Adaptations to a period of exercise training are mediated by the accumulation of proteins due to each acute exercise bout, and so an acute intervention was postulated to be indicative of changes expected over the long term. A stable isotope infusion combined with sampling of breath, blood, and muscle was used to determine the rate of whole body protein synthesis in 12 older adults. Intracellular signalling for mitochondrial and whole body protein synthesis was examined using RT-quantitative PCR and Western blotting in eleven young adults. The rate of post-exercise whole body protein synthesis was 19% greater over the first four hours of post-exercise recovery, in subjects receiving a protein-plus-carbohydrate drink immediately after a bout of cycling than in those receiving a carbohydrate-only drink ($p = 0.001$). The same trend was revealed in signalling for whole body protein synthesis and the abundance of cytochrome c, a mitochondrial protein, although these results were not statistically significant ($p = 0.2$). In contrast there was a strong, albeit also statistically insignificant, tendency for signalling for mitochondrial protein synthesis to be higher in the skeletal muscle of subjects receiving a carbohydrate-only drink after a bout of cycling ($p = 0.06$). The exercise and feeding intervention described in this thesis may provide a means to enhance the rate of mitochondrial protein synthesis in older individuals and, in so doing, improve the quality of their old age.

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- Participated in experimental design and sample collection for Anna Rolleston's (PhD) Tour of Southland study.

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List of Publications

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3-MH	3-methylated histidine
4E-BP1	eIF binding protein 1
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
α-KIC	alpha-ketoisocaproate
AMPK	adenosine monosphosphate kinase
APE	atom percent excess
APS	ammonium persulphate
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
ATF2	activating transcription factor 2
AUC	area under the curve
A-V	arterio-venous
β2m	beta 2-microglobulin
BCAA	branched chain amino acid
BMI	body mass index
BSTFA-TCMS	N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane
CV	coefficient of variation
C_T	threshold cycle
CHO	carbohydrate-only drink
COXI	cytochrome c oxidase subunit I
COXIV	cytochrome c oxidase subunit IV
DEPC	diethyl pyrocarbonate
DEXA	dual-energy x-ray absorptiometry
dNTP	deoxyribonucleotide triphosphate
ECL	electrochemiluminescence

eEF2	eukaryotic elongation factor 2
eEF2K	eEF2 kinase
eIF	eukaryotic initiation factor
ETC	electron transport chain
EtOH	ethanol
FFM	fat-free mass
FSR	fractional synthesis rate
GC-C-IRMS	gas chromatography - combustion - isotope ratio mass spectrometry
HR_{max}	heart rate maximum
IMM	intermyofibrillar mitochondria
IRS-1	insulin receptor substrate 1
LDH	lactate dehydrogenase
LS-TTBS	low sodium Tween-20 in Tris-buffered saline
MEF2	myocyte-specific enhancer factor 2
MPB	muscle protein breakdown
MPE	molar percent excess
MPS	muscle protein synthesis
MTBSTFA	N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NFDM	non-fat dry milk
NOLD	non-oxidative leucine disposal
NRF	nuclear respiratory factor
NTC	no template control
OPD	1,2-phenylenediamine

$p70^{S6K}$	70 kDa ribosomal protein S6 kinase
PCA	perchloric acid
PDK-1	phosphoinositide-dependent kinase 1
PGC-1α	peroxisome proliferation-activated receptor gamma co-activator - 1 alpha
PI3kinase	phosphatidylinositol-3-OH kinase
PPARα	peroxisome proliferation-activated receptor alpha
PPARγ	peroxisome proliferation-activated receptor gamma
PRO	protein-plus-carbohydrate drink
PVDF	polyvinylidene difluoride
R_a	rate of appearance
R_d	rate of disappearance
RER	respiratory exchange ratio
Rheb	ras homolog enriched in brain
ROS	reactive oxygen species
ROX	6-carboxyl-X-rhodamine
RPE	rating of perceived exertion
SSM	subsarcolemmal mitochondria
TA	*tibialis anterior*
t-BDMS	*tert*-butyldimethylsilyl
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamene
TOP	tract of pyrimidine
TFam	mitochondrial transcription factor A
tRNA	transfer RNA
TSC2	tuberous sclerosis complex 2

TTBS	Tween-20 in Tris-buffered saline
VDAC	voltage-dependent anion channel
VPDB	Vienna PeeDee Belemnite
VO_2	rate of oxygen consumption
VO_{2max}	maximal rate of oxygen consumption
WBPB	whole body protein breakdown
WBPS	whole body protein synthesis
WBPT	whole body protein turnover
W_{max}	maximum power output

1 Introduction

Elderly physical disability is a growing public health concern due to an ageing population placing a progressively greater strain on public health resources and the economy, and as such, requires scientific attention . One of the contributors to increasing disability with advancing age is sarcopenia. Sarcopenia is a term used to describe the involuntary loss of muscle mass and function that occurs with advancing age . Maintenance of muscle mass is critically important to good health at any age . Along with a loss of mass, ageing muscle also loses mitochondrial protein content. Mitochondria provide muscles with the ability to generate energy for repetitive daily activity, and their loss leads to reduced endurance capacity. The practical implications of this loss may be that an activity as simple as a walk to the letterbox becomes an ordeal. Declining activity levels and poor nutrition, habits often characteristic of older individuals, are some factors that can lead to a decline in the rate of mitochondrial protein synthesis. As proteins become damaged and require repair, a higher rate of synthesis could help to maintain function . Damage to mitochondrial protein often comes in the form of reactive oxygen species, which have long been implicated in the ageing process .

Regular exercise, which mediates skeletal muscle adaptation, has been shown to have a significant positive effect on such pathological conditions of ageing as sarcopenia . Aerobic exercise training increases mitochondrial content, which delays the onset of muscle fatigue at sub-maximal workloads . A bout of aerobic exercise exerts a demand which leads to adaptive events that reprogram gene expression and ultimately lead to accrual of new mitochondrial protein. Intracellular signals are the early molecular events that occur in response to this functional demand and indicate the potential for an adaptive response. Higher mitochondrial protein content allows muscle to more easily maintain cellular homeostasis during any

subsequent bouts of exercise . Appropriate post-exercise nutrition can have an influence on these adaptations.

From Holloszy's early work in the 1960s and '70s , reporting that chronic endurance exercise increases the concentration of mitochondria in human skeletal muscle, through findings about ultrastructural changes in muscle , to today's work on elucidating signalling pathways, mitochondrial adaptation has long been a subject of interest. While it is now known that mitochondrial biogenesis is a result of endurance exercise training, elucidation of the underlying signalling mechanisms is ongoing.

The adaptations described in the preceding two paragraphs are triggered by exercise but accumulate during the post-exercise recovery period, with transient post-exercise changes mediating long-term adaptations. While adaptations to a period of chronic training are a response to cumulative effects, initial signalling responses are likely to occur after each acute bout. Thus, the study of acute responses at both the molecular and the metabolic level, provide an indication of training adaptations. In this thesis, a detailed examination of the responses to an acute intervention is made at the level of the whole human, muscle tissue, and the muscle cell, using an integrative biology approach (Figure 1-1).

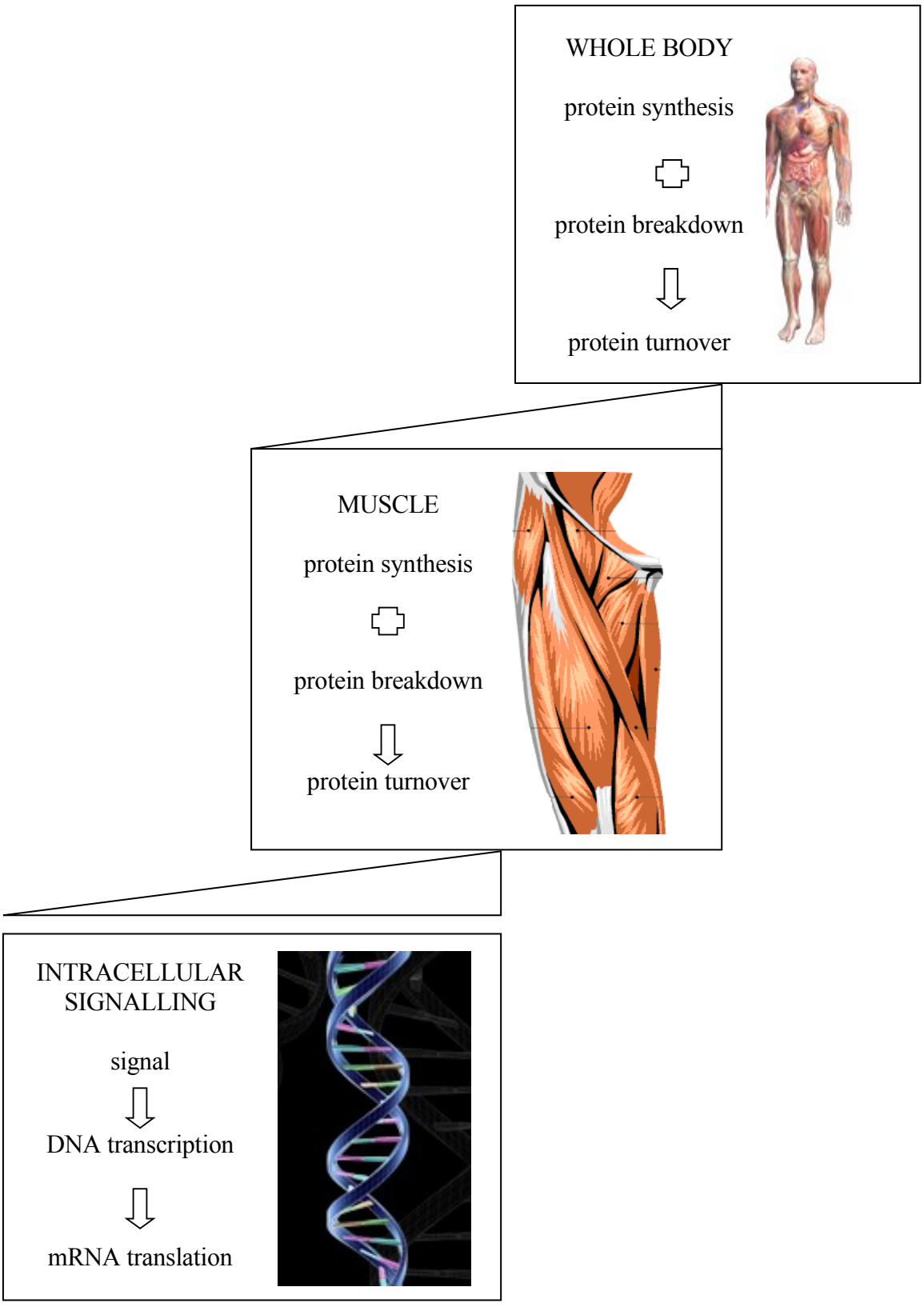


Figure 1-1 Overview of levels of body systems studied in this thesis
This thesis examines the effects of post-aerobic exercise nutrition on whole body protein synthesis (WBPS), skeletal muscle protein synthesis (MPS), and some of the intracellular signals regulating these processes. WBPS and whole body protein breakdown (WBPB) together comprise whole body protein turnover (WBPT). One component of WBPT is muscle protein turnover, which is comprised of MPS and muscle protein breakdown (MPB). Intracellular signalling cascades regulate transcription of DNA into messenger RNA and its subsequent translation to protein.

This thesis seeks to determine, at multiple levels (Figure 1-1), the effects of an acute bout of aerobic exercise followed by nutrition. The post-aerobic exercise effects of a protein-plus-carbohydrate drink are compared to those of a carbohydrate-only drink, on whole body protein synthesis, skeletal muscle mitochondrial protein synthesis, and cellular signalling for mitochondrial protein synthesis. There is currently limited evidence detailing the continuity between cellular signalling pathways, mRNA expression, and resulting protein synthesis, particularly concerning the effects of post-aerobic exercise nutrition. This thesis attempts to investigate this link using a wide scope of techniques to study transcription, translation, phosphorylation, and the rate of protein synthesis *in vivo* from disciplines encompassing chemistry, molecular biology, and exercise physiology.

In Chapter 2, a review of literature is presented. First protein turnover is discussed at the whole body and muscular levels, and the current knowledge regarding the effects of exercise and nutrition on turnover is detailed. Next, the intracellular signals that lead to synthesis of mitochondrial protein are described, and the current understanding of the ways exercise and nutrition can affect these signals is reviewed. Finally, the effects of age on muscle protein metabolism and the response to exercise and nutrition are presented.

In Chapter 3, an overview of the research is provided. The hypotheses tested and the studies and experiments performed in this thesis are described.

The experimental work is reported in Chapters 4 through 7. Chapter 4 describes the effects of aerobic exercise and nutrition on the rate of whole body protein synthesis (WBPS) in older individuals, while Chapter 5 reports the resulting rates of mitochondrial protein synthesis in the same population. Chapter 6 examines signalling for the formation of new skeletal muscle mitochondria in response to the same stimuli in healthy adults, and Chapter 7 measures signalling for skeletal muscle protein synthesis (MPS) in the same population. Finally, Chapter

8 draws the work together, discusses limitations and potential applications, and proposes directions for future research.

2 Review of literature

Protein breakdown and synthesis are continuous processes that are together known as turnover. Because proteins continually become damaged and require repair, a high rate of turnover maintains protein quality and function. Protein metabolism can be studied at the level of the whole body, mixed muscle, or specific sub-fractions of muscle. If muscle protein breakdown (MPB) exceeds synthesis over the long term, muscle loss will occur, while if the latter exceeds the former, muscle protein will accrue. Signalling in the mTOR (mammalian target of rapamycin) pathway leads to the translation of all skeletal muscle proteins, while signalling in the PGC-1α (peroxisome proliferation-activated receptor gamma co-activator - 1 alpha) pathway induces mitochondrial biogenesis. Both exercise and nutrition affect signalling in the mTOR and PGC-1α pathways.

Exercise on its own, either resistance or aerobic, can increase muscle protein turnover above resting levels . Over time aerobic exercise training may stimulate net synthesis of the mitochondrial proteins involved in aerobic respiration .

Combining exercise with well-timed and carefully formulated nutrition can create a situation of net protein accrual. Consumption of protein provides the body with amino acids, the building blocks of muscle protein. Ingestion of carbohydrate provides energy and also causes a rise in plasma insulin. Amino acids, energy, and insulin each increase rates of MPS and decrease rates of breakdown, and, in combination, provide a synergistic effect . Without carefully formulated nutrition the body cannot take full advantage of the stimulation to protein metabolism that exercise provides. It may be that the timing of nutrient ingestion in relation to an exercise bout plays a part in the efficacy of that nutrition .

In sarcopenia, ageing muscle declines in quantity and quality, due to decreased rates of MPS and turnover, resulting in losses of strength and aerobic capacity . The causes of sarcopenia include oxidative damage by free radicals, a decreased level of activity, and poor nutrition . Mitochondria are both the site of free radical generation, and their target . As the quality of mitochondrial protein declines due to decreased turnover, so the generation of free radicals increases which, in turn, causes oxidative damage and further reductions in rates of mitochondrial protein turnover and synthesis. The sarcopenic progression can be slowed by increasing physical activity level and ensuring adequate energy and protein intake, in order to stimulate muscle protein metabolism.

2.1 The effects of exercise and nutrition on protein turnover

Protein breakdown and synthesis are continuous processes that are known together as turnover. Skeletal muscle protein turnover accounts for between 33% and 50% of the body's protein turnover . The single pool model is regularly used in studies of whole body and muscle protein turnover (see Appendix A for a discussion of this model). As its name suggests, the single pool model assumes that the whole body's store of a compound, for example amino acids, are contained in a single, homogeneous and instantaneously mixed pool . In the single pool model, tracee enters the pool from one or more sources. For example, sources of amino acids are infusion, ingestion, and protein breakdown (Figure 2-1). Substrates may disappear from the pool for such fates as oxidation, excretion, and protein synthesis.

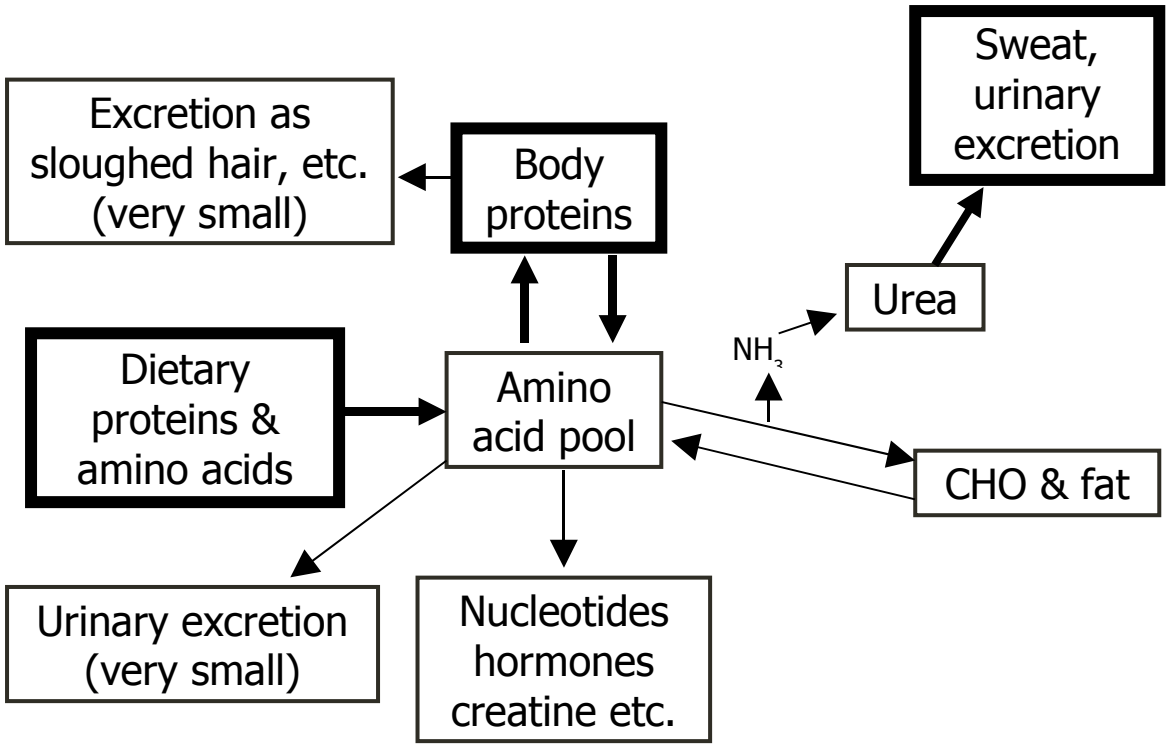


Figure 2-1 Appearance to, and disappearance from, the body's amino acid pool

The store of amino acids in the body is depicted as a single, homogenous pool. Amino acids enter the free pool by ingestion and as a result of protein breakdown. Amino acids leave the free pool when they are used as substrates for oxidation or are oxidised themselves, when they are excreted via the urine, faeces and sweat, and when they are used to synthesise body proteins such as hormones, skin and hair, and muscle proteins. The bold outlines indicate major routes into (ingestion and protein breakdown) and out of (oxidation and protein synthesis) the pool. CHO, carbohydrate. Adapted from .

Amino acids are constantly being removed from the body's free pool for use in MPS and returned as a result of MPB . If MPB exceeds MPS over the long term, a situation termed negative balance, a net loss of muscle protein will occur, as is the case during muscle wasting. Net loss may be the result of either an increased rate of MPB, a decreased rate of MPS, or both . The reverse situation, a positive balance, will result in a net gain, as is the case during muscle growth. Even in the absence of net loss or gain of muscle protein, maintaining a high rate of protein turnover is desirable because proteins continually become damaged and require repair . Increasing the rate of muscle protein turnover therefore maintains the quality and function of that muscle. As will be reviewed in this section, combining exercise with well-timed and carefully formulated nutrition can create a situation of increased turnover and/or positive balance.

2.1.1 The effects of exercise on protein turnover

At rest, in the fasted state, there is net release from leg muscle of 13 of the 20 amino acids, including leucine and net release from arm muscle of all but glutamate and serine . Because amino acids are released from muscle due to MPB, net release indicates a catabolic state, which will ultimately lead to a loss of muscle protein.

Ahlborg et al., , using the arterio-venous (A-V) balance method (Appendix A3), measured an exercise-induced stimulation of leg amino acid uptake. Net uptake indicates that amino acids are being used for either MPS or oxidised as fuel. The latter case is more likely because, during exercise, rates of MPS are depressed below those at rest due to the energy needed for this process being diverted for muscular contraction. 3-methylated histidine (3-MH) release, a marker of MPB, is decreased during exercise, which suggests that rates of MPB are also decreased. Restriction of ATP availability dramatically affects rates of turnover because both MPS and breakdown are energy-consuming, and are the most sensitive to energy supply of all cellular processes . Decreased rates of both processes combine to result in lower protein turnover, a situation observed by Stein et al. during eight hours of aerobic exercise at roughly 50% VO_{2max}. Protein turnover stabilised during the exercise bout at a level 20% lower than resting, supporting the notion that energy availability is a limiting factor in protein turnover.

After a bout of exercise, rates of both protein synthesis and breakdown are stimulated over those at rest. Exercise stimulates the subsequent rate of MPS via cellular signalling cascades (section 2.2) that initiate transcription and translation, leading to increased protein expression in skeletal muscle. The rate of MPB is also stimulated after exercise, although often to a lesser extent than MPS. When the rate of MPB is elevated less than that of MPS protein, net balance is increased but may not necessarily become positive, simply less negative. The exact nature of

the protein response can be influenced by the mode of exercise, its intensity and duration, and the training history of the individual.

Resistance and aerobic exercise both lead to a more positive, or less negative, muscle protein balance by stimulating post-exercise synthesis and/or decreasing breakdown, but do so in different muscle sub-fractions. Resistance exercise is characterised by multiple repetitions of lifting a load which requires muscles to move at a high force output over a brief period of time . Repeated bouts of resistance exercise result in muscular hypertrophy through increased myofibrillar protein synthesis . In contrast, aerobic exercise is characterised by the repeated application of a small amount of force over a much longer duration in a cyclic and dynamic fashion, such as occurs in cycling, running, and swimming . Stimulation of rat muscle *in vitro* has shown that aerobic exercise-type patterns stimulate myofibrillar MPS far less than resistance exercise-type ones, and it is generally accepted that aerobic exercise does not result in muscular hypertrophy . In the case of aerobic exercise, stimulation of protein synthesis is specific to the proteins of the aerobic respiratory chain, that is, mitochondrial enzymes comprising the Krebs cycle and electron transport chain (ETC). Mitochondrial size, enzyme activity, enzyme protein content, and muscle oxidative capacity all increase after a period of aerobic exercise training comprised of repeated acute bouts . Such mitochondrial adaptations result in increased fatigue resistance and changes in substrate metabolism without a dramatic change in muscle size .

The intensity of aerobic exercise, defined as a percentage of VO_{2max} (Table 2-1), as well as its duration, influences the effect it has on muscular adaptation. It is important to understand that there are not distinct and separate pathways being up-regulated by the different modes of exercise, but that the end results are on an adaptive continuum. Any form of exercise leads to

intracellular signalling, which leads to the synthesis of new proteins, and the parameters of the exercise determine which proteins are synthesised.

Intensity	% VO_{2max}
mild	< 50
moderate	50 - 70
moderately high	70 – 85
high	> 85

Table 2-1 Intensity of aerobic exercise as a percentage of VO_{2max}
The ranges described have been selected based on common usage of terms in current literature. The descriptors specified in this table are used throughout the thesis. VO_{2max}, maximal rate of oxygen consumption.

During prolonged aerobic exercise (longer than 45 minutes) there is little or no change in muscle protein turnover in rats until at least a moderate intensity is reached, at which point there is a net loss of amino acids from muscle due to protein catabolism . Rats also oxidise leucine at a greater rate with increasing exercise intensity . In rats exercising at moderately high intensity, the rate of MPS varies inversely with the intensity and duration of the exercise bout while no change is seen in those running at a mild intensity . In humans running on a treadmill, the rate of leucine oxidation increases with increasing intensity . In rats run to exhaustion, MPS decreases during exercise, and decreases more with increasing exercise intensity . Taken together the above results indicate that mild-intensity aerobic exercise has no effect on muscle protein metabolism, but at or above a moderate intensity, the rate of MPS decreases and that of MPB increases.

The effect of an acute bout of exercise tends to vary inversely with training history in that the more trained a person is, the less of an effect exercise will have on their muscle protein turnover. For example, Tipton et al. reported no increase from resting values of whole body protein breakdown (WBPB) following either resistance exercise, aerobic exercise, or a combination of the two, and no increase in MPS, following resistance exercise in highly trained subjects. Further, Devlin et al. found a significant correlation between post-exercise non-

oxidative leucine disposal (NOLD, a proxy for WBPS) and subjects' VO_{2max}, suggesting an attenuation of the effect of exercise with training. Phillips et al. found that mixed-muscle protein fractional synthesis rate (FSR, Appendix A2) was elevated by 118% by a bout of resistance exercise in untrained subjects, but by only 48% in trained subjects. Roy et al. also reported no increase in trained subjects' MPS following resistance exercise, indicating that the use of untrained subjects may be preferable when trying to detect small changes due to exercise interventions.

As well as the magnitude of the response to an exercise stimulus, training history may also affect the time course of this response. Lamont et al. compared endurance-trained and sedentary individuals after one hour of exercise at 50% VO_{2max}, and found that WBPB decreased in the trained subjects from exercise to recovery, while values remained elevated for some time in the sedentary group. Furthermore, NOLD remained depressed post-exercise for much longer in the untrained than the trained group indicating that training provides an adaptive response in terms of quicker recovery from the depression in MPS induced by moderate-intensity aerobic exercise.

Some of the variability in reported values may also be explained by the time course over which MPS is stimulated. Chesley et al. used a leucine infusion to measure changes in the FSR of the *biceps brachii* and found a 50% elevation on the day of exercise but a 109% elevation the following day. MacDougall et al. found a 129% increase in MPS between 22 and 24 hours after a bout of resistance exercise. MacDougall et al. have shown that the rate of MPS in the *biceps brachii* is then returned to baseline by 36 hours post-exercise. The above results indicate that the sampling time after cessation of exercise is an important variable to control when conducting studies on the effect of exercise on rates of MPS.

2.1.1.1 Whole body protein turnover

Evidence suggests that rates of WBPS decline during aerobic exercise. In humans cycling at a mild intensity for 105 minutes, the rate of WBPS decreased roughly 50% during the exercise bout . Similarly, Rennie et al. determined that the rate of WBPS fell 14-18% over nearly four hours of walking at a moderate intensity. In contrast, Phillips et al. observed no change in NOLD in humans running for 90 minutes at a moderate intensity. The fact that the subjects in Phillips et al.'s study were on a protein-deficient diet may be a confounding factor in these findings. It is possible that a low-protein diet may induce a protein-sparing effect as the body attempts to preserve its stores of lean mass in the absence of sufficient free amino acids for further MPS. It is most likely, therefore, that the findings of Phillips et al. can be disregarded and the conclusion drawn that the rate of WBPS does indeed fall during aerobic exercise.

Unlike protein synthesis, the rate of WBPB is stimulated considerably during aerobic exercise. Rennie et al. measured a 50-75% increase in WBPB during four hours of treadmill walking at a moderate intensity. Wolfe et al. also found that WBPB was increased during 105 minutes of cycling at a mild intensity, as did Phillips et al. and Millward et al. during running. In contrast to these findings, Stein et al. used dilution of plasma leucine enrichment to measure WBPB and found no change during exercise. The problem with Stein et al.'s measure, however, is that plasma leucine does not take into account the amount of unlabelled leucine released due to MPB and transaminated to alpha-ketoisocaproate (α-KIC) in the intracellular compartment, (Figure 2-2, see Appendix A1 for explanation of the use of stable isotope methodology in the determination of whole body protein turnover (WBPT)) thus underestimating protein breakdown. Wolfe et al. and Phillips et al. both used plasma α-KIC enrichment in their calculations of protein breakdown and, therefore, likely yielded more accurate results thus suggesting that WBPB does increase during aerobic exercise.

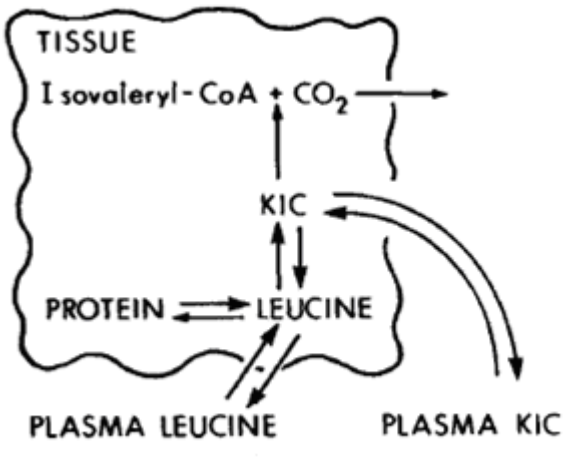


Figure 2-2 Model of leucine metabolism

Leucine is transaminated to its alpha-ketoacid alpha-ketoisocaproate (α-KIC) in both the plasma and intracellular fluid. α-KIC can then be de-carboxylated and carbon dioxide (CO_2) released, or re-aminated back to leucine. α-KIC and leucine are both in equilibrium between the plasma and intracellular fluid compartments. Adapted from .

Sustained aerobic exercise stimulates the oxidation of amino acids which may be a cause of net protein loss. Refsum et al. noted an increase in urea production and a decrease in circulating amino acid concentration following six hours of high-intensity cross-country skiing, findings consistent with increased protein catabolism and amino acid oxidation. Rennie et al. determined that leucine oxidation increases two- to three-fold during moderate-intensity aerobic exercise. Muscle catabolism may provide the starting point for leucine oxidation as de-aminated leucine released from muscle is oxidised as α-KIC (Figure 2-2). There is some question of whether leucine oxidation is a reliable measure of MPB however, because as one of the branched chain amino acids (BCAAs), leucine is oxidised to a greater extent than most other essential amino acids (phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, and lysine) and so may overestimate muscle protein use .

More likely contributors to the increase in WBPB seen during aerobic exercise are liver and gut proteins. Ahlborg et al. used the A-V balance method to measure protein turnover in humans during four hours of exercise at a mild intensity. They found that, as the exercise progressed,

catabolism of liver protein increased to provide gluconeogenic precursors, while that of muscle protein did not. Further support for this hypothesis is provided by Felig & Wahren , who, using A-V balance across the leg and the splanchnic bed, measured no consistent difference in uptake and release of amino acids across the leg, and significant net release from the splanchnic bed during exercise. Felig & Wahren's findings indicate that net WBPB during high intensity aerobic exercise is attributable to changes in splanchnic rather than peripheral exchange.

The majority of findings regarding the rate of WBPB after aerobic exercise, using a variety of methodologies, indicate an increase. Rennie et al. , using an oral dose of [^{15}N]glycine and subsequent ^{15}N production as a measure of protein oxidation, found the rate of WBPB was increased 25-35% after a bout of running. Fielding et al. found the rate of WBPB, measured by unlabelled leucine release into plasma, was increased immediately after a 45 minute bout of eccentric cycling exercise and was still elevated when measured ten days later. Devlin et al. measured whole blood amino acid concentrations after an exhaustive bout of cycling and found concentrations of leucine and isoleucine elevated above resting values, indicating a net release due to protein breakdown. Two studies found that protein breakdown was unchanged after exercise. Devlin et al.'s study used the dilution of plasma α-KIC to estimate WBPB, a reliable measure. Both studies, however, used highly trained study populations so their results may not be relevant to the general population, as training status may affect the response of muscle protein metabolism to exercise as already reviewed.

2.1.1.2 Muscle protein turnover

In agreement with whole body results, van Hall et al. found increased MPB during a bout of aerobic exercise, measured by net amino acid release from the leg, during 90 minutes of one-legged kicking exercise. In contrast, Millward et al. and Rennie et al. , using 3-MH release as a marker, concluded that MPB actually decreased during two hours, and three and three quarter

hours respectively, of treadmill running at a moderate intensity. Different again, Carraro et al. , using 3-MH excretion to assess MPB, found no significant increase during a four-hour bout of exercise. Keeping in mind the limitations of the 3-MH release and A-V balance methods (discussed in Appendix A3), it is fair to say that it is currently unclear whether MPB increases or decreases during aerobic exercise.

An acute bout of aerobic exercise has been shown, by several groups of researchers using stable isotope infusions, to stimulate post-exercise rates of MPS in human subjects. The results of these studies are summarised in Table 2-2 and show that the rate of MPS is stimulated by a bout of aerobic exercise to an extent dependent on both the intensity and duration of the exercise. The results in Table 2-2 also indicate that the rate of MPS increases as post-exercise recovery progresses.

exercise parameters	change in protein synthesis	reference
4h walking, mild	↑ 25% (4h)	
3h cycling, moderately high	↑ 12% (3h)	
45min walking, mild	↑ 3% (1h)	
1h dynamic kicking, moderate	↑ 125% (6h)	

Table 2-2 Changes in the rate of skeletal muscle protein synthesis in humans after a bout of aerobic exercise
Exercise intensity is described using the definitions in Table 2-1. Change in protein synthesis is indicated as a percent change from baseline values; value in parentheses is time in hours after cessation of exercise when measurements were made. h, hours; min, minutes.

In contrast to the above findings, one group of researchers found no change in MPS, measured by infused stable isotope incorporation into protein, after 45 minutes of cycling. It is possible that, had sampling occurred later in the recovery period, a significant result may have been found. In rat skeletal muscle, Dohm et al. found that the rate of MPS was decreased following aerobic exercise, with the magnitude of the reduction varying directly with the severity of the exercise. Dohm et al.'s study was performed *in vitro* on rat muscles using a radioactive isotope perfusion, a variation in methodology that may explain the conflicting results due to possible differences in muscle metabolism when studied *in vitro*. Dohm et al. followed up their 1980

study with an *in vivo* investigation and still found a post-exercise depression in MPS . Again, the use of a radioactive tracer and non-human subjects renders the application of these findings to humans questionable due to possible differences in rat and human skeletal muscle metabolism.

Stimulation of MPS following a bout of aerobic exercise appears to be specific to the mitochondrial sub-fraction. Aerobic exercise and low-frequency electrical stimulation designed to simulate aerobic exercise both lead to increases in mitochondrial volume, protein content, and enzyme activity and/or content . Aerobic exercise training has been shown to increase mitochondrial volume , which may be due to an increase in the size of existing mitochondria or an increase in their number, indicating enhanced mitochondrial biogenesis. McKenzie et al. found that mitochondrial enzyme activity (citrate synthase and complexes I-III) increased after 38 days of cycle training, indicating an increased oxidative capacity. Since mitochondria are comprised mostly of enzymatic proteins , an increase in mitochondrial volume or enzyme activity indicates enhanced mitochondrial protein synthesis and/or turnover.

The rate of MPB also increases after a bout of aerobic exercise. Carraro et al. , using 3-MH excretion to assess MPB in humans, found an 85% increase in 3-MH excretion in the four hours following cessation of exercise. Dohm et al. , using a radioactive tracer, found a 55% increase in MPB in perfused rat hind limbs following one hour of swimming.

In summary, it seems that during aerobic exercise rates of protein synthesis are depressed and rates of breakdown are elevated when compared to resting values. After exercise rates of protein synthesis and breakdown are both elevated above resting values, with the former being stimulated more than the latter, leading to a positive net protein balance. If these changes persist over a period of chronic exercise, net accrual of protein should result.

2.1.2 The effect of nutrition on protein turnover

Consumption of protein and carbohydrate, either individually or in combination, has a positive effect on protein balance. Protein provides amino acids while carbohydrate ingestion provides energy, and both cause a rise in plasma insulin . Amino acids, energy, and insulin each increase the rate of MPS and decrease that of MPB. Exercise and nutrition have similar effects on the rates of MPS and MPB to nutrition, and a combination of the two has a synergistic effect. It is also possible that the timing of nutrient ingestion relative to exercise may further enhance protein balance.

2.1.2.1 The effect of amino acids on muscle protein turnover

The availability of amino acids, the building blocks of muscle protein, depends on the body's nutritional state. In the absence of an exogenous source, the rate of MPB will increase in order to replenish the free pool, thus making muscle protein balance less positive or more negative. At rest after a mixed meal, the availability of amino acids can increase mRNA translation into protein in a dose-responsive fashion . Several studies, summarised in Table 2-3, have shown that rates of protein synthesis in humans are increased after either ingestion or infusion of amino acids.

As time passes after a meal, the rate of protein synthesis is slowed. Bohe et al. studied the effects of a six-hour amino acid infusion and found that MPS increased to nearly three times the basal value by the two hour mark and then returned to baseline quite quickly despite the continued availability of amino acids. This suggests that there is a limit to the time course of the response to amino acid delivery and that there are other rate-limiting factors in MPS.

experimental condition	results	reference
3 x hourly PRO feed	↑ WBPS, -- WBPB	
4h AA infusion	↑ NOLD, ↑ FSR, ↓ WBPB	
flooding AA infusion	↑ MPS	
3h AA infusion		

3h AA infusion	↑ FSR
8h AA infusion	↑ FSR
	↑ MPS

Table 2-3 Effect of amino acid availability on rate of protein synthesis in humans

Experimental condition is compared to baseline measures prior to the start of infusion/ingestion. An increase in circulating amino acid concentration results in an increase in the rate of whole body and muscle protein synthesis, resulting in a more positive protein balance. AA, amino acid; PRO, protein; WBPS, whole body protein synthesis; WBPB, whole body protein breakdown; NOLD, non-oxidative leucine disposal, a proxy for WBPS; FSR, fractional synthesis rate; ↑, increase; --, no change; ↓, decrease; h, hours.

2.1.2.2 The effect of insulin on muscle protein turnover

Insulin, released after carbohydrate and amino acid consumption , has an inhibitory effect on MPB. Many researchers have found that, at rest, an insulin infusion decreases the rate of appearance (R_a) of leucine, indicating decreased MPB . MPB also responds in a dose-dependent manner to insulin, as shown by several groups of researchers who infused insulin at a range of rates and found that MPB in human skeletal muscle decreased more at the higher infusion rates. It appears that the presence of amino acids augments this inhibitory effect of insulin on MPB, with lower rates being measured than with insulin alone, suggesting that circulating amino acids reduce the need for MPB to supply the body's free pool .

There is evidence from rats and pigs that insulin can stimulate MPS . Several studies suggest that this situation is not replicated in resting humans. McNurlan et al. , using a flooding dose of stable isotope, found no measurable difference in FSR in the m. vastus lateralis both before and after a euglycemic insulin clamp. Similarly, Meek et al. and Biolo et al. found no effect of an insulin infusion on MPS using the A-V balance method. Finally, Pacy et al. (563) found no effect of insulin on FSR.

The confounding factor in the above human studies is that insulin in plasma decreases concentrations of circulating and intracellular amino acids due to reduced splanchnic release . A decrease in amino acid availability could theoretically negate any stimulatory effect insulin might have on MPS. The studies described in Table 2-4 were designed, therefore, to measure

the effect of an insulin infusion on MPS in human skeletal muscle in the presence of physiological concentrations of circulating amino acids.

experimental condition	results	reference
AAs + glu + insulin	↑ MPS, ↓ MPB	
AA clamp + insulin	↑ MPS, -- MPB	
AAs + insulin	↑ MPS	
AA clamp + insulin	↑ MPS	
AA clamp + insulin	↑ MPS	

Table 2-4 Effect of insulin infusion on human skeletal muscle protein synthesis in the presence of amino acids

Experimental condition is compared to baseline measures prior to the start of infusion or compared to an infusion of only amino acids. Insulin, in the presence of sufficient plasma amino acids, facilitates a rise in the rate of MPS resulting in a more positive muscle protein balance. AAs, amino acids; glu, glucose; MPS, muscle protein synthesis; MPB, muscle protein breakdown ↑, increase; ↓, decrease; --, no change.

Taken together the results in Table 2-4 suggest that, in the presence of sufficient amino acids, insulin can increase the rate of MPS in human skeletal muscle. A recently published study by Greenhaff et al. however, indicates that insulin does not exert a stimulatory effect, but that its presence is required to allow amino acids to exert their stimulatory effect. In other words, it appears that the effect of insulin on MPS is permissive rather than stimulatory. Indeed, evidence suggests that the presence of insulin allows translation initiation to proceed, as will be reviewed in section 2.2 . Greenhaff et al. steadily increased the concentration of infused insulin while holding circulating amino acid concentrations constant, and measured no increase in the rate of MPS beyond that observed at an insulin concentration of 5 mU/L. It appears then that this is the minimum concentration above which insulin exerts its permissive effect on MPS in human skeletal muscle in that its presence allows synthesis to progress independently of dosage .

2.1.3 The interactive effect of exercise and nutrition on muscle protein turnover

After exercise protein balance becomes more positive, or less negative, due to post-exercise stimulation of the rate of protein synthesis, and a decrease in the rate of protein oxidation.

Although these factors combine to result in a higher net protein balance, values may remain negative in the fasted state . Maximum positive protein balance following exercise is only achieved with adequate nutrition . Another way of interpreting this is that maximal MPS responses to exercise are only obtained in the fed state when the nutrients are available to take advantage of the exercise stimulus.

Recent research in the area of post-exercise nutrition has focused on resistance exercise and mixed MPS. Ingestion of amino acids after resistance exercise, either in isolated form or in protein , stimulates the rate of MPS more than exercise alone . Table 2-5 summarises the results of several studies examining the effects of providing nutrition immediately after a bout of resistance exercise.

experimental condition	results	reference
RE + AA infusion	MPS ↑ 2-fold from rest	
RE + AAs and CHO ingestion	MPS ↑ 4-fold from rest	
RE + PRO and CHO ingestion	FSR ↑ 2.5-fold from rest	

Table 2-5 Effect on human skeletal muscle protein synthesis of provision of nutrition immediately after a bout of resistance exercise

Experimental condition is compared to baseline measures prior to the start of exercise. The combination of resistance exercise and post-exercise nutrition causes a rise in the rate of skeletal MPS. RE, resistance exercise; AA, amino acid; CHO, carbohydrate; PRO, protein; MPS, muscle protein synthesis; FSR, fractional synthesis rate; ↑, increase.

The results in Table 2-5 suggest that availability of amino acid has a marked effect on protein balance after resistance exercise. This conclusion is further supported by a 14-week resistance training study , in which subjects were given either protein or carbohydrate nutrition before and after each exercise bout. Only the group receiving protein supplementation experienced muscle hypertrophy, the result of a prolonged period of positive protein balance.

As well as stimulating rates of protein synthesis, the availability of amino acids has an inhibitory effect on rates of protein breakdown after a bout of exercise. Because exercise stimulates MPS, if the size of the amino acid free pool is small, the main source of amino acids for MPS is MPB. This intramuscular recycling of amino acids is increased by exercise .

Tipton et al. and Biolo et al. both found that amino acid infusion after resistance exercise prevented the normal post-exercise rise in MPB. The decreased breakdown observed in these studies was likely due to an increased availability of amino acids for MPS, thereby reducing the need for MPB to supplement the free pool.

On its own, insulin has a positive effect on protein balance after resistance exercise due to inhibition of MPB. Roy et al. found that 3-MH release after a bout of resistance exercise was lower in a group ingesting carbohydrate after the bout than in those consuming a placebo. The insulin released due to the carbohydrate consumption would have inhibited WBPB and thus created a more positive protein balance. Both Biolo et al. and Borsheim et al. , using the A-V balance technique, also found a decreased response of exercise-induced MPB in the m. vastus lateralis with either a local insulin infusion or carbohydrate ingestion after resistance exercise. The above findings indicate that the presence of plasma insulin, either from infusion or as a response to feeding, has an inhibitory effect on the rise in MPB caused by resistance exercise.

Regarding the effect of protein supplementation on muscle protein balance after aerobic exercise, only two studies have been done. Using a stable isotope tracer, Koopman et al. found that during six hours of cycling and running the consumption of carbohydrates alone left subjects in negative whole body protein balance. Adding protein to the carbohydrate beverage resulted in a more positive, or less negative, whole body protein balance during both the exercise and the post-exercise recovery period. The improvement in protein balance was the result of a combination of increased protein synthesis and decreased breakdown. Also using stable isotope tracers, Howarth et al. found that consuming a combination of protein and carbohydrate after two hours of cycling resulted in a higher rate of mixed muscle FSR than consuming carbohydrate alone. Howarth et al. also found that whole body protein balance was positive only in the protein-plus-carbohydrate condition. Unlike Koopman et al., Howarth et al. determined that the improved protein balance was due mainly to a decreased rate of WBPB.

Post-aerobic exercise protein nutrition is an area that requires considerable further investigation.

Insulin also appears to have a positive effect on protein balance during and after aerobic exercise. Millward et al. found that glucose given during exercise prevented a rise in WBPB, presumably by causing an increase in plasma insulin. It is possible however that the effect of carbohydrate ingestion during and after aerobic exercise, on protein balance may have less to do with insulin and in fact be an issue of energy supply. Because MPS is an energy-consuming process, protein balance and energy balance are inextricably linked. Exercise is more anabolic when energy and protein intakes are adequate, and more catabolic when energy is inadequate, while consuming additional protein in an energy-deficient state improves protein balance . Roy et al. provided either carbohydrate only, or a combination of carbohydrate, protein, and fat, to subjects after a bout of resistance exercise, and found that both meals increased NOLD by an equal amount when compared to subjects consuming a placebo. Roy et al.'s findings suggest that absence of energy was the factor limiting the exercise-induced increase in protein synthesis. As it is impossible to provide energy in the form of carbohydrates without causing a rise in plasma insulin, it is unlikely that this dilemma will be resolved. Regardless of the specific effect of insulin, the above research findings indicate that a combination of carbohydrates and amino acids or protein, has the most positive effect on MPS after a bout of aerobic exercise.

2.1.3.1 Post-exercise nutrient timing and muscle protein turnover

Evidence also suggests that the timing of post-exercise nutrition may affect its efficacy. Nutrient intake immediately following resistance exercise has a greater effect on the rate of protein synthesis in human skeletal muscle than that delayed by two or three hours . Ingestion of a protein-plus-carbohydrate supplement immediately following each bout of resistance

exercise over a 12 week training programme, resulted in greater increases in muscle strength and mass, indicating chronically positive muscle protein balance when compared to ingestion delayed by two hours . A confounding factor in Esmarck et al.'s study is that the group receiving the delayed treatment drink did not receive any food energy at all until two hours post-exercise. Delayed energy provision makes it difficult to separate the effect of the amino acids from that of the energy needed for MPS. Rasmussen et al. used the three-pool model of muscle protein kinetics (Appendix A3) to compare the effect of essential amino acids and carbohydrate consumed either one or three hours after a bout of resistance exercise. Rasmussen et al. detected no difference in the rate of MPS between the timing conditions, suggesting that even a one-hour delay is too great to provide maximal stimulation to the rate of post-exercise MPS. Furthermore, Tipton et al. provided carbohydrate and essential amino acids or whey protein either just before, or just after, a bout of resistance exercise, and measured the effect on phenylalanine uptake by the leg, an indicator of MPS. The authors found a greater stimulatory effect on MPS when the amino acid mixture, but not the whey protein mixture, was provided before exercise than afterwards. As Tipton et al. have been the only group to consider pre-exercise feeding, and their results were obtained using the relatively inaccurate three-pool model (see Appendix A3 for discussion), it seems that feeding post-exercise is the appropriate method. It appears that immediate post-exercise protein nutrition has a greater stimulatory effect on MPS than that which is delayed, a factor that should be incorporated into the design of studies investigating the effect of post-exercise nutrition on muscle protein kinetics.

2.2 Intracellular signalling in skeletal muscle: selected pathways

The intracellular signals leading to mitochondrial biogenesis will now be considered. There are three types of transcriptional regulators in the intracellular signalling process; transcription factors that bind to the promoter regions of DNA, co-activators that integrate signals and affect

transcription without binding directly to DNA, and proteins of the transcriptional machinery such as ribosomes and RNA polymerase . During an exercise stimulus, signals are localised to the contracting muscles and lead to the adaptive changes described in 2.1 . The PGC-1α pathway is involved in signalling for up-regulation of mitochondrial proteins. An increase in mitochondrial protein expression occurs as an adaptive response to aerobic exercise training, therefore study of the PGC-1α pathway is relevant to this thesis. A PGC-1α-independent signal for mitochondrial biogenesis is the presence of reactive oxygen species (ROS), a topic not covered in this thesis. The mTOR pathway signals for the translation of many skeletal muscle proteins; up-regulation of this pathway is required for many proteins, including mitochondrial, to be synthesised.

2.2.1 The PGC-1α pathway: from exercise to mitochondrial protein

The PGC-1α pathway begins with contractile activity setting off intracellular events leading to kinase and phosphatase activation by phosphorylation. Subsequently activated transcription factors and transcriptional co-activators then up-regulate the transcription of nuclear and mitochondrial DNA, which leads ultimately to increased translation and expression of mitochondrial protein. Various gene knockout studies have shown that the absence of a single step in the pathway does not eliminate this increase in expression completely, suggesting that there is considerable redundancy and/or synergism in the pathway . Transcriptional co-activators, such as PGC-1α, affect transcription without direct DNA binding; instead they bind to, or interact with, transcription factors bound to the promoter regions of the ultimately targeted genes to form a protein complex . In contrast, transcription factors bind directly to specific DNA sequences .

2.2.1.1 The PGC-1α pathway and mitochondrial protein

Among the myriad biochemical effects of exercise, three have been shown to increase mitochondrial biogenesis either directly or indirectly (Figure 2-3). These effects are: an increase in intracellular calcium ion concentration , a decrease in the ATP/ADP ratio, and an increase in cyclic GMP.

The release of calcium by the sarcoplasmic reticulum into the cytosol causes the activation of CaMK which subsequently activates p38 MAPK . Activation of p38 MAPK in turn increases the transcription of PGC-1α in one of several ways, leading to an increased concentration of PCG-1α mRNA . Activation of p38 MAPK leads to activating transcription factor 2 (ATF2) phosphorylation , inhibition of $p160^{MBP}$, and direct activation of PGC-1α . ATF2 phosphorylation stimulates the cyclic adenosine monosphosphate (AMP)-responsive element-binding protein on PGC-1α, thereby providing transcriptional activation . Inhibition of $p160^{MBP}$ relieves this protein's inhibitory effect on PGC-1α thereby effectively stimulating it . Calcium release can also directly activate p38MAPK without acting through CaMK . Calcium release also activates the calcium/calmodulin-activated protein phosphatase calcineurin . Calcineurin activation leads to an increase in myocyte-specific enhancer factor 2 (MEF2) expression, a factor that can stimulate PGC-1α expression . MEF2 is also activated by p38MAPK . PGC-1α is involved in a number of auto-regulatory positive feedback loops; with MEF2, with ATF2, and with itself, the details of which are beyond the scope of this review . This high degree of redundancy and complementarity in the PGC-1α pathway provides compensatory mechanisms to enhance cell survival .

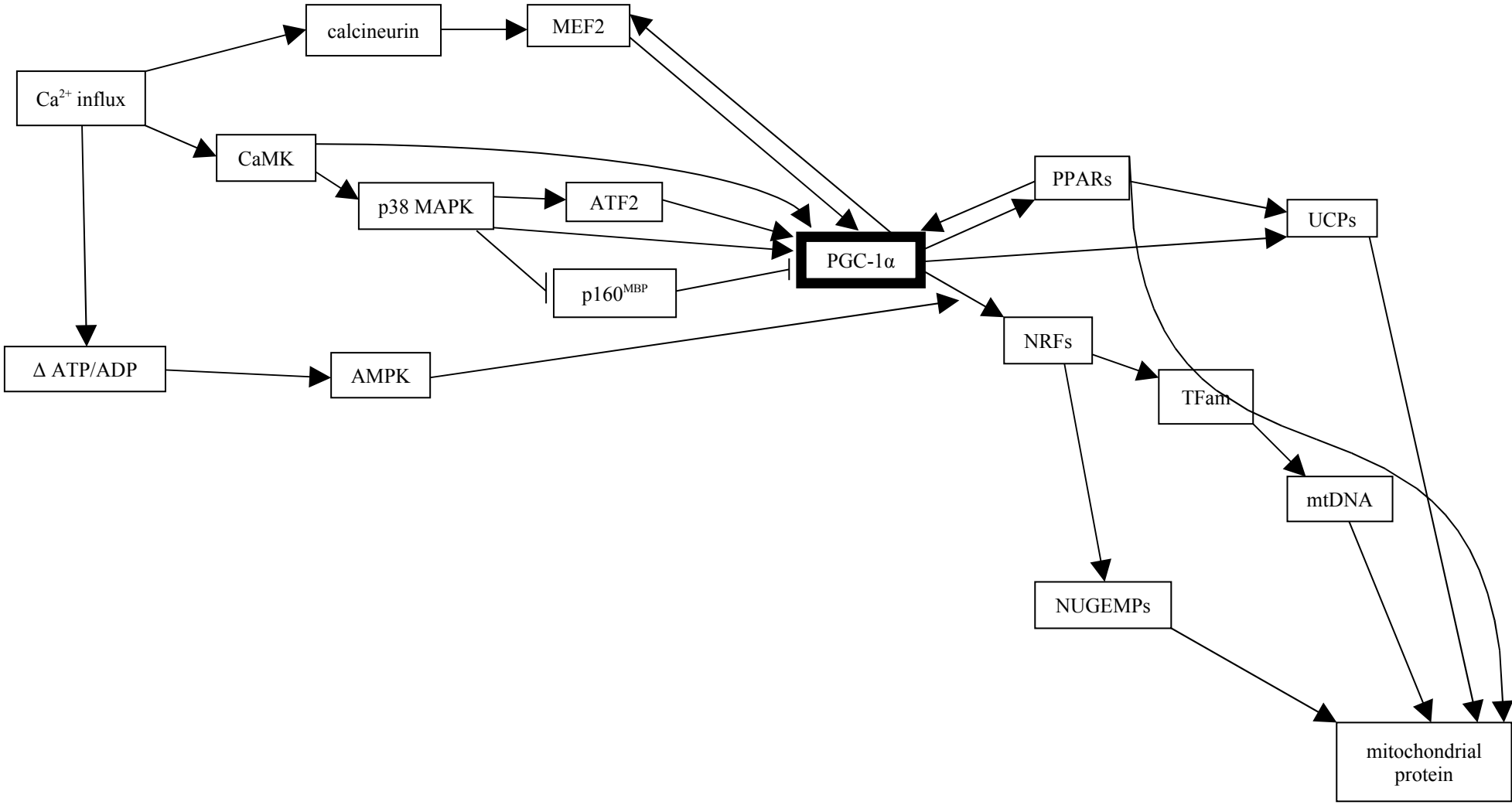


Figure 2-3 Signalling cascade resulting in increased concentrations of mitochondrial protein in mammalian skeletal muscle

Contractile activity causes a rise in intracellular calcium concentration and a decrease in the ATP/ADP ratio. Both these signals activate a cascade leading to up-regulation of PGC-1α, the master regulator of mitochondrial biogenesis. PGC-1α activates the transcription of genes encoding mitochondrial protein located in both the nucleus and mitochondrial DNA, ultimately leading to an increased expression of mitochondrial protein. → activation or up-regulation; —| inhibition; see text for abbreviations.

The ATP/ADP ratio, a measure of cellular energy status, is affected directly by exercise and nutrition, and indirectly by an increase in intracellular calcium. ATP hydrolysis leads to an increased cellular concentration of AMP, decreased phosphocreatine and subsequently increased phosphorylation of AMP kinase (AMPK) . AMPK acts as a cellular energy sensor in that, if mitochondria are not producing ATP fast enough to meet the cell's demand during a bout of exercise, then the intracellular signalling cascade to make more mitochondria is activated after the bout is finished, in order to better meet the demands of the anticipated next bout of exercise. AMPK acts in concert with PGC-1α to co-activate downstream transcription factors involved in mitochondrial biogenesis. Chemical activation of AMPK (with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)) has been shown to increase the expression of mitochondrial enzymes, while AMPK knockout transgenic mice display no up-regulation of downstream genes involved in mitochondrial biogenesis . Cyclic GMP has also been implicated in increased PGC-1α expression .

Evidence from transgenic mice over-expressing PGC-1α suggests that this gene is likely responsible for mitochondrial biogenesis . The transgenic mice in Calvo et al.'s study had lower respiratory exchange ratio values during exercise, indicating increased fat oxidation by the mitochondria. Calvo et al.'s transgenic mice also demonstrated a 20% higher VO_{2peak} than their wild-type counterparts, suggesting greater peripheral oxygen extraction and use by mitochondria in the working muscle. Presumably it is PGC-1α's downstream effects, as described in the following paragraphs, that induce this mitochondrial biogenesis.

Mitochondrial proteins are encoded by both nuclear and mitochondrial DNA (mtDNA) and therefore their synthesis requires a co-ordination of signalling to both these genomes . PGC-1α has been identified as the master regulator in the mitochondrial protein synthetic process as it co-activates a number of downstream transcription factors as well as regulating its own

transcription and translation . PGC-1α co-activates the nuclear hormone receptors peroxisome proliferation-activated receptors alpha and delta (PPARα, PPARδ) which bind to DNA and induce the expression of downstream genes such as uncoupling proteins 2 and 3 (UCP2, UCP3) respectively . Increased expression of the UCPs has been associated with increases in such mitochondrial proteins as cytochrome c, cytochrome c oxidase, citrate synthase, and succinate dehydrogenase . There is evidence that the PPARs may also independently control the expression of mitochondrial oxidative enzymes , as PPARδ up-regulates oxidative capacity in mice . Furthermore, evidence from murine skeletal muscle myocytes suggests that PPARδ plays a regulatory role in PGC-1α and mitochondrial transcription factor A (TFam) transcription , while PPARα is involved in the expression of enzymes involved in fatty acid oxidation .

PGC-1α also co-activates the upstream transcription factors nuclear respiratory factors 1 and 2 (NRF1 and 2) and enhances their binding to DNA . NRF activation increases both the activation of TFam and nuclear genes encoding mitochondrial proteins (NUGEMPs) . NRF2 promotes both its own expression as well as that of NRF1 . These post-translational changes in transcription factor phosphorylation status increase the gene expression of such downstream mitochondrial protein targets as cytochrome c, cytochrome c oxidase, NADH dehydrogenase, citrate synthase, and succinate dehydrogenase . Activated TFam translocates into the mitochondria where it binds to the D-Loop of mtDNA , thereby inducing mtDNA transcription and replication and ultimately translation of mitochondrial protein . TFam is the sole transcription factor interacting directly with mtDNA , and mtDNA transcription cannot be initiated correctly without its action . The NRFs also regulate transcription of the mtDNA transcription initiation factors TFB1m and 2m . In addition to their role as upstream transcription factors, the NRFs also directly regulate the NUGEMPs .

2.2.1.2 Effects of exercise and nutrition on the PGC-1α pathway

As explained in 2.2.1.1, the onset of exercise increases concentrations of intracellular and mitochondrial calcium and causes AMPK activation . Low-frequency stimulation, designed to mimic the effects of aerobic exercise, results in a roughly two-fold increase in phosphorylation of AMPK in rat and murine skeletal muscle that remains elevated for several hours following cessation of stimulation . In humans, an hour of moderately high-intensity cycling elicits a slightly lower and more transient increase in AMPK phosphorylation . This increase may or may not be enough to trigger the signalling cascade, ultimately resulting in an expansion of the mitochondrial reticulum, and further work is needed in human skeletal muscle to determine this .

Up-regulation of kinase and phosphatase activity has been demonstrated following bouts of aerobic exercise. Calcineurin activation has been shown to increase up to three-fold, peaking at one hour after a bout of cycling or one-legged kicking . Phosphorylation and subsequent activation of p38MAPK has been shown to increase up to four-fold, in both rat and human skeletal muscle, immediately after swimming, running or one-legged cycling at moderately high intensities . Electrically-induced contraction of muscle cells and murine skeletal muscle results in activation of both p38MAPK and its downstream target ATF2 , while a bout of swimming resulted in an immediate three-fold increase in ATF2 phosphorylation in rat skeletal muscle . These changes in activation signal directly to PGC-1α and would be expected to precede increases in PGC-1α mRNA and its downstream targets.

Table 2-6 provides a summary of findings on the effects of aerobic exercise on the expression of PGC-1α mRNA in skeletal muscle. The magnitude of the effect depends on the intensity and duration of the exercise, as well as the time following cessation of exercise at which the muscle is sampled. Baar et al. measured an increase in PGC-1α protein at 18 hours post-

exercise, thus demonstrating that an increase in PGC-1α mRNA does precede an increase in PGC-1α protein translation in rat skeletal muscle. Furthermore, Berardi et al. found that chronic contractile activity led to a higher steady state concentration of PGC-1α protein content, indicating that measures of PGC-1α mRNA do provide an informative proxy for protein translation. While an increase in PGC-1α mRNA does suggest a subsequent increase in its protein concentration, the initial downstream effects of PGC-1α activation may be post-translational in that the phosphorylation of existing PGC-1α protein leads to the transcriptional activation of its downstream targets . Over-expression of PGC-1α in murine skeletal muscle has been shown to lead to increased mitochondria gene expression and enzyme activity as well as enhanced muscle oxidative capacity .

animal	exercise parameters	change in expression	reference
mice	12h voluntary wheel running	↑ 2-fold	
mice	90min treadmill running	↑ 4-fold (3h)	
rats	2 x 3h swimming, 45min rest	↑ 95% (6h)	
rats	2 x 3h swimming, 45min rest	↑ 8-fold (0h)	
rats	2 x 3h swimming, 45min rest	↑ 2-fold (6h)	
humans	3h leg extension; 50% Wmax	↑ 8.5-fold (3h)	
humans	3h 2-legged kicking	↑ 10-fold (2h)	
humans	45min 1-legged kicking	↑ 2.5-fold (2, 6h)	
humans	1h cycling; 70% VO_{2peak}	↑ 8.5-10-fold (3h)	

Table 2-6 Changes in PGC-1α mRNA expression in untrained mammalian skeletal muscle following a bout of aerobic exercise

An acute bout of aerobic exercise leads to an increase in PGC-1α mRNA expression in mammalian skeletal muscle. Expression is indicated as a -fold change from baseline values, time in hours is that at which expression was highest following cessation of exercise. VO_{2peak}, peak rate of oxygen consumption during a maximal test; ↑, increase; h, hours; min, minutes.

Aerobic exercise also increases the mRNA expression of genes downstream of PGC-1α, such as the PPARs. PPARα mRNA abundance in human skeletal muscle increases slightly in response to an acute bout of aerobic exercise as well as a period of aerobic exercise training . Aerobic exercise training and a period of electrical stimulation designed to mimic the effects of aerobic exercise have both been shown to increase the expression of PPARδ mRNA in rat and

mouse skeletal muscle . Investigation of the effects of aerobic exercise on PPARδ mRNA in post-absorptive human skeletal muscle is yet to be done.

Further downstream, aerobic exercise-induced increases in TFam mRNA expression have been measured in humans at six, but not two, hours post-exercise . In rats, swimming and running have been shown to elicit increases in the mRNA expression of NRF-1, and of such mitochondrial proteins as citrate synthase and cytochrome c . Clearly, more work in the area of PGC-1α signalling to its downstream effectors after aerobic exercise in humans is required.

To summarise, exercise induces activation of gene expression in the PGC-1α signalling pathway, which leads to post-transcriptional changes ultimately resulting in the structural and biochemical adaptation of the mitochondrial reticulum . Repeated bouts of exercise over time have been shown to lead to an accumulation of mitochondrial protein, with Williams et al. being the first to show that the mRNA of both NUGEMPS and mtDNA increase with an exercise training programme.

The only work regarding the direct effect of nutrition on the PGC-1α signalling pathway has been in mice, demonstrating activation of both the α and δ PPAR isoforms with fatty acid ingestion, and of the δ isoform during starvation . All three isoforms (α, δ, and γ) have been implicated in fatty acid metabolism, the regulation of which is beyond the scope of this review. There appears to be no information whatsoever regarding the effect of carbohydrate or protein nutrition on the PGC-1α pathway and its downstream effects on the synthesis of the mitochondrial proteins involved in oxidative metabolism; this is one area warranting considerable investigation.

Previous work in the area of post-aerobic exercise nutrition and the PGC-1α signalling pathway has focused on carbohydrate ingestion. Cluberton et al. provided a carbohydrate drink before

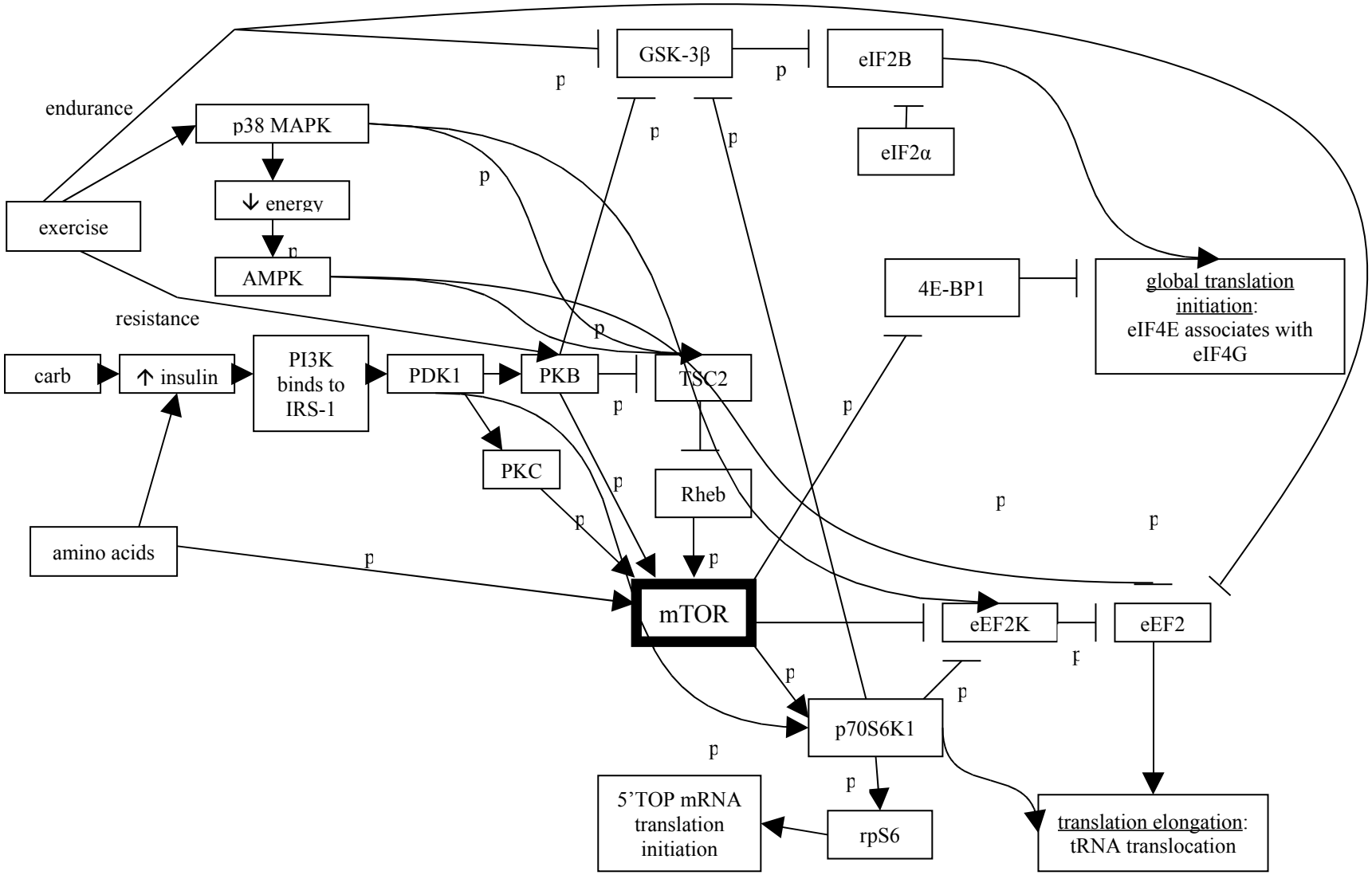
one hour of cycling at 75% VO_{2max}, while Russell et al. provided carbohydrates before and during a two hour bout of cycling at 50% W_{max}. Both studies measured an up-regulation in PGC-1α and PPARδ mRNA which was not different from subjects receiving a no-energy placebo. Pilegaard et al. and Mathai et al. provided either a low- or a high-carbohydrate meal after a high- or moderate-intensity bout of cycling respectively. After the moderate-intensity bout, both the carbohydrate-fed and the placebo groups showed three- and six-fold increases in PGC-1α mRNA expression immediately following, and two hours after, the cessation of exercise respectively. There was a 23% increase in PGC-1α protein expression at both time points in both groups. The findings of Cluberton et al., Russell et al., and Mathai et al. all suggest that the ingestion of a carbohydrate-only meal has no additive effect on signalling for mitochondrial biogenesis after aerobic exercise. Only Pilegaard et al.'s high-intensity bout of cycling resulted in a difference between the carbohydrate conditions, presumably because of stronger AMPK signalling due to greater energy depletion, with the low-carbohydrate meal inducing a more prolonged up-regulation of PGC-1α mRNA expression. A five-fold increase in PGC-1α mRNA was observed in both feeding conditions at five hours post-exercise. This increase returned to baseline by eight hours post-exercise in the high-carbohydrate condition but was maintained at the same five-fold increase in the low-carbohydrate condition. Both conditions stimulated a 2.5-fold increase in PPARα mRNA at eight hours post-exercise. Pilegaard et al. and Neufer et al.'s findings suggest that the energy restriction imposed in the low-carbohydrate condition may up-regulate AMPK and therefore increase signalling for mitochondrial biogenesis. Mathai et al.'s findings are consistent with this hypothesis, as restricting carbohydrate intake after a bout of aerobic exercise would limit glycogen repletion. The authors found a significant negative correlation between muscle glycogen and PGC-1α protein expression. The findings in this paragraph were kept in mind in designing the study, undertaken in this thesis, of the effect of post-aerobic exercise nutrient consumption on

signalling in the PGC-1α pathway.

To date there have been no studies looking at the effect of feeding protein after aerobic exercise on signalling for mitochondrial biogenesis.

2.2.2 The mTOR pathway: signalling for protein translation

The mTOR pathway (Figure 2-4) is responsible for the translation of mRNA into protein. This is the pathway by which most protein synthesis (including mitochondrial) occurs. mTOR, a serine-threonine protein kinase, is a convergence point for a variety of signals coming from amino acids, insulin, and various types of exercise, to a plethora of downstream effectors. Phosphorylation status affects binding of transcription factors to DNA .


endurance
p38 MAPK
↓ energy
exercise
AMPK
resistance
carb
↑ insulin
PI3K binds to IRS-1
PDK1
PKB
TSC2
PKC
Rheb
amino acids
mTOR
GSK-3β
eIF2B
eIF2α
4E-BP1
global translation initiation: eIF4E associates with eIF4G
eEF2K
eEF2
p70S6K1
5'TOP mRNA translation initiation
rpS6
translation elongation: tRNA translocation
p

2.2.2.1 mTOR signalling and protein translation

Translation initiation begins with the binding of eukaryotic initiation factor 2 (eIF2) to met-$tRNA_i$, a process that involves a guanine nucleotide exchange that is mediated by eIF2B (Figure 2-5). This major regulatory point in global translation initiation is mediated by eIF2α via its inhibitory effect on eIF2B . Subsequent binding of the met-tRNA ternary complex to the 40S ribosomal subunit forms the 43S pre-initiation complex. Binding of eIF4G and eIF4E to form the active eIF4F complex depends partly on the phosphorylation and inactivation of 4E-BP1 . Formation of the eIF4F complex allows binding of mRNA and the 43S complex to form the 48S pre-initiation complex, a step that can be affected by leucine . The final step in translation initiation is the formation of an 80S translation-capable ribosome complex which is also comprised of 60S ribosome subunits and ribosomal proteins .

Elongation, when the ribosome moves along the mRNA strand , involves binding of amino acids to the tRNAs for incorporation into a polypeptide chain, and is controlled by the eukaryotic elongation factors (eEFs) . Finally, translation is terminated when the completed protein is released from the ribosome, an event mediated by a stop codon .

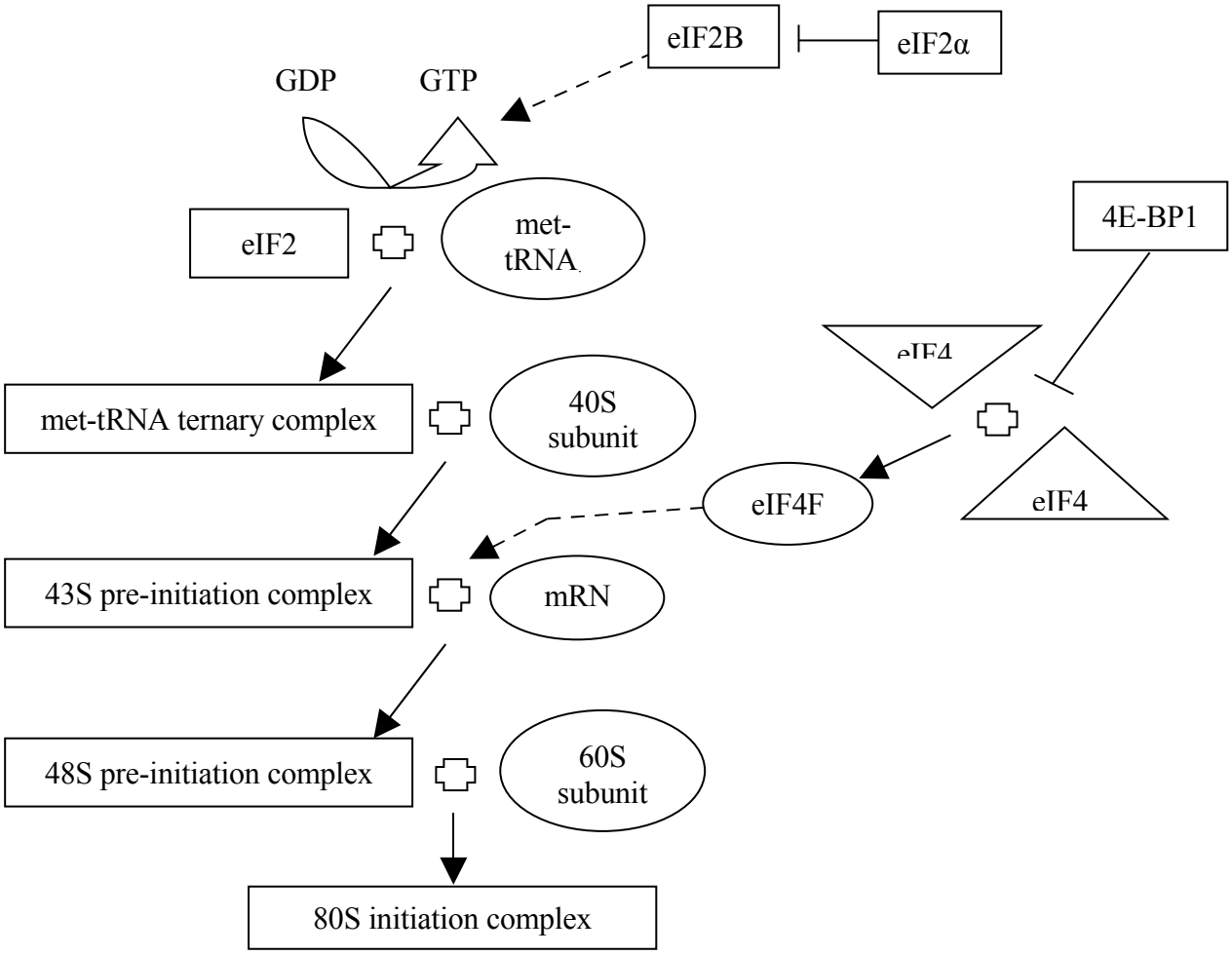


Figure 2-5 Steps in the initiation of protein translation in mammalian skeletal muscle

Phosphorylation of 4E-BP1 is required to release its inhibitory effect on eIF4F complex formation and for translation initiation to proceed. See text for further explanation. GDP, guanine diphosphate; GTP, guanine triphosphate; eIF, eukaryotic initiation factor; tRNA, transfer RNA; 4E-BP1, eIF binding protein; ⌬ binds with; → to form; - - → mediates. Adapted from .

Up-regulation of the mTOR pathway mediates both translation initiation and elongation. Phosphorylation of mTOR leads to a number of downstream events (Figure 2-4), including phosphorylation and inhibition of 4E-BP1, phosphorylation and inactivation of eEF2 kinase (eEF2K), and phosphorylation and up-regulation of 70 kDa ribosomal protein S6 kinase 1 ($p70^{S6K1}$) . Inhibition of 4E-BP1 is required for translation initiation as, in its active form, 4E-BP1 forms an inactive complex with eIF4E . Phosphorylation of $p70^{S6K1}$ has a myriad of effects on translation elongation, including release of inhibition on eEF2 and phosphorylation of ribosomal protein S6 (rpS6). The phosphorylation of rpS6 increases the rate of translation

initiation of 5'TOP (tract of pyrimidine) proteins . The 5'TOP proteins are mainly those of the protein synthetic machinery, such as ribosomes and elongation factors, the translation of which increases the capacity of the cell to synthesise protein .

The mTOR pathway is stimulated by insulin, amino acids, and both aerobic and resistance exercise (Figure 2-4). Insulin acts via phosphatidylinositol-3-OH kinase (PI3kinase) and IRS-1 (an insulin receptor substrate) to phosphorylate and activate mTOR via a phosphoinositide-dependent kinase 1 (PDK-1) and PKB (aka Akt, a serine-threonine kinase) -dependent pathway. PDK-1 can affect translation by either phosphorylating and activating $p70^{S6K1}$ directly, or via PKC and mTOR. PKB can also activate mTOR either directly by phosphorylation or indirectly by inhibiting tuberous sclerosis complex (TSC2). Inhibition of TSC2 relieves inhibition on ras homolog enriched in brain (Rheb), which then activates mTOR. Some amino acids are sensed by a new class of intracellular pI3 kinases that phosphorylate mTOR directly and thereby stimulate translation initiation and elongation . Only the BCAAs are specific regulatory proteins of the mTOR pathway . Although the specific mechanism by which amino acids signal mTOR is unclear, the receptor hVps34 has been identified . Amino acids also stimulate a rise in plasma insulin and, hence, stimulate mTOR by the insulin-dependent pathways described above . Resistance exercise sends stimulatory signals to PKB, while aerobic exercise can have a positive effect on translation initiation by relieving GSK-3 inhibition on eIF2B .

2.2.2.2 Effects of exercise and nutrition on the mTOR pathway

Exercise has an up-regulatory effect on signalling for protein synthesis. Resistance exercise activates signalling for translation via PKB phosphorylation and its subsequent downstream effectors mTOR, 4E-BP1, $p70^{S6K1}$, and eEF2 . Meanwhile, the specific effects of aerobic exercise remain unclear. Aerobic exercise appears to phosphorylate and inhibit both the α and

β isoforms of GSK-3 , which subsequently relieves inhibition of eIF2B and promotes translation initiation . In contrast, Atherton et al. found that an aerobic exercise-type stimulus of isolated rat skeletal muscle resulted in decreased 4E-BP1 phosphorylation, which would increase inhibition of active eIF4F complex formation and subsequent translation initiation, suggesting that aerobic exercise may inhibit mTOR-mediated translation. Furthermore, Rose et al. found that eEF2 phosphorylation increases during a bout of moderately high-intensity cycling, which would cause an inhibition of translation elongation (Figure 2-4). Signalling in the mTOR pathway following aerobic exercise is an area of research that is currently expanding.

Until recently, it was thought that only resistance exercise stimulates the mTOR pathway and only aerobic exercise stimulates the PGC-1α pathway . Recent evidence has demonstrated a departure from this dichotomy in that there is some crossover between the type of exercise and the pathway it affects. TSC2 appears to be a point of crossover between the PGC-1α and mTOR pathways as it is influenced by both insulin (via PKB) and p38MAPK and AMPK (Figures 2-3, 2-4). Insulin signalling increases inhibition on TSC2, and hence releases inhibition on mTOR, while the up-regulatory effect of p38MAPK and AMPK is a down-regulation of mTOR and its downstream effectors . As reviewed in 2.2.1.1, p38MAPK phosphorylation and activation is increased by calcium flux into the sarcoplasmic reticulum as a consequence of muscular contraction , while AMPK phosphorylation and activation is a consequence of the altered energy status following a sustained bout of aerobic exercise. Both p38MAPK and AMPK activation up-regulate the PGC-1α pathway. The effect of AMPK activation on inhibition of downstream targets PKB, mTOR, $p70^{S6K1}$, and rpS6 has been shown in rat, but not human, skeletal muscle . It stands to reason that AMPK activation would inhibit protein synthesis as the former indicates restricted energy levels while the latter is an energy consuming process . It may be surprising then that increases in PKB and mTOR

phosphorylation in human skeletal muscle after a bout of moderately high-intensity cycling have been measured . Widegren et al. however, reported no change in PKB activation after an hour of single-leg cycling at the same intensity. More extensive investigation into the combined effect of nutrition and aerobic exercise on signalling for translation initiation merits attention.

An inhibitor of translation elongation, eEF2K, is also activated directly by AMPK . Thus one would expect that during times of energy depletion, translation elongation would be limited. However, Coffey et al. and Wilkinson et al. demonstrated that a moderately high-intensity bout of cycling increased $p70^{S6K1}$ phosphorylation, suggesting an enhancement of translation elongation via relief of eEF2 inhibition, and initiation of translation of the proteins of the synthetic machinery via rpS6 (Figure 2-4). In addition, Mascher et al. reported a decrease in eEF2 phosphorylation, and hence relief of inhibition, after a similar hour of cycling, further supporting increased translation elongation following aerobic exercise. Contrary to expectations, translation elongation appears to be up-regulated following a bout of aerobic exercise. It is possible that the demand placed on the musculature by the exercise creates an anabolic signal that results in enhanced translation elongation using amino acids already present in the body's free pool. Whether this adaptive response could be maximised through the provision of appropriate post-exercise nutrition is a worthwhile area for future investigation.

Evidence suggests that translation initiation, rather than elongation, is the primary regulatory step in the response of protein synthesis to feeding. Jeyapalan et al. fed neonatal pigs either glucose with both insulin and amino acid concentrations clamped, or a combination of glucose, insulin and amino acids, and found that, while both conditions increased the formation of the

active eIF4F-eIF4G complex which precedes translation initiation, neither had an effect on eEF2, a mediator of elongation.

It has been shown, in cell culture and in rat skeletal muscle, that the presence of insulin above a concentration of 5 μU/mL increases the phosphorylation of several proteins in the mTOR pathway that lead to increased translation such as Akt , $p70^{S6K1}$, and 4E-BP1 , and also enhances eIF4F complex formation . Similarly, an insulin infusion in humans up-regulated the expression of those genes involved in the regulation of transcription and translation via rpS6 . Previous findings regarding the effect of insulin on mTOR signalling in human skeletal muscle provide equivocal results, and further investigation is needed .

Humans receiving an oral dose of essential amino acids experience a five- to eight-fold increase in skeletal muscle mTOR, 4E-BP1, and $p70^{S6K1}$ phosphorylation . Feeding amino acids causes the release of insulin into plasma, which sets off the signalling cascade described above . In addition, the BCAAs (leucine, isoleucine, and valine) exert a specific regulatory control on translation initiation by stimulating mTOR, with leucine exerting the majority of this effect . An oral dose of BCAAs has been shown to increase phosphorylation of both 4E-BP1 and $p70^{S6K1}$ in human skeletal muscle, thus demonstrating their role as direct nutrient signals . In rat skeletal muscle, leucine may also exert an mTOR-independent stimulatory effect on the rate of mixed MPS, although results in this area are equivocal . Furthermore, while rat skeletal muscle exposed to leucine displays increased mTOR phosphorylation, phosphorylation of its downstream effectors 4E-BP1 and $p70^{S6K1}$ does not increase, suggesting that amino acids signal to mTOR in a different way to insulin . It has been shown, in rat skeletal muscle, that the presence of leucine increases $p70^{S6K1}$ phosphorylation and reduces the formation of the inactive 4E-BP1-eIF4E complex, thus increasing eIF4E availability for active eIF4G-eIF4E complex formation . Furthermore, increased circulating concentrations of the essential amino acids may

enhance the rate of translation elongation by increasing the supply of required substrate for this process . In Chinese hamster ovary cells, increasing the availability of the essential amino acids resulted in decreased phosphorylation of eEF2 and, hence, increased up-regulation of elongation . To better understand the effect of amino acid feeding on signalling for protein translation in human skeletal muscle, particularly on mTOR's downstream effectors, more work is needed.

The concentration of circulating insulin does influence the effect that provision of essential amino acids has on mTOR signalling in human skeletal muscle. At insulin concentrations below 5 μU/mL, the provision of amino acids causes an increase in the rate of MPS with no change in mTOR signalling . Presumably, this increase in protein translation occurs at the elongation stage, with the extra amino acids providing more building blocks for the lengthening polypeptide chain. At insulin concentrations above 5 μU/mL, the provision of essential amino acids increases both the rate of MPS, and mTOR signalling .

It is currently unclear whether the provision of amino acids and carbohydrates together exerts a synergistic effect on signalling in the mTOR pathway in human skeletal muscle. Fujita et al. provided healthy humans with a leucine-enriched meal of amino acids and carbohydrates, and measured an increase in signalling for protein translation at PKB, mTOR, 4E-BP1, $p70^{S6K1}$, and eEF2, as well as decreased AMPK phosphorylation, which would have had positive downstream effects on translation. The provision of any nutrient would increase ATP availability, thus decreasing AMPK phosphorylation. Phosphorylation of the above proteins has been measured with the provision of either amino acids or carbohydrates alone. Since the authors did not compare the effect of providing carbohydrate-plus-amino acids with that of providing only amino acids or only carbohydrates, it is difficult to determine whether the nutrients provided were acting in concert or independently. In rat muscle, feeding a

combination of leucine and carbohydrate causes a greater increase in circulating insulin concentration that feeding carbohydrate alone, but the same increase in 4E-BP1 phosphorylation and eIF4F complex formation . As discussed in section 2.1.2, the anabolic effect of insulin could differ between rats and humans, so caution is warranted in extrapolating Anthony et al.'s findings to human skeletal muscle.

Interestingly, Fujita et al. measured no change in TSC2 phosphorylation, a factor downstream of both PKB and AMPK (Figure 2-2), while Jeyapalan et al. did measure a change in TSC2 phosphorylation in neonatal pigs following intravenous administration of a glucose, insulin, and amino acid mixture. In contrast to Fujita et al., Jeyapalan et al. found no change in eEF2 phosphorylation status. There are several explanations for this difference in findings, including the method of nutrient provision, the dose, and the fact that neonatal development is often different from normal adult growth. Clearly the effects of providing a combination of carbohydrate and amino acids on signalling for protein translation in human skeletal muscle is an area that requires further research.

The combination of resistance exercise and post-exercise nutrition has been shown to increase activation in the mTOR pathway more than exercise on its own. Phosphorylation of Akt, mTOR, 4E-BP1, $p70^{S6K1}$, and rpS6 were all increased to greater amounts and/or for longer when post-exercise nutrition was provided . Evidence also indicates that the provision of a carbohydrate-plus-protein meal following a bout of resistance exercise has a greater effect on signalling for protein translation in human skeletal muscle at 4E-BP1, $p70^{S6K1}$, and rpS6 than does a carbohydrate-only meal .

In rats and humans, work has shown that post-aerobic exercise nutrition elicits a greater increase in mTOR signalling than exercise on its own. Phosphorylation of PKB during a hyperinsulinemic and euglycemic clamp was higher in young healthy subjects following a one-

hour bout of moderately high-intensity cycling than in the same subjects at rest . Phosphorylation of PKB, 4E-BP1, $p70^{S6K1}$, and rpS6 are all greater when receiving a carbohydrate-plus-protein meal after exercise than a placebo . In rat skeletal muscle harvested after three hours of swimming, phosphorylation in muscle from rats in an unfed condition did eventually reach the same degree as that in muscle from rats fed immediately after they had finished swimming, but with considerable time delay to allow for gluconeogenesis to restore plasma insulin concentration . This finding suggests that insulin is the major signal to the mTOR pathway in rats, but it is unknown whether this situation is replicated in humans. Also in rats, provision of a carbohydrate-plus-protein meal has been shown to result in greater 4E-BP1 phosphorylation and active eIF4F complex formation than a carbohydrate-only meal after a high-intensity bout of running, suggesting that adding protein to post-exercise carbohydrate enhances mTOR signalling in rats . Whether this situation is replicated in humans is currently unknown, although it stands to reason that a combination of carbohydrate, to increase circulating insulin concentration, and essential amino acids, would have a synergistic effect due to multiple points of stimulation of the mTOR pathway . There is scope for investigation with regards to elucidating the roles of insulin and amino acids on signalling for MPS after aerobic exercise.

2.3 Ageing muscle and the effects of nutrition and exercise

Sarcopenia is a phenomenon whereby ageing muscle loses both mass and functional capacity, leading to frailty, disability, metabolic deterioration, and, ultimately, a loss of functional independence and quality of life . Skeletal muscle strength, aerobic capacity, and protein quality are all compromised. The quality of a protein can be defined as its ability to carry out its designated function, and the efficiency with which it does so per gram or litre or cross-sectional area . Some examples of muscle function are enzymes catalysing reactions, the oxidation of fuel, and the production of force. Some causes of sarcopenia are declining activity

levels, inadequate nutrition, decreased protein synthesis and turnover, and damaged DNA . While some of these causes are inevitable, the sarcopenic progression can be slowed with exercise and nutrition so that older people can retain their independence and quality of life for longer.

A loss of muscle mass could be explained by a reduction in protein synthesis, while reduced protein turnover can lead to a decline in muscle quality since damaged proteins are removed by protein breakdown . At the whole body level, both protein synthesis and turnover have been reported to decline with age . As fat-free mass (FFM) declines with age it is more appropriate, when comparing groups of young and old subjects, to use values of protein synthesis and turnover that have been adjusted for FFM. Table 2-7 defines a series of age ranges that will be used throughout this thesis.

Descriptor	Age Range (years)
young	18 – 34
middle-aged	35 – 55
older	56 – 69
elderly	> 70

Table 2-7 Definitions of age descriptors used in this thesis
The ranges described have been selected based on common usage of terms in current literature. The descriptors specified in this table are used throughout the thesis.

Young et al. were the first to report that protein synthesis per kilogram of body mass declines with age in humans, finding that WBPS in the elderly was 63% lower than in the young. Similarly, Balagopal et al. examined 24 subjects and found that the rate of WBPS decreased from that in the young to that in the middle-aged with no further decline in the elderly, even after adjustment for FFM. Using a much larger number of subjects than the above studies (n = 78), Short et al. measured whole body protein kinetics in individuals aged from 19 to 87. The authors found a 3 to 4% per decade decline in measures of whole body protein turnover, breakdown, and synthesis, with more of the variance in these measures being explained by age

than by FFM. Taken together, the above results indicate that whole body protein turnover and synthesis decline with age even following adjustment for FFM. A decline in protein turnover means decreased remodelling which will lead to accumulation of damaged proteins and a decrease in protein quality, while a decline in synthesis will lead to a decrease in protein quantity.

In contrast to the results in the preceding paragraph, several studies have found no difference in whole body protein kinetics between the young and the elderly. Winterer et al. and Benedek et al. both found no effect of age on whole body protein turnover per kg of FFM. Using the same tracer methodology as the above two authors, Morais et al. detected no difference in WBPS or breakdown per litre of lean tissue between young and elderly subjects after seven days on a 1.27 g protein/kg/day diet. Similarly, Welle et al. found no difference in WBPS between older people and the young when adjusted for FFM. The results in this paragraph suggest that the age-related decline in muscle quantity cannot be explained by differences in protein kinetics at the whole body level. So does WBPS decline with age or not?

It is possible that nutritional state may explain some of the discrepancies in the previous two paragraphs. Boirie et al. compared whole body protein kinetics in groups of young and older subjects and found no difference between the groups in leucine oxidation per kilogram of FFM in the post-absorptive state, while after a meal the older group had lower oxidation values than the young at similar rates of WBPS. According to the standard equations used to calculate whole body protein kinetics (Appendix A1), if synthesis remained constant then WBPB must have been reduced in the older group to retain a steady state. Lower WBPB suggests a reduced opportunity for remodelling and, therefore, reduced protein quality . Further, Boirie et al. found a greater splanchnic extraction of the leucine ingested with the meal, which suggests a

reduced availability of ingested amino acids to skeletal muscle, which could have implications for MPS, and hence muscle quantity.

Regardless of whether or not whole body protein turnover remains constant as the body ages, it is entirely possible that the contribution that muscle protein makes to this value may decline, a topic that is addressed in section 2.3.1.

2.3.1 Skeletal muscle mass and function declines with age

Body composition changes with age in that a decline in muscle mass is usually matched by an increase in fat mass. So even if people stay the same weight they have more adipose tissue . An early study found that the percentage of body weight that is muscle decreased by 10% from a young group to an elderly group . Similarly, Benedek et al. found that the percentage of body mass as FFM was significantly lower in older men than in young healthy men. Measures at the level of the muscle support the above statements. Short et al. measured a 37% decrease in leg muscle cross-sectional area between the ages of 19 and 87, while Welle et al. found 12% less FFM and 21% less muscle mass in older people than in young. Clearly, skeletal muscle mass declines with increasing age.

Evidence suggests that, while the overall amount of muscle protein may decrease, the quality of that muscle does not decline with age. While less muscle translates into less absolute strength , it appears that strength per unit of muscle mass may not be compromised. Frontera et al. found no effect of age on the peak torque produced by elbow and knee flexors and extensors in 200 healthy adults ranging from middle-aged to elderly. Retention of strength suggests that the quality of actin and myosin, key muscle proteins responsible for force production, is not compromised with age. In contrast, Balagopal et al. found that leg extensor strength declined with age independently of muscle mass in 24 subjects aged from young to elderly. In keeping with Balagopal et al.'s findings, both Lindle et al. and Lynch et al. found an age-related

decline in peak torque per kilogram of FFM in both arm and leg muscles in over 650 subjects aged from young to elderly. Resolution of the conflict in the above results seems to have been provided by Metter et al. who conducted a 25-year longitudinal study of over 400 men and found no age-related changes in isometric arm strength per unit of muscle. While a cross-sectional study cannot account for such factors as generational differences in environment, diet, and physical activity levels across a lifetime, a longitudinal study uncovers true age-related trends by eliminating inter-generational and inter-individual differences. Taken together, the above results suggest that muscle strength per gram may not decline with age.

In contrast, indicators of muscle protein quality other than strength suggest a definite decline with age. For instance, older muscle has decreased protein content both per kilogram and per unit of DNA . Decreased muscle protein means less storage of amino acids which means that, on degradation, fewer amino acids are released for use in MPS. A decline in amino acid availability may lead to a decline in muscle protein turnover, a process that maintains muscle quality . Such a decrease in protein content and quality would set up a downward spiral of diminishing muscle protein quality.

Evidence suggests that anabolic signalling is diminished in elderly skeletal muscle. With respect to synthesis of new mitochondrial proteins, the expression of PGC-1α in human skeletal muscle decreases with age , as do the mRNA levels of genes encoding mitochondrial protein . Signals for protein translation are also diminished in elderly rat and/or human muscle, as this tissue contains less total IRS-1, mTOR, S6K1, and eIF2B, and has lower resting levels of IRS-1 and PKB phosphorylation . Older rat skeletal muscle also has a higher RNA-to-protein ratio which suggests a lower efficiency of protein translation with age .

2.3.1.1 Muscle protein metabolism in ageing

Specific information regarding muscle protein metabolism at rest provides insight into the processes underlying the age-related decline in muscle quantity and quality. Two studies using [1-^{13}C]leucine infusions found a decline in resting muscle FSR with age. Short et al. determined that mixed muscle protein FSR decreases 3.5% per decade between the ages of 19 and 87. Similarly, Balagopal et al. , using tissue fluid as the precursor pool, found that the FSR of mixed muscle protein decreases by 12.5% from youth to middle age with no further decline in the elderly. Furthermore, lower mixed muscle FSRs have been measured in the elderly as compared to the young . Taken together, the above results indicate a decreased capacity to synthesise new muscle proteins with ageing. Decreased MPS sustained over time will result in lower muscle mass and quality.

A reduction in protein synthesis is also observed when measurements are made at the level of individual muscle sub-fractions. Welle et al. found a 28% lower myofibrillar FSR in older people than young, and found that myofibrillar protein synthesis made a 7% smaller contribution to WBPS in the older group. A decrease in myofibrillar protein synthesis rate will lead to strength losses. Supporting the above statement, Balagopal et al. measured an 18% decline in myosin heavy-chain FSR from youth to middle age, and a further 24% decline in the elderly. The subject of mitochondrial protein synthesis will be addressed in section 2.3.3.

In contrast to the findings of the previous two paragraphs, Volpi et al. found increased rates of MPS in older and elderly subjects, as compared to the young, when using the R_d of phenylalanine in the A-V balance method. Strangely, when using the three-pool model (Appendix A3) in the same study, the rate of mixed muscle FSR was similar between the old and young. Similarly, no difference in resting measures of MPS between groups of young and older subjects were found by other researchers using the A-V balance and three-pool model methods . Using more convincing methodology, Cuthbertson et al. determined FSR using

intracellular leucine as the precursor pool, and again found no difference between young and elderly men. The conflicting results described in this and the preceding two paragraphs highlight a current controversy surrounding the effect of age on the resting rate of MPS.

The other side of the muscle protein turnover story is MPB. Using nitrogen balance techniques, Winterer et al. and Uauy et al. were among the first to report a decreased resting rate of MPB in the elderly, as compared to the young, and a lesser contribution of muscle to whole body protein breakdown with advancing age. Morais et al. found MPB to be roughly 30% lower in the elderly than in the young, a difference that persisted when MPB was expressed per litre of muscle. In the same subjects, Morais et al. reported a lesser contribution of MPB to WBPB in the elderly group, measured by 3-MH excretion. Another measure of MPB was made by Trappe et al. in young and elderly subjects. The authors found that the rate of breakdown of actin and myosin, determined by 3-MH release, was higher at rest in the elderly. Similarly, Volpi et al. found higher values of MPB in their elderly subjects than in their young subjects. Over time, higher MPB coupled with lower MPS will most certainly result in lower muscle mass.

2.3.1.2 Causes of age-related decline in skeletal muscle

A number of factors outside a person's absolute control contribute to the decline in skeletal muscle mass and quality observed with ageing, such as oxidative damage caused by free radicals, a situation addressed in section 2.3.3, and changes in circulating hormones . The loss of functional capacity observed with increasing age is not, however, simply due to the process of growing older. Decreased activity levels and muscular de-conditioning lead to muscular atrophy , while muscular disuse and inactivity reduce the activities of mitochondrial enzymes . It is not simply a case of older people doing less exercise, but less activity in general as older people lead increasingly sedentary lifestyles .

Another cause of muscle mass loss under voluntary control is food intake. When muscle protein balance is chronically negative, as is the case during periods of inadequate nutrition, muscle loss occurs. Protein content, as well as overall energy content, of nutritional intake regulates the rates of MPS and breakdown, and also provides a source of scavengers for free radicals which, left unchecked, can cause muscle damage and atrophy . Bartali et al. conducted an epidemiological study of 802 people over 65, and found significant correlations between those in the lowest quintile of energy, and protein intake and low muscle strength. Furthermore, Winterer et al. found a strong positive correlation ($r = 0.72$) in the elderly between protein intake per kilogram of FFM and whole body protein kinetics. The above findings indicate that inadequate protein and overall energy intake can both play a significant role in skeletal muscle loss with ageing.

2.3.2 Aerobic function declines with age

Whole body aerobic capacity, measured as VO_{2max}, can decline with age to values below the aerobic demands of activities of daily living . VO_{2max} typically declines 10% per decade of life . VO_2 measures oxygen utilisation and, during exercise, active muscle can account for greater

than 95% of oxygen consumption so, given the loss of skeletal muscle with age, some decline with ageing in VO_{2max} is to be expected. The decline in VO_{2max} persists following adjustment for remaining FFM, indicating a concomitant decline in specific muscle quality.

Evidence from both cross-sectional and longitudinal studies indicates a decline in muscle oxidative capacity with ageing. Proctor and Joyner measured VO_{2max} in young and older endurance-trained subjects and found that the older group had scores 13% lower after adjustment for differences in muscle mass. Similarly, Houmard et al. measured a 0.4 mL/kg/min/yr decline in sedentary men aged 18 to 80. In a more comprehensive, cross-sectional study of untrained individuals aged 19 to 87, Short et al. observed an 8% loss of VO_{2max} per decade after adjustment for FFM. Results from longitudinal studies support the above findings. Fleg et al. followed subjects for eight years and measured VO_{2peak} in each decade of life. Per kilogram of FFM there was an accelerating, rather than linear, rate of decline, with a 3-5% loss per decade in young subjects, 7% per decade in middle-aged subjects, and a greater than 20% loss per decade in the elderly. Similarly, Hollenberg et al. followed subjects over the age of 55 for six years and found that their aerobic capacity declined 21% per decade and that this decline was independent of changes in body composition. Clearly, there is a loss of aerobic capacity with increasing age that cannot be explained entirely by the loss of skeletal muscle mass.

One factor in the age-related decline in VO_{2max} per kilogram of FFM is a loss of skeletal muscle's ability to extract and use oxygen from the circulation, that is, its oxidative capacity. VO_2 is a product of heart rate, stroke volume, and A-V oxygen difference and isolation of each variable indicates that the latter does decline with increasing age. Hollenberg et al. adjusted their measurements of VO_{2max} for declines in lung capacity and maximal heart rate and found that VO_{2max} still declined 10% per decade indicating that there is some peripheral cause for the

decline. Fleg et al. found that the decline in oxygen pulse, a product of stroke volume and arterio-venous oxygen difference, followed a similar trend to that of VO_{2max}. This reduction in arterio-venous oxygen difference is attributable to peripheral changes in oxygen uptake or extraction leading to decreased oxygen utilisation. Finally, Pedersen et al. used hyperoxic conditions to determine that VO_2 in the exercising muscle of young men is not limited by blood flow and oxygen delivery by measuring VO_{2peak} of the *quadriceps femoris* in normal air and 60% O_2. The authors found that, despite lower blood flow in the high oxygen condition, oxygen delivery was not different between the conditions. Taken together, the above results indicate that the decline in VO_{2max} with advancing age is in part due to a peripheral limitation in oxygen extraction.

The above conclusion is supported by the findings of Pogliaghi et al. , who subjected previously sedentary subjects to 12 weeks of either cycle ergometer or arm-crank training. Following the training period, the subjects' VO_{2max} were tested on both machines, with measured improvement on the non-specific apparatus being only half that of the machine they had been training on. Pogliaghi et al.'s findings indicate that only half of the subjects' adaptation to training was central and the other half, most likely, peripheral. If some of adaptation is peripheral then it stands to reason that some limitation would also be peripheral.

A reduction in skeletal muscle oxidative capacity underlies the above-mentioned peripheral limitations as maximal arterio-venous oxygen difference is a reflection of the ability of active muscle to extract and use oxygen to produce ATP . Conley et al. compared the change in VO_2 from unloaded cycling to maximum in groups of middle-aged and older subjects. The authors found that the change in VO_2 in the older group was less than half that of the middle-aged group and that whole body oxidative capacity, even when adjusted for the elderly group's diminished muscle volume, was reduced by nearly half. Finally, Conley et al. determined that the

oxidative capacity of the quadriceps in the older group was only one third of the middle-aged value and that this decline matched the decline in $VO_{2max.}$ Conley et al.'s result indicate that, although skeletal muscle quantity does decrease with increasing age, so does oxidative capacity per unit volume, indicating a decline in muscle quality.

The action of mitochondrial oxidative enzymes determines muscle oxidative capacity therefore the decline in this latter variable is likely due to a loss of muscle mitochondria protein content and function . Mathieu et al. measured mitochondrial volume density and VO_{2max} in a range of mammals and found a direct, linear relationship between these variables. Hoppeler et al. compared cat *gracilis* (glycolytic) and *soleus* (oxidative) muscles and found a significant correlation between mitochondrial volume density and muscle VO_{2max} indicating that, regardless of muscle type, equal amounts of oxygen are consumed by equal amounts of mitochondria. Coggan et al. measured concomitant increases in VO_{2max} and oxidative enzyme activity in response to exercise which suggests that VO_{2max} is associated with mitochondrial content.

2.3.3 Role of mitochondria in ageing

As explained in section 2.3.2, the age-related decline in VO_{2max} is primarily influenced by the quality and quantity of mitochondria in skeletal muscle protein, specifically, the muscle's capacity to extract oxygen from circulation, and mitochondrial oxidative capacity . Rates of mitochondrial protein synthesis and VO_{2max} both decline with advancing age , suggesting a link between these two variables. The quantity and quality of mitochondria affect both oxygen utilisation by working muscles, and the generation of ATP for muscular contraction .

Mitochondria are the site of energy production for muscular contraction via action of the Krebs cycle and the ETC, the latter being located on the inner mitochondrial membrane. The Krebs cycle generates some of the energy used for muscular contraction via the action of such oxidative enzymes as citrate synthase and succinate dehydrogenase. The most important

function of the Krebs cycle, however, is the production of the high-energy, reduced compound NADH. Oxidation of NADH is the first step in the ETC's five complexes, the last step being phosphorylation of ADP by ATP synthase to produce ATP. Oxidative capacity is defined as the ability to move electrons through the ETC and thereby generate ATP, which is then hydrolysed to provide energy for muscular contraction . This production of ATP is tightly linked to muscular aerobic capacity .

2.3.3.1 Mitochondrial protein quantity and quality

The quantity of mitochondrial protein in skeletal muscle is reduced with advancing age. Conley et al. measured a 19% lower mitochondrial volume density, estimated morphometrically using magnetic resonance spectroscopy, in the *m. vastus lateralis* of older subjects as compared to middle-aged subjects. The quantity of mitochondrial protein in skeletal muscle is a reflection of the balance between its synthesis and breakdown. Rooyackers et al. found that the rate of mitochondrial protein synthesis declines by 40% from youth to middle age, with no further declines into older age. Rooyackers et al. also measured a lower rate of mitochondrial protein synthesis relative to that of mixed muscle, indicating that mitochondrial protein synthesis declines proportionally more than the synthesis of all other muscle proteins. The results from Rooyackers et al.'s study provide a likely reason for the loss of mitochondrial protein with increasing age.

The quality, and hence function, of mitochondrial protein is also compromised in ageing skeletal muscle . A definition of mitochondrial quality is the rate of production of ATP via oxidative phosphorylation per milligram of mitochondrial protein, a variable which is closely associated with VO_{2max} . Many studies have compared activity levels of the mitochondrial enzymes succinate dehydrogenase and citrate synthase, in groups of young and older subjects, and found an age-related decrease . To measure mitochondrial function, Conley et al.

estimated oxidative capacity from post-exercise changes in muscle creatine phosphate in the *m. vastus lateralis* in middle-aged and older subjects. The rate at which creatine phosphate recovers after exercise varies in direct relation to the muscle's oxidative enzyme activity. Conley et al. found a 50% lower oxidative capacity in the older group's muscles, and also a reduced oxidative capacity per unit mitochondrial volume, indicating a decline in mitochondria protein quality.

Just as oxidative enzyme activity is impaired in ageing skeletal muscle, so too is ETC activity. Boffoli et al. and Cooper et al. both measured greater than 50% decreases in the activity and function of complexes I, II and IV of the ETC, from the young through to the elderly. Short et al. measured a progressive decline of 5% per decade in ATP production per milligram of mitochondrial protein, in subjects ranging in age from 18 to 89. The older group in Conley et al.'s study, mentioned in the previous paragraph, had a lower resting ATP concentration than the middle-aged group. As ETC activity contributes to ATP production, a lower concentration of ATP may indicate reduced ETC activity. As the older group also had reduced oxidative capacity, Conley et al.'s findings suggest that reduced oxidative capacity is related to reduced ETC activity. Supporting this notion are the findings of Kwong & Sohal , who compared the activities of complexes II, III and IV of the ETC in mitochondria isolated from young, adult, and old mice and found that the efficiency of the ETC decreased with advancing age. Taken together the above results indicate a decline in the oxidative enzyme activity, and hence function, of ageing skeletal muscle. As mitochondrial function is an indicator of muscle quality, the above findings suggest a mechanism for the decline in muscle quality with age.

In contrast to the above findings, a number of studies have detected no difference in indicators of mitochondrial function with increasing age. Kent-Braun & Ng found no difference in the post-exercise recovery time of creatine phosphate in the *tibialis anterior* between groups of

young and elderly men. Similarly, Houmard et al. found no difference in citrate synthase activity in the *m. vastus lateralis* between their groups of young and old men. Both Kent-Braun & Ng and Houmard et al. studied sedentary subjects with habitually low activity levels, whereas the studies in the previous two paragraphs made no mention of matching their subjects for activity status. It may be that activity status, rather than age, is the key variable in maintenance of mitochondrial function. In other words, the young may be just as susceptible to the deleterious effects of inactivity as the elderly, and the decline in function with age described in the previous paragraph may be a consequence of the elderly's natural tendency to decrease their activity levels rather than simply their advancing age. Such a conclusion is supported by Barrientos et al. who found no age effect on ETC activity after including physical activity status as a confounding variable in multivariate analysis. It is the opinion of the author that normalising for physical activity status would eliminate the decline in mitochondrial function with age. The notion of physical activity maintaining mitochondrial function and aerobic capacity will be addressed in section 2.3.4.

Decreases in the quantity and quality of mitochondrial protein lead to a decline in oxidative enzyme activity and the efficiency of ATP production. Restriction of ATP availability affects rates of protein turnover because both MPB and synthesis are energy-consuming . ATP is used in a hierarchical manner with synthesis of macromolecules, such as protein, being most sensitive to energy supply . Therefore, in times of restricted ATP availability, muscle protein turnover will be reduced. Reduced turnover results in poorer quality protein as damaged proteins are not replaced as expediently. Furthermore, a decrease in the rate of mitochondrial protein turnover has been related to a decline in oxidative capacity . Muscular endurance capacity also decreases with decreasing ATP availability, leading to a decrease in functional capacity . Loss of mitochondrial protein quantity and function therefore compromises muscular endurance capacity and strength , and therefore exercise tolerance. In this way, a

vicious cycle creates further inactivity and dysfunction , which result in increased susceptibility to fatigue and decreased quality of life.

2.3.3.2 Oxidative damage and mitochondria

Oxidative damage is caused by ROS, produced by mitochondria, predominantly in the ETC . When the ETC passes electrons from complex to complex, there is some inefficiency where some leaked electrons may react with other species or oxygen leading to the formation of superoxide molecules that in turn bond with hydrogen atoms to form hydrogen peroxide (H_2O_2). The H_2O_2 diffuses out of the mitochondria where it is usually converted to water, but occasionally releases ROS instead . When the rate of ROS production exceeds the cell's antioxidant capacity, oxidative damage can occur . Ageing leads to intracellular metabolic imbalances, such as decreased activity of glutathione peroxidase - the enzyme which removes H_2O_2, changes in electron transport chain stoichiometry, and changes in membrane potential and pH gradient across the inner mitochondrial membrane . Furthermore, a lack of exercise slows the transfer of electrons along the ETC due to an increase in the ATP/ADP ratio . Such imbalances increase the oxidant-producing potential of the mitochondria, thus increasing oxidative stress. Furthermore, antioxidant activity does not increase concomitantly with increasing age , so ROS damage tends to accumulate.

Mitochondrial DNA is particularly susceptible to ROS damage because it is very physically close to the ETC and therefore chronically exposed to mitochondrial ROS, is not protected by a histone sheath as is nuclear DNA, and possesses an insufficient repair system . Muscle mitochondrial dysfunction, to which ROS damage may be causative , is characterised by decreases in mtDNA copy number and mRNA concentration in genes encoding muscle mitochondrial proteins. These decreases in mtDNA and mRNA accumulate with advancing age , and lead to a subsequent decrease in the rate of mitochondrial protein synthesis due to

decreased amounts of DNA and mRNA available for transcription and translation of mitochondrial protein . Tissues with higher oxidative capacity show a greater decrease in mtDNA copy number with age, which lends credence to this notion of oxidative damage . ROS damage also results in an increased incidence of mutations and deletions in mtDNA, another factor which increases with age . More defective mtDNA, in turn causes synthesis of defective, poorly functioning proteins , leading to a reduction in VO_{2max} due to impaired peripheral oxygen extraction as already reviewed. Oxidative damage sets up a vicious cycle in that the presence of defective proteins and mtDNA mutations creates defects in the oxidative phosphorylation process, which leads to further increased ROS production and further protein damage .

There is evidence of increased oxidative damage to skeletal muscle mitochondria with advancing age. The first theory of ageing postulated that this damage was in fact a contributor to the ageing process . Fano et al. found that oxidative damage to DNA in the *m. vastus lateralis* was 40% higher in elderly than young humans. Supporting evidence is provided by Kovalenko et al. , who found that deletion mutations in mtDNA accumulate in ageing human muscle, and that the corresponding decrease in the amount of full-length amplifiable mtDNA correlates with a decrease in cytochrome c oxidase (COX) activity, an indicator of mitochondrial oxidative capacity. mtDNA mutations can be pathogenic and lead to mitochondrial disease, along with being a cause of ageing . Finally, Barazzoni et al. found decreased mtDNA copy number in the skeletal muscle of old rats, the greatest decline being found in the oxidative *soleus* muscle. Barazzoni et al's findings suggest a link between oxidative metabolism and mtDNA damage, and also a link between this damage and oxidative capacity as the two variables were found to have declined proportionally with increasing age.

To summarise, oxidative damage contributes to cellular ageing and it is likely that age-related mitochondrial dysfunction plays a role in the aetiology of sarcopenia . As mitochondria are the main source of ROS, and also their main target , oxidative stress is a vicious cycle. Certainly the more damaged mitochondrial protein is, the more dysfunctional it is likely to be.

2.3.4 Successful ageing with post-exercise nutrition

Increasing physical activity levels by doing more deliberate exercise and leading a less sedentary lifestyle may have a number of positive health outcomes, including increased aerobic and functional capacities, lower blood pressure, improved quality of life, and decreased frailty and disability . There is evidence that increasing activity levels will also increase muscle protein turnover, leading to improved muscle quality and strength . Ensuring adequate energy and protein intakes is also likely to maintain or improve muscle mass, strength, and quality .

2.3.4.1 Increasing activity levels

Deliberately increasing activity levels can attenuate the loss of physical capacity that accompanies growing older. Regular exercise has been found to combat sarcopenia and increase both muscular endurance capacity, defined as the time taken to reach fatigue at a given workload, and the ability to perform the activities of daily living . Even an increase in the amount of activities of daily living is positively associated with mitochondrial enzyme activity . Quality of life also seems to be improved as shown by Wang et al's 13-year longitudinal study of 649 people over 50, roughly half of whom belonged to a running club. Wang et al. found that the club members experienced postponement of disability and protection against early morbidity and mortality, when compared to their sedentary counterparts. Furthermore, VO_{2max} and oxidative enzyme activity both improve with regular activity, presumably due to stimulation of muscle protein turnover.

VO_{2max} declines with age regardless of exercise status, as stated in section 2.3.2, but absolute values remain higher, and decline less, in active people compared to their inactive counterparts . An elderly active person can therefore have a VO_{2max} similar to, or even higher than, a much younger, sedentary person . A similar trend exists with regards to muscle oxidative capacity as revealed by Proctor et al. , who found that older trained men had the same amount of succinate dehydrogenase activity in the *m. vastus lateralis* as young trained men, and double that of their age-matched, healthy counterparts. These data suggest that endurance training can prevent or slow the age-related decline in oxidative enzyme activity.

It seems that it is never too late to begin an exercise programme or improve VO_{2max}. In three separate studies , previously sedentary older subjects took part in aerobic exercise programmes lasting between 12 and 16 weeks. In every case, absolute measures of whole body VO_{2max} or VO_{2peak} increased at least 14% from baseline. Coggan et al. demonstrated that VO_{2max} continues to improve over the long term, by exercising initially sedentary older men and women for nine to 12 months and measuring a 28% average increase in VO_{2max} relative to body mass. In another study of the same duration , previously sedentary older subjects trained four times a week and experienced an average improvement in VO_{2max} in mL/kg/min of 24%. The improvement in Kohrt et al.'s subjects was independent of their initial degree of fitness. The subjects in Kohrt et al.'s study attained a VO_{2max} commensurate with sedentary individuals 20 years their junior. The above results show that the elderly can experience improvements in VO_{2max} in response to exercise programmes, and that this improvement is independent of both age and starting level of aerobic fitness. An otherwise healthy, sedentary person of any age can, therefore, increase their aerobic capacity by following an appropriate, prescribed exercise programme.

Not only do older individuals experience gains in aerobic capacity, their relative increases in VO_{2max} as a result of aerobic exercise training are similar to those experienced by young and middle-aged adults . Table 2-8 summarises the results of studies undertaken to compare adaptations in older and younger subjects to such training. Taken together, the results suggest that the elderly have the same, or greater, response to aerobic exercise training as the young.

subjects	training parameters	increase in VO_{2max}	reference
young v old M	3-5 bouts/wk; 8 wks	11% in both groups	
young v old M, F	3 bouts/wk; 12 wks	12% in young; 19% in old	
aged 19 - 87	3-4 bouts/wk; 16 wks	9% in all subjects	

Table 2-8 Change in maximal rate of oxygen consumption of older and younger subjects as the result of an aerobic exercise training programme

In all cases, subjects adapted to a higher maximal rate, with older subjects demonstrating the same, or greater, increase as the young. VO_{2max}, maximal rate of oxygen consumption (mL/kg/min); M, men; F, women; wk, week.

The response to aerobic exercise of oxidative enzyme activity also remains sensitive in elderly muscle . Table 2-9 summarises the results of studies undertaken to determine adaptations to such training of mitochondrial enzymes in older subjects. In all cases, subjects adapted to a considerably higher level of skeletal muscle oxidative enzyme activity. Furthermore, age-related changes in mitochondrial function disappear when adjustment is made for physical activity status , suggesting that it is mitochondrial protein content that is impaired with age, rather than function/quality. Taken together these findings indicate that aerobic training can improve muscle quality in older individuals, and that the age-related decline in oxidative enzyme activity is due mainly to inactivity, rather than ageing per se.

subjects	training parameters	increase in enzyme activity	reference
elderly	4 bouts/wk; 14 wks	citrate synthase 33%	
older	4 bouts/wk; 9 – 12 mo	succinate dehydrogenase 51%	
		citrate synthase 23%	
older	4-6 bouts/wk; 12 wks	succinate dehydrogenase 62%	

Table 2-9 Changes in skeletal muscle mitochondrial enzyme activity in older or elderly subjects as the result of an aerobic exercise training programme

In all cases, subjects adapted to a higher level of enzyme activity. Wk, week; mo, month.

Just like VO_{2max}, factors associated with muscle quality appear to improve by the same amount relative to baseline values in response to aerobic training in older and younger people. In an eight-week aerobic exercise training study of older, previously sedentary men, the activities of malate and succinate dehydrogenases increased an average of 11%, a similar increase to that in young men . In their 16-week aerobic training study of 19 to 87 year olds, Short et al. found the same increase in oxidative enzyme activity regardless of age. In a 12-week aerobic training study comparing young and older subjects, the older subjects showed a 128% increase in muscle oxidative capacity, while the young group did not make a significant change, suggesting that peripheral adaptation is a big part of the response to aerobic training in the elderly . Further evidence that peripheral adaptation is unimpaired comes from Heath et al. , who found that the majority of the age-related decline in VO_{2max} in masters athletes can be attributed to a decline in maximal heart rate. Also, Seals et al. trained older subjects for one year and measured an increase in VO_{2max} with no concomitant change in maximal cardiac output. What did increase was the ability of the muscle to extract oxygen, as measured by the change in arterio-venous oxygen difference, indicating a peripheral adaptation. The above findings demonstrate that the quality of older skeletal muscle adapts to training just as well, if not better than, that of the young. Hence, chronic aerobic exercise could be used to slow the age-related decline in muscle functional capacity.

The mechanism by which regular exercise improves muscle protein quality may be by increasing rates of MPS and turnover. Increased turnover means faster repair after injury and faster replacement of damaged or defective proteins. Evidence from studies using [1-^{13}C]leucine infusions suggests that muscle protein metabolism in the elderly responds to resistance training with an increase in mixed muscle FSR of the same magnitude as in the young , although perhaps over a different time course . The similar results between the age

groups in all of these studies augur well for the positive effects of exercise on healthy elderly muscle.

Given the similar muscle protein metabolic response to resistance training in the elderly and the young, it seems likely that muscle protein metabolism also responds to aerobic training in a similar fashion regardless of age. The stimulatory effect of aerobic exercise on rates of WBPS, explained in section 2.1.1.1, persists in older subjects, but at a reduced level when compared to the young . To date, the response of mixed muscle FSR to a bout of aerobic exercise has only been studied once , and with somewhat inconclusive results. Sheffield-Moore et al. compared mixed muscle FSR between older and young men after a bout of mild-intensity aerobic exercise. Using the 3-pool model, the authors found that mixed muscle FSR was increased more in the older men at ten minutes post-exercise, but remained elevated at one hour post-exercise in the young men only. Strangely, when using the A-V balance method, the authors found exactly the opposite results, that is, that MPS remained elevated at one hour post-exercise in the older men only. While these contradictory findings highlight the shortcomings of the two methods used (see Appendix A3 for discussion), they also indicate that there may be some stimulation of MPS in the elderly after aerobic exercise and that more work is needed in this area.

The fraction of mixed muscle protein which is most likely affected by aerobic exercise is the mitochondria. Short et al. found no change with age in the stimulatory effect of exercise on mitochondrial enzyme activity. As explained in section 2.1.1.2, aerobic training can stimulate mitochondrial biogenesis and function which will theoretically lead to increased ATP production and, hence, energy for muscular contraction. An increase in mitochondrial protein turnover may also help in the repair of oxidative damage. More studies examining the response of mitochondrial protein synthesis to aerobic exercise are needed.

Post-exercise signalling for mitochondrial biogenesis and protein translation also appears to be unimpaired in elderly skeletal muscle. Short et al. found no change with increasing age in the effect of a bout of aerobic exercise on the rate of transcription of mitochondrial genes, and the transcription of genes involved in signalling for mitochondrial biogenesis in humans. In rats and mice, signalling for protein translation continues to be stimulated by exercise. In ageing mouse skeletal muscle, three months of aerobic exercise training resulted in increased resting PKB phosphorylation and concentrations of total mTOR and PKB . Furthermore, young and old rats completing a bout of resistance exercise experienced the same increase in skeletal muscle S6K1 phosphorylation . Furthermore, a group of healthy elderly men experienced the same magnitude of increase in $p70^{S6K1}$, 4E-BP1, and eEF2 phosphorylation following a bout of resistance exercise as a group of healthy young men participating in the same study .

From the literature reviewed in this section, it appears that increasing physical activity levels will improve aerobic capacity, muscle function, and quality of life in ageing humans.

2.3.4.2 Nutrition

As explained in section 2.1.2.1, ingestion or infusion of amino acids or protein in the rested state increases rates of protein synthesis. This effect persists in older individuals. Volpi et al. found that an intravenous amino acid infusion in elderly subjects increased mixed muscle FSR with no concomitant change in MPB, indicating an improvement in muscle protein balance.

Evidence suggests that the increase in protein synthesis in response to feeding amino acids is the same in young and older individuals. Volpi et al. provided groups of young and elderly subjects with 40 g of oral amino acids over three hours and found that MPS was stimulated to a similar extent in both groups. Similarly, Paddon-Jones et al. measured a comparable increase in mixed muscle FSR in young and older subjects after feeding 15 g of essential amino acids.

In contrast, Cuthbertson et al. observed that the rates of both myofibrillar and sarcoplasmic MPS in elderly muscle did not respond to the ingestion of essential amino acids to the same extent as in the young. Perhaps the situation in the sub-fractions measured by Cuthbertson et al. differs from the average of that in the whole mixed muscle. In aged rats, a one-month nutritional intervention consisting of increased ingestion of essential amino acids resulted in greater endurance capacity and faster post-exercise recovery of ATP concentration than in healthy adult control rats . Chen et al.'s findings suggest that the acute responses to amino acid ingestion measured in the other studies described in this paragraph do accumulate into long-term changes, at least in rat skeletal muscle.

Some findings make it tempting to speculate that leucine may be a critical amino acid for stimulation of protein synthesis in the elderly. Katsanos et al. provided young and older subjects with an oral essential amino acid mixture containing either 26% or 41% leucine. They found that the FSR of the *m. vastus lateralis* increased to a similar extent in both young groups but only in the older group receiving the higher proportion of leucine. Similarly, Rieu et al. found that leucine supplementation in the presence of a mixed meal increased the rate of myofibrillar protein synthesis more than the meal alone in older men. It appears, however, that it is simply the quantity of available essential amino acids that is the key factor in this response, as Koopman et al. found no additive effect of leucine on mixed meal stimulation of post-exercise MPS in elderly men when ample protein (0.88 g/kg over six hours) was ingested.

To determine the effect of protein consumption, rather than that of isolated amino acids, Young et al. compared the efficiency of WBPS per gram of protein consumed in young and elderly subjects, and found that one gram of protein consumed supported the synthesis of four to five grams of body protein in both age groups. To determine the long-term effect of protein intake on whole body protein metabolism, Gersovitz et al. provided young and elderly subjects with

1.5 g protein/kg/day for seven days and found no difference between the groups in markers of either whole body protein turnover or synthesis. More recently, Welle et al. provided young and older subjects with small meals consisting of 15% protein every half an hour for five hours, and measured 25% and 50% increases in WBPS and myofibrillar FSR respectively, in both groups. Further, Benedek et al. provided groups of young and older subjects with eight meals over 14 hours, each containing 14% protein, and found that WBPS exceeded WBPB by a similar amount in both groups, again showing a more positive protein balance with feeding. The above results indicate that there is no impairment with age on the stimulatory effect of feeding amino acids, either in isolated or whole protein form, on protein synthesis. Such a conclusion suggests that ensuring adequate dietary protein is a key step in ageing successfully.

It appears that human skeletal muscle experiences a deficit in anabolic signalling by amino acids with increasing age. Elderly muscle has roughly half the total resting protein concentration of mTOR, $p70^{S6K1}$, 4E-BP1, and eIF2B as is present in the young . Cuthbertson et al. compared the effect of amino acid ingestion on phosphorylation in the mTOR pathway in groups of young and older subjects. The older subjects demonstrated phosphorylation of mTOR, 4E-BP1, and eIF2B to a degree roughly half that of the young, and showed no phosphorylation of $p70^{S6K1}$ at all. Guillet et al. also found no increase in $p70^{S6K1}$ phosphorylation during a hyperinsulinemic and hyperaminoacidemic clamp but, in contrast to Cuthbertson et al., found that phosphorylation of PKB, mTOR and 4E-BP1 all increased to the same degree in elderly and young subjects. An absence of $p70^{S6K1}$ phosphorylation would limit translation of proteins of the synthetic machinery (Figure 2-4, section 2.2.2) and could be one factor explaining the loss of skeletal muscle protein with age.

The presence of insulin, either alone or in combination with amino acids, has the same inhibitory effect on MPB in the fasted and rested elderly as it does in the young. Fukagawa et

al. measured equal decreases in the R_a of leucine indicating equal decreases in the rate of MPB in young and elderly subjects in response to an insulin infusion. Even after a week of bedrest, a situation of extreme disuse that could reflect an elderly lifestyle, the response of WBPB to an insulin infusion is unimpaired in human skeletal muscle .

In combination with amino acids, insulin continues to have an inhibitory effect on MPB, as demonstrated by Short et al. who administered an amino acid infusion in the presence of a euglycemic insulin clamp to young and elderly subjects. Short et al. found that MPB was inhibited by the same amounts in both groups. Co-ingestion of amino acids and carbohydrate has a similar effect as an infusion, as demonstrated by Volpi et al. who provided 40 g each of oral amino acids and glucose over a three-hour period to young and elderly subjects. Volpi et al. found that MPB was inhibited to the same extent in both groups.

The effect of insulin on MPS, however, does not appear to be the same in ageing muscle as it is in young. In Volpi et al.'s study described in the previous paragraph, MPS was stimulated by oral glucose only in the group of young subjects. Similarly, Rasmussen et al. found that local hyperinsulinemia in young and older subjects led to a stimulation of leg MPS in the young group only. It appears that ageing muscle may exhibit some insulin resistance as some effectors downstream of insulin, such as IRS-1, demonstrate decreased phosphorylation in response to insulin in ageing rat muscle . It may also be that the release of insulin stimulated by carbohydrate consumption blunts elderly muscle's anabolic response to amino acids . Altogether the results in this and the preceding paragraphs indicate that muscle protein metabolism responds to both amino acids and insulin in many, but not all, of the same ways in the old as in the young.

The interactive stimulatory effect of exercise and nutrition on muscle protein metabolism does persist in older adults. Holm et al. provided oral protein and carbohydrates or a low-energy

placebo to early post-menopausal women after an acute bout of resistance exercise. They found that net protein balance was improved in the protein-plus-carbohydrate group compared to the placebo group via an initial increase in the rate of disappearance (R_d) of infused [*ring*$^2H_5$]phenylalanine, an indication of increased MPS, followed by a decrease in the R_a of unlabelled phenylalanine, an indication of suppressed MPB (see Appendix A1 for a discussion of stable isotope methodology). Koopman et al. measured the effect of consuming a combination of protein, carbohydrate and added leucine or carbohydrates only after mild aerobic exercise in elderly and young subjects. The authors found that the protein-plus-carbohydrate treatment stimulated FSR and leucine incorporation into muscle which improved whole body protein balance equally in both age groups. In contrast, Fiatarone et al. found no additive effect of supplementation with a meal containing a combination of carbohydrate, protein, and fat during a ten-week resistance exercise training programme, on muscle functional capacity in elderly subjects. The fact that Fiatarone et al.'s subjects received their nutrition supplement in the evening, rather than immediately after their bouts of exercise, may have reduced the anabolic effect of the nutrition. To summarise, it appears that the synergistic effect of exercise immediately followed by nutrition that is observed in healthy young and middle-aged adults, persists in older individuals.

With respect to signalling for protein translation, Drummond et al. measured the same increase in phosphorylation of mTOR, $p70^{S6K1}$, 4E-BP1, and eEF2 following post-resistance exercise provision of essential amino acids in both young and older subjects. The only differences between the subjects in Drummond et al.'s study were that the young group demonstrated more PKB phosphorylation than the older, which would have stimulated translation, while the older group demonstrated greater AMPK activation, which would have inhibited translation via inhibition on mTOR (Figure 2-4). It has also been shown that young and elderly men experience the same increase in Rheb protein, which up-regulates mTOR, from a bout of

resistance exercise followed by amino acid ingestion . Furthermore, Fujita et al. found that an acute bout of walking at 70% VO_{2max} eliminated the afore-mentioned insulin resistance in elderly skeletal muscle, by measuring increases in Akt, mTOR, and $p70^{S6K1}$ phosphorylation. In conclusion, the results in this and the preceding paragraph suggest that provision of amino acids and carbohydrate after exercise stimulates the rate of MPS more than exercise alone in older individuals, in much the same way as it does in the young.

In summary, the age-related decline in VO_{2max} can be attenuated with the introduction and/or maintenance of an aerobic exercise training programme while the relative magnitude of the increase in VO_{2max} in response to training is unaffected by age. The key factor underlying the VO_{2max} response is likely oxidative enzyme activity, which increases in response to training in a manner that is also unaffected by age. It appears that rates of MPS in the elderly are stimulated by both aerobic exercise training and nutrition, although the magnitude of this stimulation may be diminished as compared to younger individuals. Further work is needed to definitively determine the interactive effect of exercise and nutrition on the protein quality of ageing muscle.

The aims of the studies within this thesis, as outlined in Chapter 3, are to determine the specific effects of aerobic exercise and nutrition on various aspects of mitochondrial protein synthesis, with a view to issues relating to ageing muscle. Ideally, the information gained from the studies in this thesis will contribute towards filling current gaps in understanding regarding the interactive effects of providing protein-plus-carbohydrate after a bout of aerobic exercise on whole body and mitochondrial protein synthesis, as well as on signalling for mitochondrial biogenesis and general protein translation.

3 Overview of Research

3.1 Aim

The aim of this research was to elicit an understanding of the effects of immediate post-exercise nutrition on whole body and skeletal muscle mitochondrial protein synthesis within the context of maintaining or improving skeletal muscle protein quality in older adult humans. Of specific interest was the determination of whether feeding protein-plus-carbohydrate after aerobic exercise can up-regulate mitochondrial biogenesis compared to carbohydrate alone. The rates of whole body and mitochondrial protein synthesis, and activity in signalling pathways leading to mitochondrial biogenesis in skeletal muscle, were examined using chemical and molecular biological analyses. This research consisted of two experimental studies, each consisting of an aerobic exercise stimulus and a nutritional intervention in intact, healthy adult humans.

3.2 Hypotheses

The purpose of the first study was to determine whether feeding protein in combination with carbohydrate immediately after an acute bout of moderate-intensity aerobic exercise would have an additive effect over the feeding of carbohydrate alone, on rates of whole body and mitochondrial protein synthesis.

The hypotheses for the first study were:

> A protein-plus-carbohydrate drink will increase the rate of whole body protein synthesis during the first four hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (Chapter 4).

A protein-plus-carbohydrate drink will increase the rate of skeletal muscle mitochondrial protein synthesis during the first four hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (Chapter 5).

The purpose of the second study was to investigate whether feeding protein in combination with carbohydrate immediately after an acute bout of high-intensity aerobic exercise would have an additive effect over carbohydrate alone on intracellular signalling for mitochondrial biogenesis in skeletal muscle.

The hypotheses for the second study were:

A protein-plus-carbohydrate drink will increase the mRNA expression of selected genes in the mitochondrial biogenesis signalling pathway during the first six hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (Chapter 6).

A protein-plus-carbohydrate drink will increase the phosphorylation of mTOR and 4E-BP1, and the total protein content of cytochrome c, during the first six hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (Chapter 7).

3.3 Studies and Experiments

In the first study subjects over the age of 54 were recruited, screened, and put through an exercise and nutrition protocol (Figure 3-1). Breath and blood samples were collected and analysed to enable calculations of WBPS. This portion of the study is presented in Chapter 4: Influence of post-aerobic exercise nutrient composition on whole body protein turnover in older individuals. Muscle samples were also collected and analysed to enable calculations of mitochondrial fractional synthesis rate. This portion of the study is presented in Chapter 5: Influence of post-aerobic exercise nutrient composition on skeletal muscle mitochondrial biogenesis in older individuals.

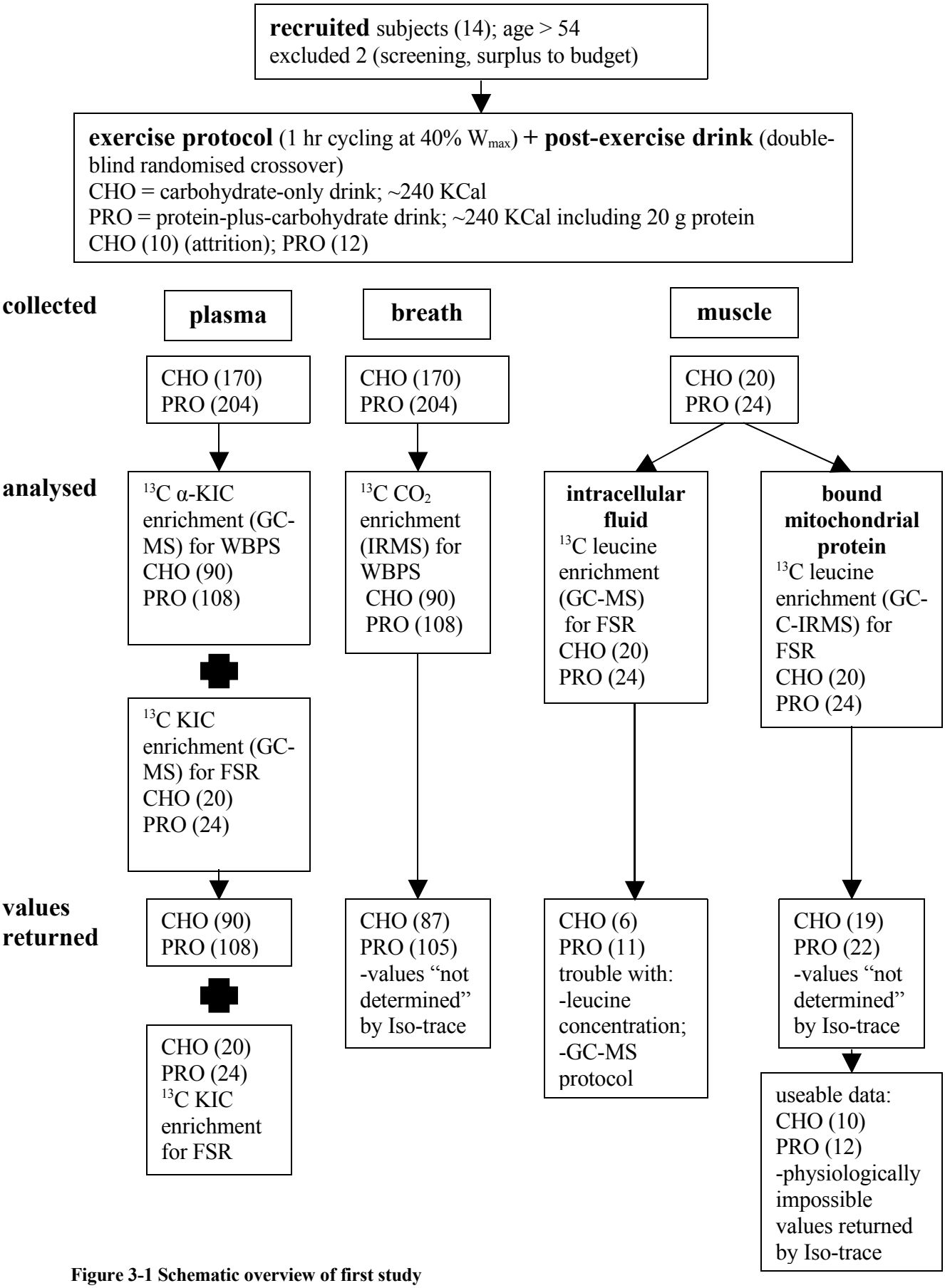


Figure 3-1 Schematic overview of first study

Numbers in parentheses indicate how many samples were available for analysis in each condition. See Chapter 4 methods for details of values returned by Iso-trace and trouble in method development. W_{max}, maximum power output; α-KIC, alpha-ketoisocaproate; WBPS, whole body protein synthesis; FSR, fractional synthesis rate; GC-C-IRMS, GC-combustion-IRMS.

In the second study subjects aged 18 to 34 were recruited, screened, and put through a slightly different exercise and nutrition protocol (Figure 3-2). For ethical reasons, young subjects were used in order to provide a "proof of concept" before investigating older individuals. This study was carried out in the United States of America, where regulations are quite stringent. In this study only muscle samples were collected. Total RNA was extracted, reverse transcribed to cDNA and then quantified to determine relative mRNA expression of six genes in the mitochondrial biogenesis signalling pathway. This portion of the study is presented in Chapter 6: Influence of post-aerobic exercise nutrient composition on signalling for skeletal muscle mitochondrial biogenesis. Total protein was also extracted and analysed to determine the magnitude of signalling by phosphorylation in the protein translation pathway. This portion of the study is presented in Chapter 7: Influence of post-aerobic exercise nutrient composition on signalling for skeletal muscle protein translation – the mTOR pathway.

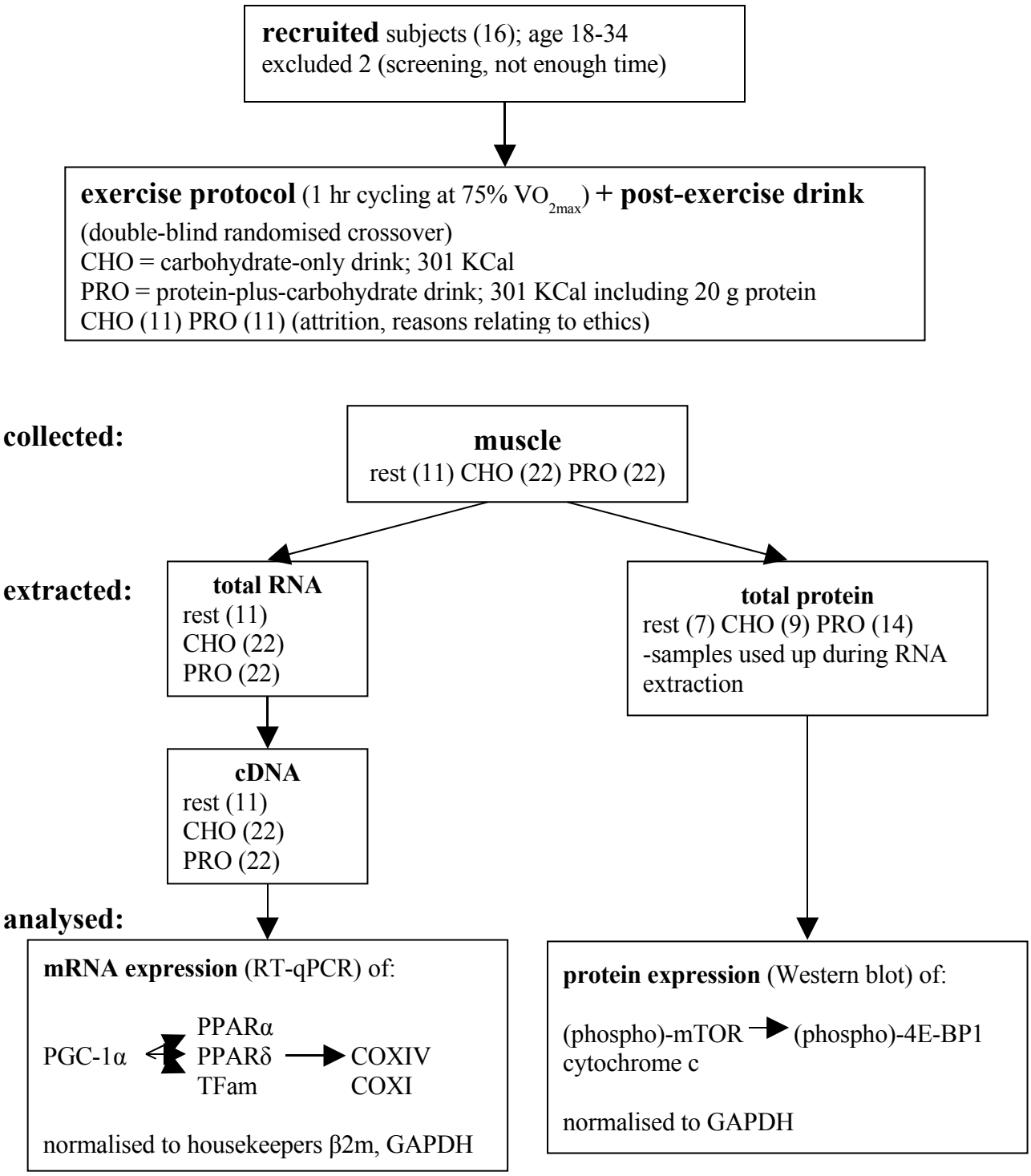


Figure 3-2 Schematic overview of second study

Numbers in parentheses indicate how many samples were available for analysis in each condition. See Chapter 6 methods for details of sample use in RNA extraction. VO_{2max}, maximal rate of oxygen consumption; PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; PPARα, peroxisome proliferation-activated receptor alpha; PPARδ, peroxisome proliferation-activated receptor delta; TFam, mitochondrial transcription factor A; COXIV, cytochrome c oxidase subunit IV; COXI, cytochrome c oxidase subunit I; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor binding protein; β2m, beta-2 microglobulin.

4 Influence of post-aerobic exercise nutrient composition on whole body protein synthesis in older individuals

4.1 *Introduction*

One contributor to elderly disability, a growing public health concern, is the progressive loss of muscle termed sarcopenia. The word sarcopenia comes from the Greek and means a paucity of flesh . Sarcopenia in ageing humans is characterised by a reduction in the quantity and quality of muscle, leading to a variety of health concerns. As described in 2.1, the rate of protein synthesis affects the quantity of that protein while the rate of protein turnover affects the quality . WBPT incorporates both synthesis and breakdown of all the body's proteins, one of which is muscle.

[1-^{13}C]leucine infusions are commonly used to make measures of whole body and muscle protein turnover. [1-^{13}C]leucine is a particularly useful tracer during exercise studies as it measures factors involved in the impact of exercise on skeletal muscle as opposed to other tissues . Infused leucine is transported from the blood into the intracellular fluid of muscle tissue where it undergoes transamination to alpha-ketoisocaproate (α-KIC, Figure 4-1) . α-KIC can either stay in the muscle, where it meets one of two fates, or return to the bloodstream. α-KIC remaining in the muscle tissue is either reaminated back to leucine and used in MPS, or decarboxylated to release CO_2. Decarboxylation of α-KIC may also occur in the blood . α-KIC is, therefore, a representative precursor of WBPS.

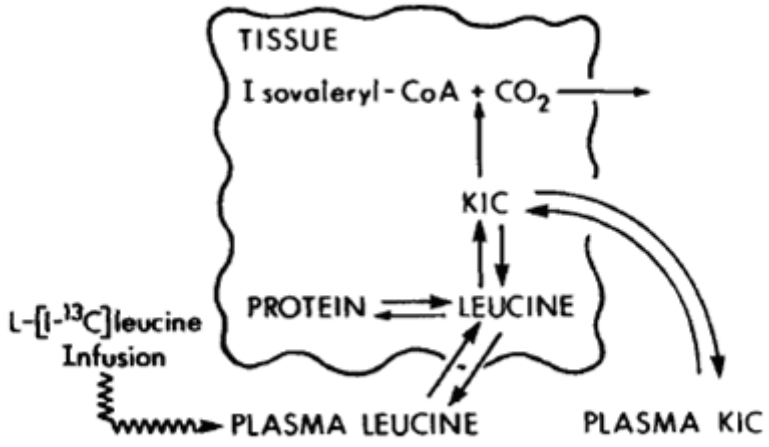
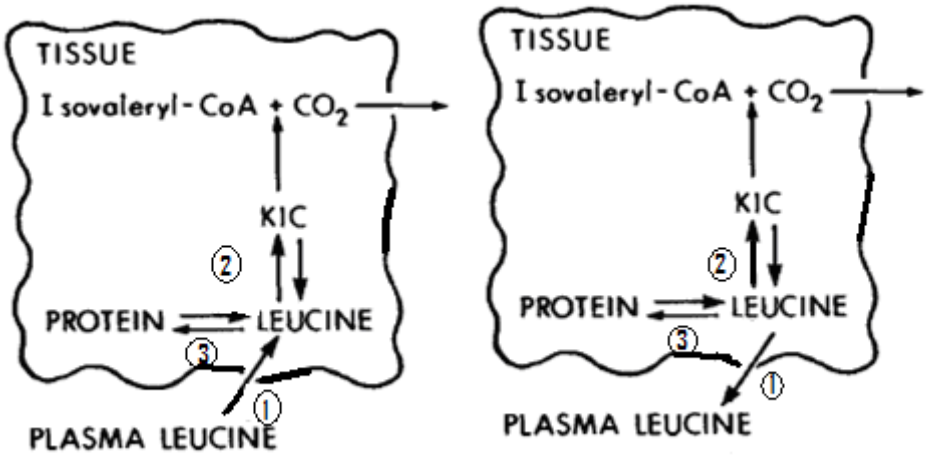


Figure 4-1 Model of leucine metabolism

Leucine is transaminated to its alpha-ketoacid alpha-ketoisocaproate (α-KIC) in both the plasma and intracellular fluid. α-KIC can then be de-carboxylated and carbon dioxide (CO_2) released, or re-aminated back to leucine. α-KIC and leucine are both in equilibrium between the plasma and intracellular fluid compartments. Leucine appears in plasma from either infusion or release from the intracellular compartment. Adapted from .

Whole body calculations use plasma ^{13}C-KIC enrichment rather than ^{13}C-leucine enrichment because all α-KIC appears in plasma from leucine in the intracellular fluid (Figure 4-1), making α-KIC the true precursor for leucine oxidation, regardless of where decarboxylation takes place . MPB, a component of WBPB, releases leucine first into the intracellular compartment and then into the bloodstream. Use of plasma leucine as the precursor pool for oxidation does not take into account oxidation of intracellular leucine and so will cause error in whole body calculations (Figure 4-2). Furthermore, Schwenk et al. demonstrated, using radioactive tracers, that whole body leucine metabolism is more accurately measured when using plasma α-KIC specific activity. It can therefore be inferred that the same is true for enrichment.



WBPS = (1 – 2 + 3)/590μmol/g protein WBPB = (1 + 2 + 3)/590μmol/g protein

Figure 4-2 Model for indirect estimation of whole body protein synthesis and breakdown from [1-^{13}C]leucine and α-KIC data

Pathway 1 is leucine release/uptake from/into intracellular compartment. Pathway 2 is transamination between α-KIC and leucine. Pathway 3 is equivalent on both sides so effectively cancels out for calculation purposes. 590 μmol is the amount of leucine per gram of protein. WBPS, whole body protein synthesis; WBPB, whole body protein breakdown; KIC, alpha-ketoisocaproate; CO_2, carbon dioxide. Adapted from .

Amino acid ingestion contributes to the R_a of leucine into the bloodstream. If ingestion is not zero, calculation of R_a becomes unreliable. Even if the leucine content of food is known, it is not simply a matter of adding this rate of ingestion to calculations of R_a because there is a delay between ingestion and entry into general circulation while the leucine is processed in the liver . The problem of ingestion can be solved by adding a tracer to the food. Some label will be lost in the liver but, because the plasma enrichment can be measured and the ingestion rate is known, this value can be calculated and an appropriate correction made.

Interventions which result in an increase in the rate of protein turnover will help to maintain protein quality, and hence function. Increasing activity levels and improving nutritional intake can both attenuate the loss of physical function and enhance whole body protein kinetics. The rate of WBPS in healthy young and middle-aged adults is depressed during aerobic exercise and stimulated afterwards . Whether this situation persists in older individuals is currently unclear. Postprandially, in both young and old subjects, the rate of whole body protein breakdown is depressed while that of synthesis is elevated above fasting measures . Research

measuring WBPT after both exercise and nutrition has generally focused on resistance exercise . One study in healthy young adults has shown that adding protein to a post-resistance exercise carbohydrate meal results in a more positive or less negative whole body protein balance than carbohydrates alone . Despite its potential for improving protein balance, there is a paucity of data regarding the effect of post-aerobic exercise nutrition on WBPT in older individuals.

Therefore the aim of this part of the first study was to investigate whether feeding protein immediately after an acute bout of moderate-intensity aerobic exercise has an additive effect over carbohydrate alone on the rate of WBPS in older individuals. The hypothesis tested was that a protein-plus-carbohydrate drink (PRO) will increase the rate of WBPS during the first four hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (CHO).

4.2 Methods

4.2.1 Subjects

Healthy volunteers aged 54 or older were recruited by printed advertisements (Appendix D1) posted in the UniSports gym and Population Health building on the University of Auckland Tamaki Campus. The advertisement was also attached to an internal email circulated to all Tamaki Campus staff. Eligibility required non-smokers free of hypertension, obesity, and cancer and from cardiac, liver, kidney, pulmonary, autoimmune, and vascular diseases. Individuals with hypo- or hyperthyroidism, hyperlipidemia, diabetes mellitus, impaired glucose tolerance, or on metabolically active medication were also excluded. Four women and nine men met these criteria as determined by the screening procedures (4.2.2.1). One man was randomly excluded as the budget provided for only 12 subjects. During the course of the study two men withdrew due to discomfort at sampling sites. Participants were informed of the experimental procedures both verbally and in writing (Appendix D2). Subjects provided

written, informed consent before any tests were performed (Appendix D3). The study design and procedures complied with the principles of the Declaration of Helsinki and received approval from the Northern X Regional Ethics Committee (AKY/04/08/213).

4.2.2 Study design

4.2.2.1 Screening and preliminary testing

At initial contact subjects were asked a series of screening questions to determine their potential eligibility for the study (Appendix D4). Those meeting these initial criteria completed an oral PAR-Q questionnaire (Appendix D4), and then underwent a resting and treadmill exercise stress test (Bruce Protocol, Appendix B1, Trackmaster TMX425 Stress Treadmill, Full Vision, Newton KS, USA) with ECG monitoring (Schiller CardioLaptop AT-110, Schiller, Baar, Switzerland) under the supervision of a cardiac registrar. These subjects also completed a medical and exercise questionnaire (Appendix D4). Those found to be free of cardiovascular complications and not regularly participating in an aerobic training programme were asked to return for a fasting blood draw (Appendix B2). A full blood count was done as well as analysis of blood glucose, urate, creatinine, albumin, thyroid stimulating hormone, cholesterol, and triglyceride levels by LabPlus (Auckland Hospital, Auckland, NZ). The blood work provided information regarding the exclusion criteria described in 4.2.1; no subjects were excluded as a result of this data.

Those meeting the remaining eligibility requirements returned for preliminary testing which consisted of anthropometric measurements and determination of maximal rate of oxygen consumption (VO_{2max}). Height and body mass were measured to calculate body mass index (BMI) and body fat was estimated from skinfold measurements taken using calipers (SlimGuide, Linear Software, Bellbrook OH, USA) at eight sites: *triceps brachii*, *subscapularis*, *biceps brachii*, iliac crest, *supraspinale*, abdominal, mid -thigh, and medial calf

(Appendix B4). Each subject's VO_{2max} was determined during an incremental exercise test to voluntary maximum on an electrically-braked cycle ergometer (Schiller CH6340, Schiller, Baar, Switzerland, Appendix B3). Direct gas analysis was performed throughout the protocol to measure VO_2 (Oxygen Analyzer S-3A/1, Carbon Dioxide Analyzer CD-3A, Oxygen Sensor N-22M, Carbon dioxide Sensor P-61B, Applied Electrochemistry, AEI Technologies, Pittsburgh PA, USA). All subjects began at a power output of 50 Watts and power output was increased using a ramp function at a rate of 30 Watts/minute for men and 20 Watts/minute for women. Subjects cycled until volitional fatigue and the maximum power output (W_{max}) recorded. VO_{2max} was taken as the average VO_2, recorded at 15 second intervals, over the last minute of work.

4.2.2.2 Testing procedure

A minimum of seven days later, participants were studied after two separate acute bouts of exercise, each exercise test session being separated by a minimum period of two weeks. Participants reported to the laboratory overnight-fasted, and the order of the tests was randomly determined. Each test session consisted of one hour of steady state cycling at 40% W_{max} on a cycle ergometer (Monark Ergomedic 824E, Varberg, Sweden), preceded and followed by rest periods during which subjects reclined or lay supine on a bed (Figure 4-3). In one session the subject received a protein-plus-carbohydrate drink (PRO) immediately after their exercise bout, while in the other they received an isocaloric, carbohydrate-only drink (CHO) as described in 4.2.2.3.

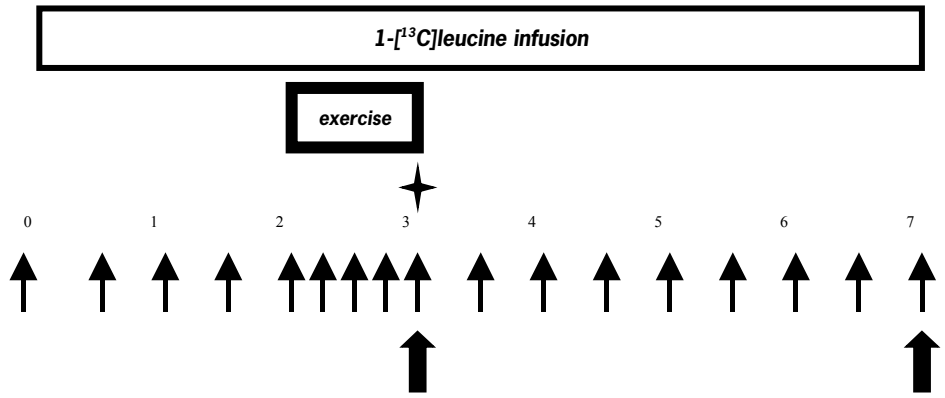


Figure 4-3 Schematic representation of the experimental protocol for the first study

Numbers indicate time in hours from the start of infusion. Baseline blood and breath samples (▲) were obtained prior to the beginning of the infusion. Blood and breath samples were then obtained every 30 minutes (every 15 minutes during exercise) until the conclusion of the experiment. Muscle biopsies (▙) were obtained immediately following cessation of exercise and four hours later. The drink (✦) was given immediately following cessation of exercise and before the biopsy was taken.

At the beginning of the pre-exercise rest period, a venous catheter was inserted into a superficial vein in each arm (Appendix B5). One arm was used to provide a priming bolus of sodium bicarbonate ($NaH^{13}CO_3$) (35 mg) and a primed-continuous infusion of [1-^{13}C]leucine (99 atom percent, Cambridge Isotope Laboratories Inc, Andover MA, USA) at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion (IMED Gemini PC-1 Volumetric Infusion Pump, Alaris Medical Systems, San Diego CA, USA). These doses are consistent with those previously used successfully in a similar study . The labelled bicarbonate was included in the priming dose to label this pool to avoid dilution of $^{13}CO_2$ enrichment as the bicarbonate pool also releases CO_2 via oxidation . Two hours were allowed to elapse between the beginning of the continuous leucine infusion and the start of exercise in order that the enrichment in the subjects' blood and intracellular fluid could reach a steady state. The catheter in the contralateral arm was used to obtain blood samples and was kept patent with a saline drip. Blood was collected in 6 mL vacutainer tubes treated with EDTA (Becton, Dickinson and Company, North Ryde NSW, Australia). At each collection, the first tube of

blood obtained was discarded as it may have been contaminated with saline while the second tube was stored on ice until six samples had been collected, then processed as detailed in 4.2.3.

Breath samples were collected for 5 minutes via a mouthpiece and Hans-Rudolph (Kansas City MO, USA) one-way valve assembly into 60 L Douglas bags. The bags were then massaged and two 10 mL air vacutainers (Labco Ltd., High Wycombe, Buckinghamshire, UK) filled for isotopic analysis. Humidity, temperature and pressure in the laboratory were recorded every two hours in order to correctly calculate VO_2.

4.2.2.3 Drinks

Subjects received either 60 g carbohydrate (maltodextrin, CHO) or 40 g carbohydrate and 20 g whey protein (PRO). Drink powders were designed by a sports supplement company (kindly provided by PRO4, New Zealand) and 60 g of each powder was mixed in 400 mL water to provide the post-exercise drink. The drinks were subsequently sent for independent analysis (Institute of Food, Nutrition and Human Health, Massey University, Auckland, NZ) to confirm the expected contents. Energy content of the drink powders was determined by bomb calorimetry and amino acid content was determined by hydrochloric acid hydrolysis followed by HPLC separation (Institute of Food, Nutrition and Human Health, Massey University, Auckland, NZ).

4.2.3 Blood processing and analysis

To calculate WBPS, ^{13}C-KIC enrichment in plasma was determined. First, plasma was separated from whole blood by spinning vacutainers at 2,000 g for 15 minutes, then aliquoted and stored at -80 °C until analysis. Plasma α-KIC was analysed for ^{13}C enrichment as its *tert*-butyldimethylsilyl (*t*-BDMS) derivative by GC-MS . Initially, the standard curve method was used. The principle of this method is to create a standard curve which contains the expected experimental enrichment (in this experiment 5%) in the middle. A graph of the expected molar

percent excess (MPE) versus M+1 should result in a straight line with an R^2 value of 0.99 or better and a slope of 1. M+1 is the ratio of the area under the curve (AUC) (the area under the GC-MS peak) for the heavier of the two monitored ions to the total AUC. R^2 is the correlation coefficient for the line of best fit. The standard curve is run at the beginning and end of each day to account for temporal variation in the GC-MS and the slope calculated. Two MPEs for each sample are then calculated by multiplying the measured value for the sample (minus background enrichment) by the slopes of the standard curves according to the equation;

$$MPE = m(M+1) + b$$

where m is the slope of the standard curve and b is the background enrichment. Background enrichment is taken as the measured M+1 for the 0 MPE sample and is the naturally occurring ^{13}C enrichment. A weighted value for each measurement is taken depending on that sample's place in the order of that day's analysis.

Stock powder was used to prepare standard solutions of ^{12}C and ^{13}C KIC and, from these, a series dilution of solutions containing 0, 2.5, 5, 7.5, and 10 MPE was prepared. Standards were derivatised using a modification of previously described methods . 1 mL of ice-cold ethanol was added to 50 µL of standard and incubated at 4 °C for 20 minutes, then 700 µL of this solution was taken and the ethanol evaporated under a stream of nitrogen gas at 90 °C. 200 µL of ddH_2O and 100 µL of 5 mg/mL 1,2-phenylenediamine (OPD) in 4 N hydrochloric acid (HCl) were added, tubes capped and then incubated for 30 minutes at 80 °C. Samples were then allowed to cool, 2 mL of ethyl acetate added and the upper layer transferred to fresh tubes. The ethyl acetate was evaporated under nitrogen gas at 90 °C, 30 µL each of N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane (BSTFA-TCMS) and pyridine were added, and the capped tubes incubated at 80 °C for a further 30 minutes. Samples were then transferred to GC-MS vials for analysis.

The whole mass spectrum was scanned using electron impact ionisation to detect the fingerprint of the α-KIC molecule (7890 GC and 5975 MS, Agilent, Santa Clara CA, USA). 1 µL of sample was injected twice into the gas chromatography (GC) column (DB-17 0.25 mm x 30 m x 0.25 µm, Agilent, Santa Clara CA, USA). The inlet temperature was set at 250 °C, the pressure at 16.35 psi, and the flow rate through the column at 1.4 mL/min. The oven temperature was initially held at 120 °C for one minute, then increased by 10 °C per minute to 200 °C, then held for 2 minutes. Between each injection the oven was held at 250 °C for 1.5 minutes to keep the column clean by driving off later eluting compounds. The mass spectrometer source was set at 230 °C and the quadrupole at 150 °C.

When a 400 µg/mL solution of [^{12}C]KIC and a 1 mg/mL solution of [1-^{13}C]KIC were run, peaks were detected at mass-to-charge ratios of 259 and 260 *m/z* with a retention time of 8:40 (Figures 4-4, 4-5) so these ions were targeted for determination of enrichment by selected ion monitoring. Although the KIC molecule also produced fragments at 217 and 232 *m/z*, the fragment at 259 *m/z* was the one that was labelled. The detected peaks were overloaded however, indicating that the solutions were too concentrated for accurate AUC calculation, so ten-fold dilutions were prepared and the whole standard curve run. All the standards produced acceptable peaks at this new dilution with $R^2 = 0.9996$ for the standard curve, so a biological sample was run (Figure 4-6) with another set of standards at this new dilution to confirm that the experimental enrichment fell in about the middle of the standard curve.

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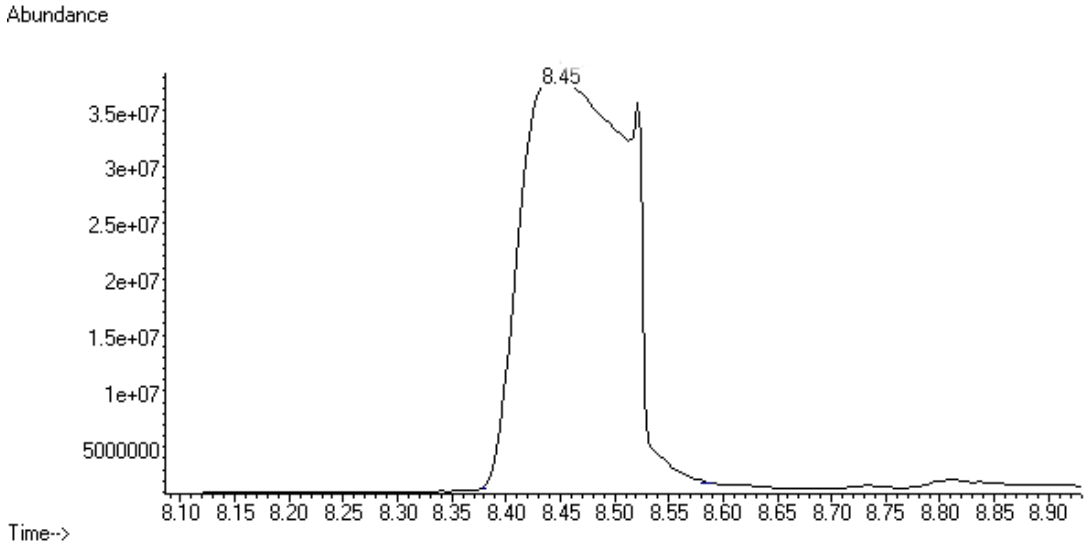

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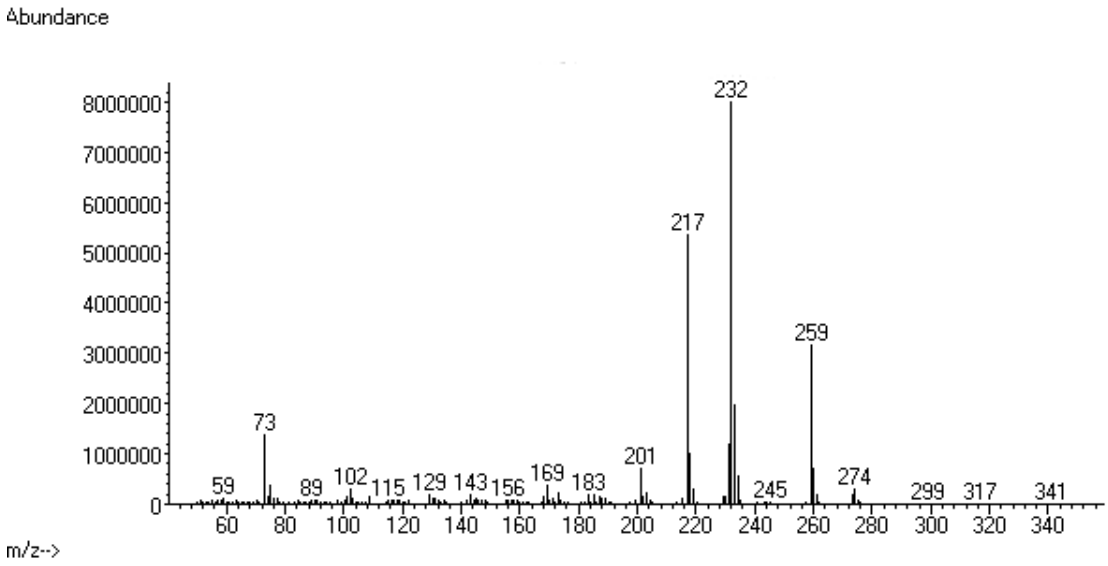

Figure 4-4 Representative GC-MS trace for 400 µg/mL solution of ^{12}C-KIC

Samples of diluted stock solution were subjected to derivatisation with BSTFA-TCMS prior to loading onto GC column. *A)* absorbance spectrum from substances eluting between 8.1 and 8.9 minutes. Elution time of KIC is 8.45 min. Asymmetrical shape of peak indicates overloading. *B)* mass spectrum showing abundant fragments at 217, 232, and 259 *m/z*. 259 *m/z* is the fragment of interest. BSTFA-TCMS, N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane.

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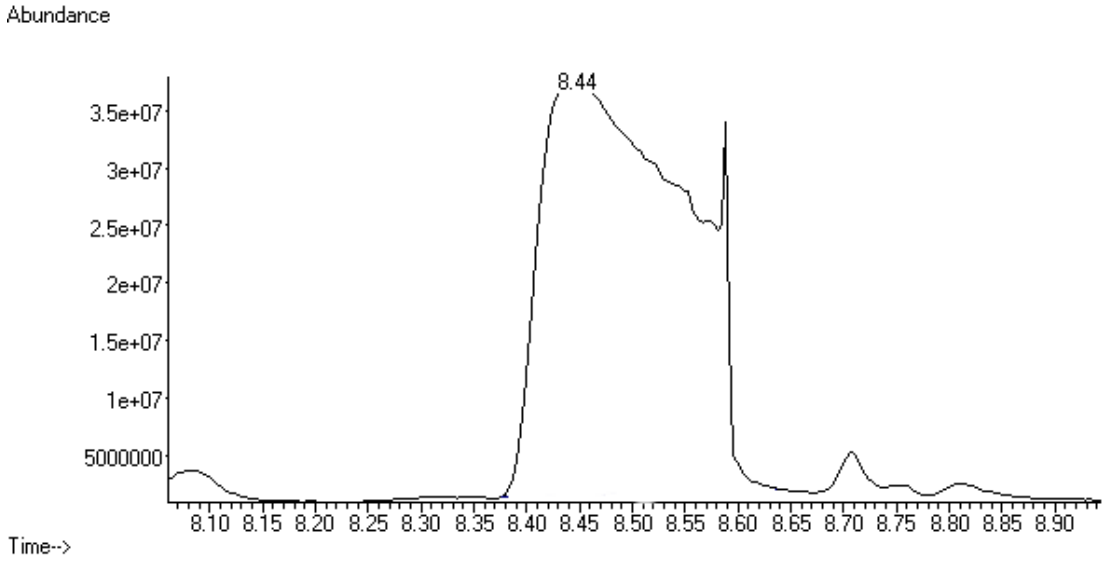

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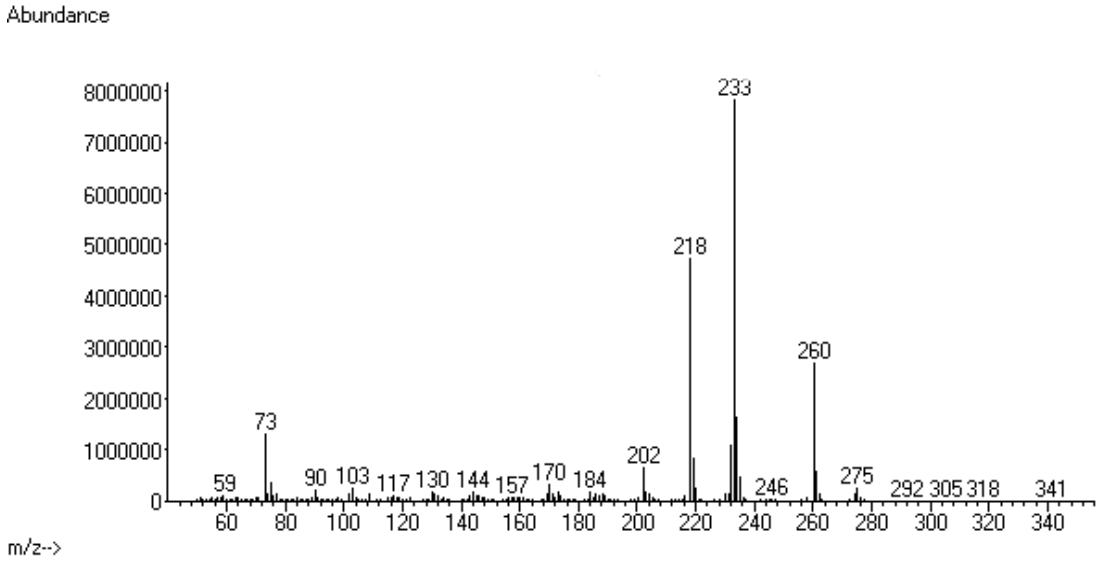

Figure 4-5 Representative GC-MS trace for 1 mg/mL solution of ^{13}C-KIC

Samples of diluted stock solution were subjected to derivatisation with BSTFA-TCMS prior to loading onto GC column. *A)* absorbance spectrum from substances eluting between 8.1 and 8.9 minutes. Elution time of KIC is 8.44 min. Asymmetrical shape of peak indicates overloading. *B)* mass spectrum showing abundant fragments at 218, 233, and 260 *m/z*. 260 *m/z* is the fragment of interest. BSTFA-TCMS, N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane.

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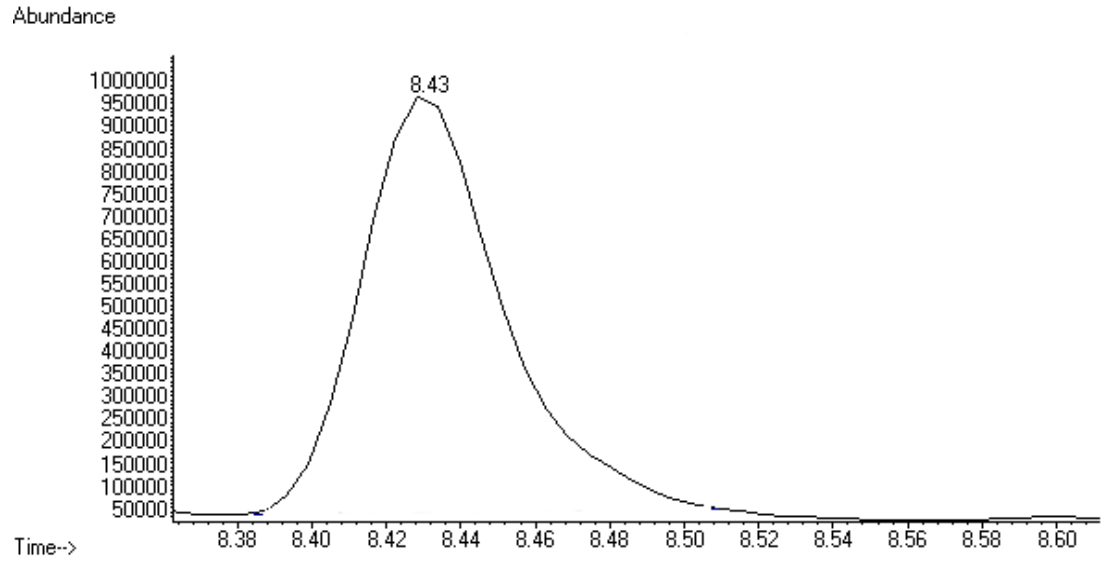

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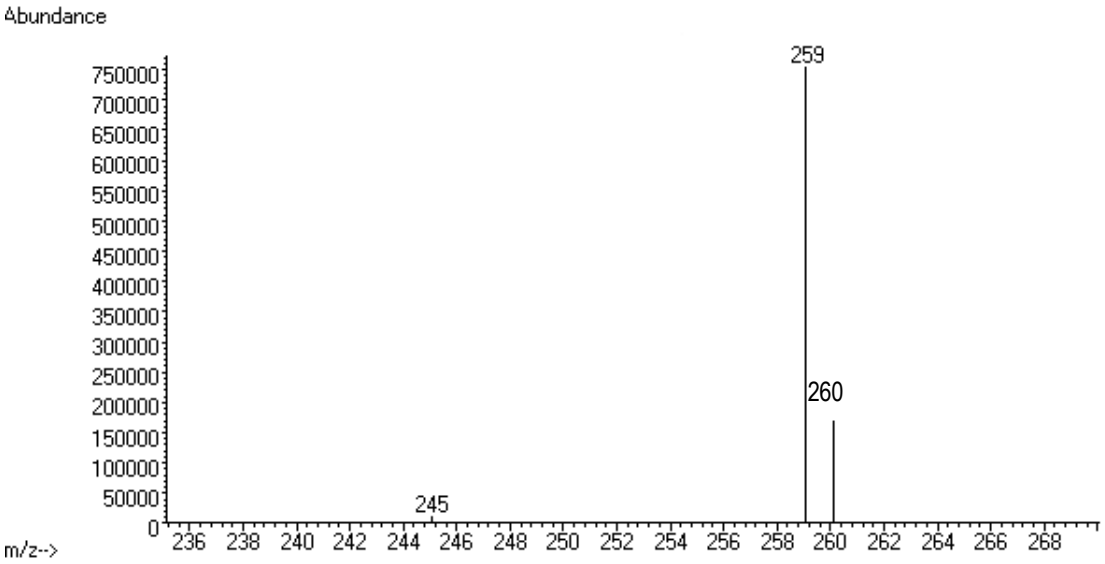

Figure 4-6 Representative GC-MS trace for plasma sample containing a natural abundance of ^{13}C-KIC

Aliquots of plasma were subjected to derivatisation with BSTFA-TCMS prior to loading onto GC column. *A)* absorbance spectrum from substances eluting between 8.3 and 8.6 minutes. Elution time of KIC is 8.43 min. *B)* mass spectrum showing results of selected ion monitoring at 245, 259 and 260 *m/z*. BSTFA-TCMS, N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane.

Once the standard curve was developed the plasma samples were analysed for tracer to tracee ratio. 100 µL of each sample was derivatised in the same way as 50 µL of the standards with a five-minute centrifugation at 3000 rpm and 4 °C added after the initial ethanol incubation. After two days of analysis it became clear that the standard curves were not meeting the quality

control criteria for values of either R^2 or slope (R^2 = 0.9338, m = 1.5254). To rule out the possibility of instability in the standard solutions, a new series dilution was prepared from fresh stock solutions. The standard curve continued to be of poor quality (R^2 = 0.8169, m = 0.4585) so a fresh OPD solution was prepared with minimal improvement in the results (R^2 = 0.9314, m = 0.7757). Finally, a new GC column was installed and conditioned and the standard curve met the quality control criteria (R^2 = 0.9979, m = 1.096). The elution time on the new column was 8.15 minutes (Figure 4-7).

To determine the reliability of the detected values for KIC enrichment, three 100 μL aliquots of a single sample were derivatised separately and each of these run through the GC-MS in a triplicate injection along with the usual standard curve. The purpose of this analysis was to determine whether the variation inherent in the detection method and instruments used was greater than the expected treatment effect and, therefore, enough to mask it.

An alternative method to the use of standard curves was ultimately used. The decision to use this method was beyond the candidate's control. Instead of standards, two or three enriched controls were run each day containing a 'normal' background ratio of ^{12}C to ^{13}C of 21.61 ± 0.4%. As long as the measured values from the zero standards did not fluctuate by more than two standard deviations from this mean value then it was assumed that the instrument's detection remained linear.

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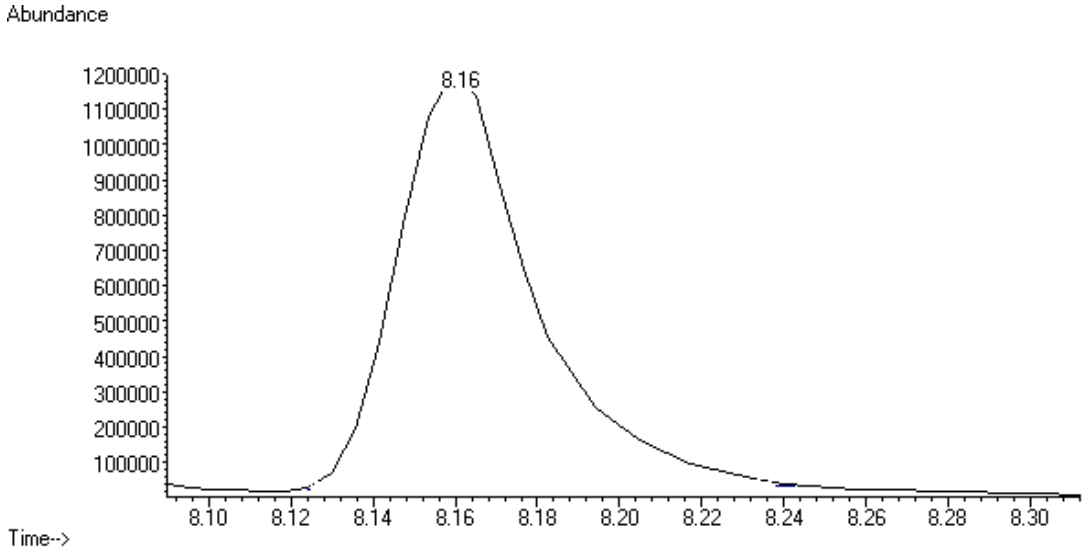

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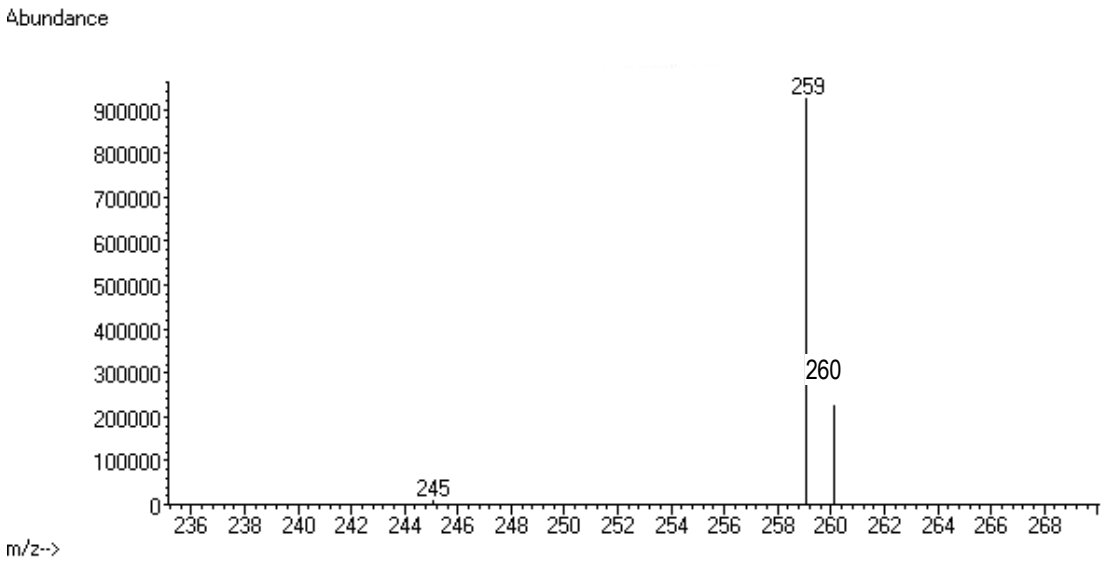


Figure 4-7 Representative GC-MS trace using new column for plasma sample containing a natural abundance of ^{13}C-KIC

Aliquots of plasma were subjected to derivatisation with BSTFA-TCMS prior to loading onto GC column. *A)* absorbance spectrum from substances eluting between 8.1 and 8.3 minutes. Elution time of KIC is 8.16 min. *B)* mass spectrum showing results of selected ion monitoring at 245, 259 and 260 *m/z*. BSTFA-TCMS, N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane.

The measured tracer to tracee ratio was adjusted by a skew correction factor according to the equation;

$$E_{plasma}*(1-0.0111)$$

where E_{plasma} is the KIC enrichment of the plasma sample .

To determine the effects of the drinks on the subjects' circulating nutrient and hormone concentrations, plasma samples were also analysed for glucose, insulin, and amino acid concentrations by the Liggins Institute at the University of Auckland. Glucose was analysed by the Roche GOD-PAP enzymatic colorimetric method on a Roche/Hitachi 902 Autoanalyser (Hitachi High-Technologies Corporation, Tokyo, Japan). Insulin was analysed by an established Microparticle Enzyme Immunoassay (IMx MEIA assay, Abbott Laboratories, North Chicago IL, USA). The inter-assay coefficient of variation for insulin is 3.4-4.5% between 8.3 and 121.7 μU/mL. Amino acid concentrations were analysed by reverse phase HPLC using pre-column derivatisation with AQC (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate,).

4.2.4 Breath processing and analysis

To obtain information required for VO_2 and leucine oxidation calculations, the oxygen and carbon dioxide content of the air remaining in the Douglas bag after the vacutainers were filled was determined using a carbon dioxide and anaesthesia monitor (Datex Normocap 200, Fisher & Paykel Healthcare, Auckland, NZ) and the volume determined by forcing the contents of the bag through a dry gas meter (Rayfield Equipment, Waitsfield VT, USA).

To calculate the rate of leucine oxidation, the ^{13}C enrichment of expired CO_2 was determined. Isotopic analysis of the 10 mL breath samples was outsourced to Iso-trace New Zealand Ltd in Dunedin. The ^{13}C enrichment of the CO_2 in the breath samples was analysed by an IRMS (Delta^{plus}Advantage, Thermo, Bremen, Germany) linked to a gasbench (Thermo Finnigan, Bremen, Germany) according to the manufacturer's standard operating conditions. The reference gas used was CO_2 which has an atom percent of 1.1112133.

Enrichment (APE, atom percent excess) was calculated as the difference between the APE of the sample and that of the reference gas. The atom percent of the sample was a value returned by the instrument, determined according to the equation;

$$\frac{100*VPDB*(\delta C_{VPDB}/1000 + 1)}{1 + [VPDB*(\delta C_{VPDB}/1000 + 1)}$$

where VPDB (Vienna PeeDee Belemnite) is the international reference value for the naturally-occurring ratio of ^{13}C to ^{12}C (0.011237), and δC_{VPDB} is the difference between the ratio of ^{13}C to ^{12}C in the sample being measured and VPDB. APE was converted to a tracer to tracee ratio according to the equation;

$$\frac{APE}{1\text{-}APE} * 100$$

and this value used in subsequent calculations.

4.2.5 Calculations and statistics

To calculate the rate of WBPS, a steady state was assumed such that the R_a of leucine into plasma was equal to its R_d. For the post-exercise recovery period, the steady state assumption did not hold, with plasma KIC enrichment declining by 25% in the final two hours of post-exercise recovery in the CHO condition, and increasing by the same percentage in the PRO condition. The R_a (μmol/kg*min) was determined according to the equation;

$$\frac{(F/BW)*0.99}{\Delta E_{plasma}}$$

where F (μmol/kg*min) is the infusion rate, BW (kg) is body mass, and ΔE_{plasma} is the difference in KIC enrichment between the plasma sample of interest and that of the baseline sample.

Oxidation was determined according to the equation;

$$\frac{\Delta E_{breath}*(VCO_2/0.81)}{\Delta E_{plasma}}$$

where ΔE_{breath} is the difference in KIC enrichment in the breath sample of interest and that of the baseline sample and VCO_2 is total CO_2 production in μmol/kg*min calculated according to the equation;

$$\frac{VCO_2 \text{ (L/min)} * 1000000}{22.4 \text{ L/mol} * BW}$$

In instances where there were no useable data for the baseline sample, as explained in Chapter 3 (Figure 3-1), an average of the baseline values for that condition across the other subjects was used. This situation arose for three of the ten subjects for the CHO condition and one of the twelve subjects for the PRO condition.

The correction factor of 0.81 was applied to the value for total CO_2 production due to not all CO_2 leaving the body in expired air. CO_2 passes through the body's bicarbonate pool before being expired and some CO_2 is retained in this pool. A standard value of 0.81 is commonly used as the correction factor , and is supported by some experimental results . A review of the literature by Leijssen & Elia revealed that appropriate values can truly range from 0.5 to 1 depending on nutritional state and recent activity levels. Methodological differences suggest that an accurate determination of CO_2 retention should be made alongside studies using ^{13}C-leucine infusions. Such a determination was not necessary in this experiment, as the CHO and PRO conditions were run identically to one another, so error in the value of the correction factor used would not have changed the measured difference between the conditions.

VCO_2 (L/min) was calculated according to the equation;

$$VCO_2 = VE_{STPD} (F_ECO_2 - 0.003)$$

where F_ECO_2 is the CO_2 production rate, determined by dividing the measured CO_2 content of the expired air by 100 and VE_{STPD} (L/min) is the volume expired at standard temperature and pressure calculated according to the equation;

$$VE_{STPD} = VE_{ATPS} * (273/(273 + t)) * ((P_B - P_{H_2O})/760)$$

where VE_{ATPS} (L) is the measured volume expired at ambient temperature and pressure, t (°C) is the ambient temperature, P_B (kPa) is the barometric pressure, and P_{H_2O} (mmHg) is the vapour pressure of water .

WBPS (μmol/kg/min) was taken as the value calculated for NOLD. The R_d is the sum of oxidation and NOLD, therefore in a steady state, NOLD is the difference between the R_a of leucine and the rate of leucine oxidation.

In performing the above calculations, a set of standard assumptions concerning the use of stable isotopes was adopted. These assumptions are generally considered acceptable , and are detailed in Appendix A.

The data was fitted to a linear mixed model and then tested for the effects of treatment and time using PROC MIXED in SAS. Significance was set at $\alpha < 0.05$.

4.3 Results

4.3.1 Physiological characteristics

The subjects' physiological characteristics are shown in Table 4-1. An average VO_{2max} value of 38 mL/kg/min for the men and 36 mL/kg/min for the women corresponds to a rating of 'very good' for healthy older adults .

parameter	men	women
Age (y)	60 ± 5	58 ± 1
Height (cm)	175 ± 4	164 ± 5
Mass (kg)	84.0 ± 10.7	66.7 ± 6.0
BMI (kg/m^2)	27.4 ± 2.4	25.0 ± 3.4
VO_{2max} (mL/kg/min)	38.4 ± 8.4	36.1 ± 12.6
W_{max} (Watts)	256 ± 57	176 ± 37
BF (mm)	143 ± 39	164 ± 46
HR_{rest}	57 ± 9	59 ± 6
HR_{max}	162 ± 15	168 ± 7

Table 4-1 Subjects' physiological characteristics in the first study

Eight men and four women were studied. BMI, body mass index; VO_2, rate of oxygen uptake; W, power output; BF, body fat, sum of 8 skinfolds; HR, heart rate. Group means ± standard deviations are shown.

4.3.2 Subjects exercised at the same intensity during both trials

Men cycled at 40 ± 1% of W_{max} and women at 38 ± 2% of W_{max}. These power outputs elicited rates of oxygen consumption and carbon dioxide production as shown in table 4-2 and caused an increase in heart rate as shown in table 4-3. VO_2 values expressed as a percentage of maximum indicate that the exercise was of a moderate intensity (46–51% VO_{2max}). Subjects exercised at 70% of their heart rate maximum.

	CHO		PRO	
	L/min	mL/kg/min	L/min	mL/kg/min
VCO_2 (males)	1.34 ± 0.13	15.78 ± 1.79	1.34 ± 0.10	16.11 ± 1.14
VCO_2 (females)	0.97 ± 0.05	14.65 ± 0.99	0.95 ± 0.07	14.16 ± 0.69
VO_2 (males)	1.56 ± 0.13	18.40 ± 1.88	1.56 ± 0.11	18.62 ± 1.04
VO_2 (females)	1.07 ± 0.07	16.16 ± 1.16	1.04 ± 0.10	15.59 ± 1.35

Table 4-2 Rates of oxygen consumption and carbon dioxide production during exercise bout in the first study for all subjects for both conditions

Subjects cycled at roughly 50% VO_{2max} for one hour. Group means ± standard errors are shown. VO_{2max}, maximal rate of oxygen consumption; VCO_2, rate of carbon dioxide production; VO_2, rate of oxygen consumption; CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition.

condition	CHO	PRO
Absolute (bpm)	114 ± 4	116 ± 5
Relative (%)	70 ± 1	70 ± 2

Table 4-3 Heart rates during exercise bout in the first study for all subjects for both conditions

Relative value is expressed as a percentage of HR_{max}. Subjects cycled at 70% of their heart rate maximum for one hour. Group means ± standard errors are shown. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition; bpm, beats per minute.

4.3.3 Energy and amino acid contents of the drink powders

The energy contents of the drink powders were 15.64 kJ/g and 17.64 kJ/g for the CHO and PRO drinks respectively, indicating that the drinks were not, in fact, isocaloric as was intended. The energy difference between the drinks was 120 kJ, a difference of roughly 12%. The amino acid content of the drinks is shown in Table 4-4. The CHO drink contained a small amount of glycine but was otherwise free of amino acids. The PRO drink contained 2.165 mg leucine.

amino acid	CHO	PRO
Essential		
Leucine	0	3.609
Valine	0	1.482
Isoleucine	0	1.506
Phenylalanine	0	1.000
Methionine	0	0.683
Lysine	0	3.030
Histidine	0	0.566
Threonine	0	1.310
Tyrosine	0	0.940
Nonessential		
Aspartic acid	0	3.695
Serine	0	0.854
Glutamic acid	0	4.881
Arginine	0	0.643
Alanine	0	1.581
Glycine	0.011	0.458
Proline	0	1.137

Table 4-4 Amino acid content of drinks used in the first study in mg/100 mg of drink powder

Subjects cycled at roughly 50% VO_{2max} for one hour before receiving a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. The CHO drink powder contained a small amount of glycine but was otherwise free of amino acids.

4.3.4 Plasma insulin and amino acids increased more with protein-plus carbohydrate feeding than with carbohydrate-only

Plasma glucose and insulin for all subjects are shown in Figure 4-8. Insulin peaked half an hour following ingestion of the PRO drink and an hour after ingestion of the CHO drink. Insulin did not return to baseline values until four hours after exercise cessation and drink ingestion in both trials. Plasma glucose values were unchanged from baseline with both treatment drinks.

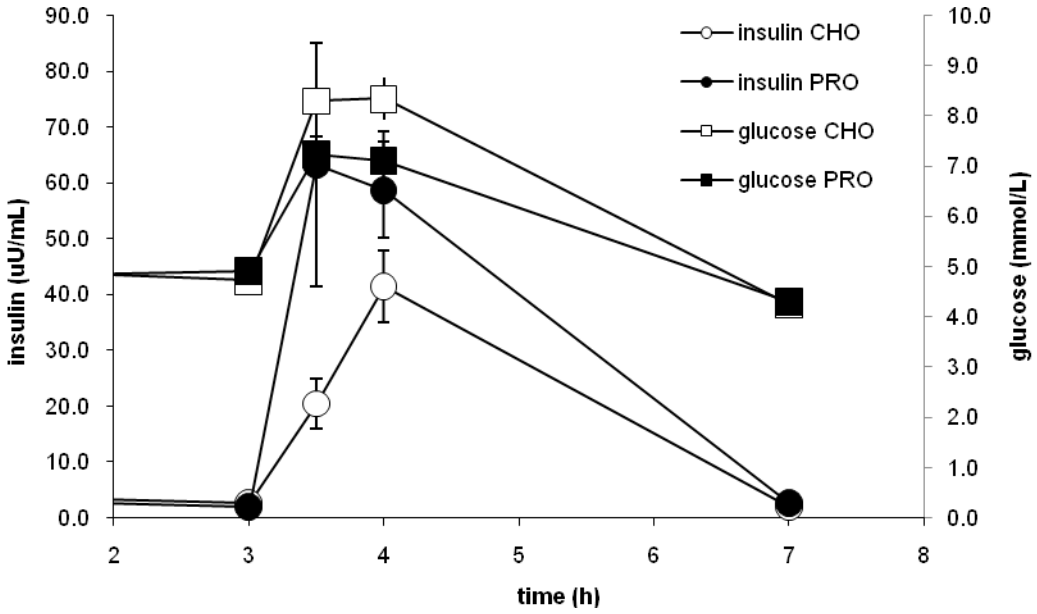


Figure 4-8 Plasma glucose (mmol/L) and insulin (μU/mL) in the first study for both treatment conditions
Subjects cycled at roughly 50% VO_{2max} for one hour before receiving a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. At time zero plasma glucose concentrations were 5.0 and 4.8 mmol/L in the CHO and PRO conditions respectively. At time zero plasma insulin concentrations were 4.6 and 3.8 μU/mL in the CHO and PRO conditions respectively. In both conditions insulin increased immediately following drink ingestion, with values increasing more sharply in the PRO condition. Plasma insulin returned to baseline values by four hours post-exercise in both conditions. Time is in hours from start of infusion. Group means ± standard errors are shown.

Plasma leucine concentrations for all subjects are shown in Figure 4-9 and all plasma amino acid concentrations in table 4-5. Plasma leucine in the PRO trial increased within 30 minutes of ingestion of the treatment drink, peaked at one hour post-ingestion, and did not return to baseline until four hours post-ingestion. Plasma leucine in the CHO trial was unchanged from baseline. At 3.5 and 4 hours, corresponding to 30 and 60 minutes post-nutrient ingestion,

plasma concentrations of all amino acids were higher in the PRO condition than the CHO condition.

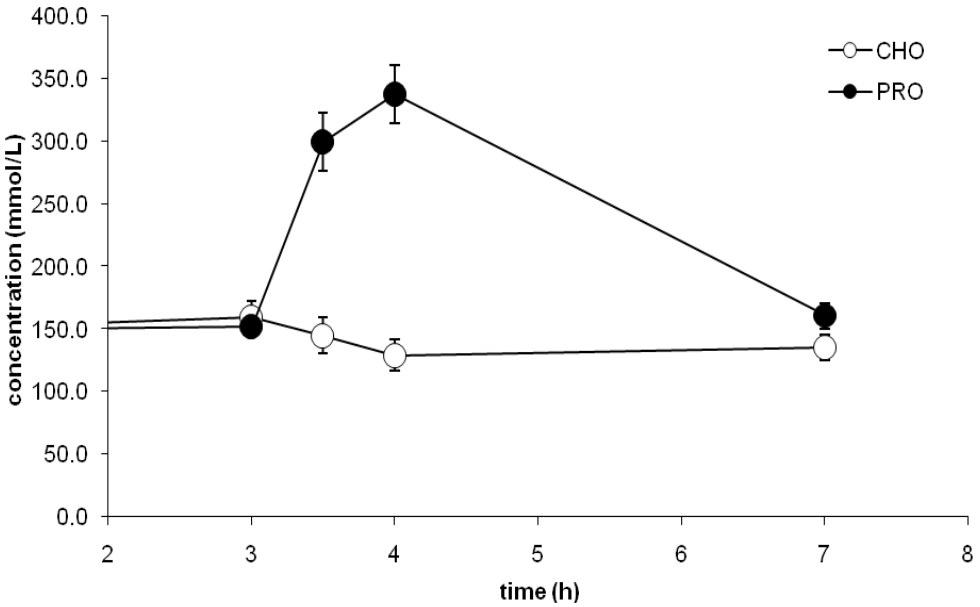


Figure 4-9 Plasma leucine concentration (mmol/L) in the first study for both treatment conditions

Subjects cycled at roughly 50% VO_{2max} for one hour before receiving a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. At time zero plasma leucine concentrations were 147.4 and 148.2 mmol/L in the CHO and PRO conditions respectively. Plasma leucine increased sharply following drink ingestion in the PRO condition only, and returned to baseline by four hours post-exercise. Time is in hours from start of infusion. Group means ± standard errors are shown.

Time (h)	0		3		3.5		4		7	
	CHO	PRO	CHO	PRO	CHO	PRO	CHO	PRO	CHO	PRO
Essential										
Leucine	147.8 ± 12.8	148.2 ± 8.6	159.4 ± 12.6	151.9 ± 7.0	144.7 ± 14.2	299.7 ± 23.1	129.0 ± 12.4	337.6 ± 23.0	149.1 ± 10.1	160.8 ± 10.2
Valine	257.5 ± 9.6	261.4 ± 12.6	225.7 ± 11.7	230.8 ± 9.6	265.8 ± 12.9	316.6 ± 17.4	269.6 ± 12.0	338.6 ± 16.4	192.0 ± 10.5	204.2 ± 10.9
Isoleucine	71.8 ± 4.7	74.1 ± 5.5	59.3 ± 4.6	61.1 ± 3.8	104.4 ± 4.8	149.4 ± 11.9	110.7 ± 4.2	170.4 ± 10.3	54.0 ± 3.3	58.7 ± 4.0
Phenylalanine	64.9 ± 5.6	64.0 ± 3.3	63.9 ± 3.3	64.7 ± 3.3	69.1 ± 3.1	80.4 ± 4.5	66.6 ± 2.7	80.4 ± 4.0	50.2 ± 2.3	49.3 ± 1.7
Methionine	20.9 ± 1.0	21.3 ± 1.1	21.0 ± 1.3	21.1 ± 1.1	26.1 ± 1.1	33.1 ± 2.2	25.5 ± 0.9	34.0 ± 1.9	14.3 ± 0.9	15.0 ± 0.8
Lysine	152.9 ± 8.7	167.5 ± 13.0	154.7 ± 11.0	174.6 ± 8.8	142.4 ± 9.4	300.6 ± 22.2	126.5 ± 10.4	329.7 ± 23.1	138.3 ± 7.9	172.7 ± 8.6
Histidine	55.1 ± 3.2	61.6 ± 4.9	56.8 ± 4.6	60.7 ± 4.2	55.6 ± 4.8	69.8 ± 5.8	52.8 ± 4.9	68.2 ± 5.7	52.8 ± 3.9	56.2 ± 4.0
Threonine	148.8 ± 9.2	151.0 ± 8.8	132.4 ± 6.4	135.0 ± 5.3	120.0 ± 8.5	174.1 ± 9.9	110.5 ± 6.0	182.8 ± 10.5	106.4 ± 6.4	123.5 ± 6.1
Tyrosine	83.7 ± 4.6	90.0 ± 5.6	78.1 ± 3.8	80.8 ± 4.6	72.8 ± 4.4	114.2 ± 8.0	65.5 ± 3.5	122.1 ± 8.9	58.3 ± 3.9	66.9 ± 3.9
Nonessential										
Asparagine	50.1 ± 1.6	52.4 ± 2.1	48.8 ± 2.3	48.8 ± 1.7	43.2 ± 2.2	66.6 ± 3.5	39.8 ± 2.2	68.0 ± 3.3	41.1 ± 2.2	42.4 ± 2.2
Serine	107.5 ± 5.9	107.7 ± 6.8	106.3 ± 6.4	107.6 ± 5.3	96.1 ± 4.9	128.5 ± 7.3	88.6 ± 6.0	124.2 ± 7.1	86.8 ± 5.0	85.3 ± 5.5
Glutamine	557.1 ± 14.9	591.2 ± 30.5	608.4 ± 18.2	696.9 ± 23.4	559.8 ± 19.0	667.7 ± 29.1	495.5 ± 56.0	662.2 ± 25.3	566.2 ± 23.4	603.1 ± 32.2
Arginine	57.0 ± 3.2	67.6 ± 5.5	60.5 ± 4.4	67.1 ± 4.6	54.2 ± 6.2	91.5 ± 8.3	49.7 ± 5.2	88.9 ± 9.3	51.2 ± 6.2	57.2 ± 4.9
Alanine	392.5 ± 26.9	422.7 ± 21.4	433.3 ± 28.4	441.5 ± 26.9	343.3 ± 18.5	456.1 ± 22.1	353.8 ± 15.6	490.8 ± 17.5	329.8 ± 21.0	353.1 ± 16.0
Citrulline	50.8 ± 7.5	48.9 ± 3.3	40.6 ± 3.1	45.9 ± 2.5	35.9 ± 3.0	43.2 ± 2.7	31.3 ± 3.0	42.8 ± 3.2	34.6 ± 2.7	42.6 ± 2.6
Glutamate	103.5 ± 9.0	83.7 ± 6.6	96.7 ± 8.8	96.7 ± 8.9	92.2 ± 8.4	117.9 ± 12.7	86.2 ± 9.8	98.4 ± 8.7	70.8 ± 8.6	67.3 ± 7.2
Glycine	235.9 ± 22.0	242.4 ± 18.1	233.0 ± 14.2	229.8 ± 13.1	201.6 ± 12.3	214.4 ± 11.3	194.4 ± 16.2	199.2 ± 11.0	205.9 ± 13.2	181.1 ± 15.3
Ornithine	54.6 ± 3.6	64.4 ± 3.8	53.4 ± 3.8	62.1 ± 3.0	50.0 ± 3.9	70.5 ± 3.9	43.3 ± 3.1	70.5 ± 3.9	38.7 ± 2.6	49.3 ± 2.7
Proline	198.7 ± 29.8	220.0 ± 20.2	176.9 ± 16.3	196.1 ± 19.3	159.3 ± 14.5	249.8 ± 20.7	149.6 ± 13.1	254.5 ± 18.3	138.0 ± 10.8	165.4 ± 14.7
Taurine	85.8 ± 4.3	80.4 ± 6.8	92.8 ± 9.2	97.4 ± 10.9	73.2 ± 6.5	91.5 ± 8.8	79.0 ± 9.0	78.1 ± 6.6	57.0 ± 3.6	60.6 ± 5.4
Aspartate	7.1 ± 0.5	7.1 ± 0.6	7.0 ± 0.5	8.7 ± 0.8	6.4 ± 0.5	9.1 ± 0.6	5.8 ± 0.5	8.7 ± 0.5	5.0 ± 0.4	5.9 ± 0.4
Hydroxyproline	11.9 ± 1.2	13.8 ± 2.3	9.7 ± 0.9	10.9 ± 1.6	9.2 ± 0.8	12.9 ± 1.9	8.7 ± 0.8	13.5 ± 1.7	8.2 ± 0.7	9.7 ± 1.1

Table 4-5 Plasma amino acids (mmol/L) over time in the first study for both conditions

Subjects cycled at roughly 50% VO_{2max} for one hour before receiving a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. At 3.5 and 4 hours, corresponding to 30 and 60 minutes post nutrient ingestion, plasma concentrations of all amino acids were higher in the PRO than the CHO condition at the corresponding time point (except for taurine at 4h post-exercise). Higher values at 3.5 and 4 hours are indicated by boxed numbers. Group means ± standard errors are shown. Time is in hours from start of infusion. Treatment drinks were given at t = 3h.

4.3.5 Plasma enrichment was higher with carbohydrate-only feeding while breath enrichment was higher with protein-plus-carbohydrate feeding

The ^{13}C α-KIC enrichment of the plasma zero standards was 21.27 ± 0.20% with a % coefficient of variation (CV) of 0.94. All of the values for the zero standards fell within the acceptable range for background enrichment of 21.61 ± 0.4%. The ^{13}C α-KIC enrichment of the baseline plasma samples was 21.32 ± 0.31% with a %CV of 1.43. Only three of the 42 values fell outside the acceptable range for background enrichment indicating that the GC-MS's detection remained sufficiently linear for accurate interpretation of the values returned.

The ^{13}C α-KIC enrichment determined for the sample analysed in triplicate for reliability was 6.66 ± 0.54 with a %CV of 8.05. A variation of this magnitude would create a %CV of 7.6 in calculated values of whole body leucine oxidation and NOLD. As the differences between the CHO and PRO conditions in this study were 45% and 84% for calculated values of oxidation and NOLD respectively, the variability introduced by the derivatisation process and the GC-MS would not have had a significant effect on the findings.

The ^{13}C α-KIC enrichment of plasma samples from the first two hours of infusion shows that an isotopic steady state was achieved prior to exercise beginning (Figure 4-10). There was no significant variation with time in the enrichment of the samples taken during the second hour of infusion ($p = 0.74$). There was a difference in the steady state enrichment between the CHO and PRO conditions ($p < 0.05$).

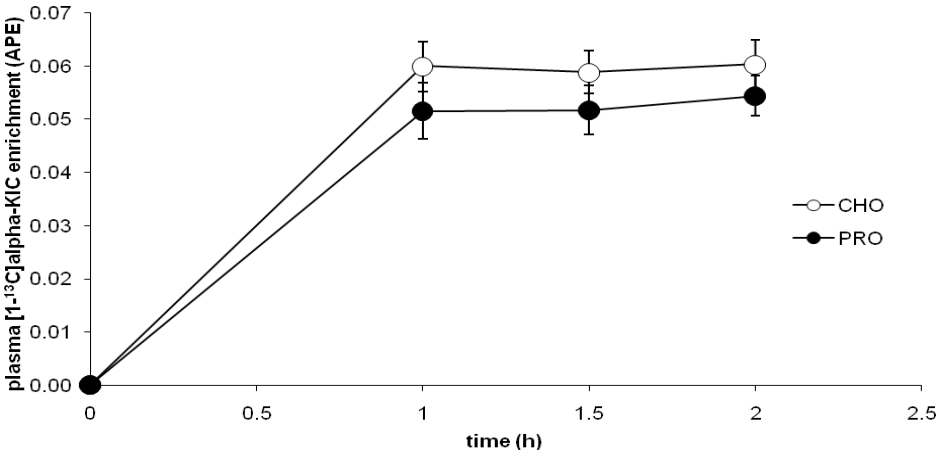


Figure 4-10 Plasma ^{13}C α-ketoisocaproate enrichment for the first two hours of infusion for both conditions
Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. Plasma enrichment increased from zero at the start of infusion to reach a steady state after one hour of infusion. Time is in hours from the start of infusion. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

The ^{13}C α-KIC enrichment of plasma samples from the final two hours of infusion is shown in Figure 4-11. Enrichment for the CHO condition was higher than that for the PRO condition ($p < 0.001$).

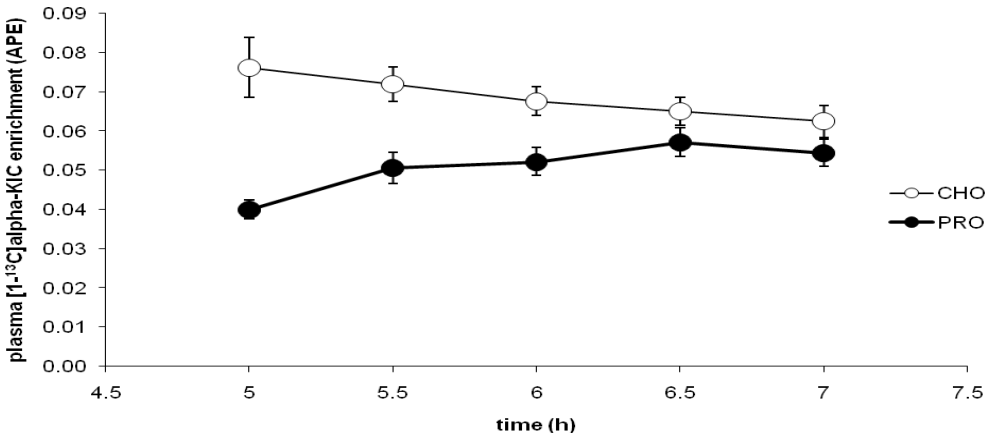


Figure 4-11 Plasma ^{13}C α-ketoisocaproate enrichment for the final two hours of infusion for both conditions
Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Blood was collected from an antecubital vein. In the PRO condition plasma enrichment increased from two to four hours post-exercise, while in the CHO condition enrichment decreased over the same time period. Time is in hours from the start of infusion. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

The $^{13}CO_2$ enrichment of the breath samples for the final three and a half hours of infusion is shown in Figure 4-12. Enrichment for the PRO condition was higher than that for the CHO condition ($p < 0.001$).

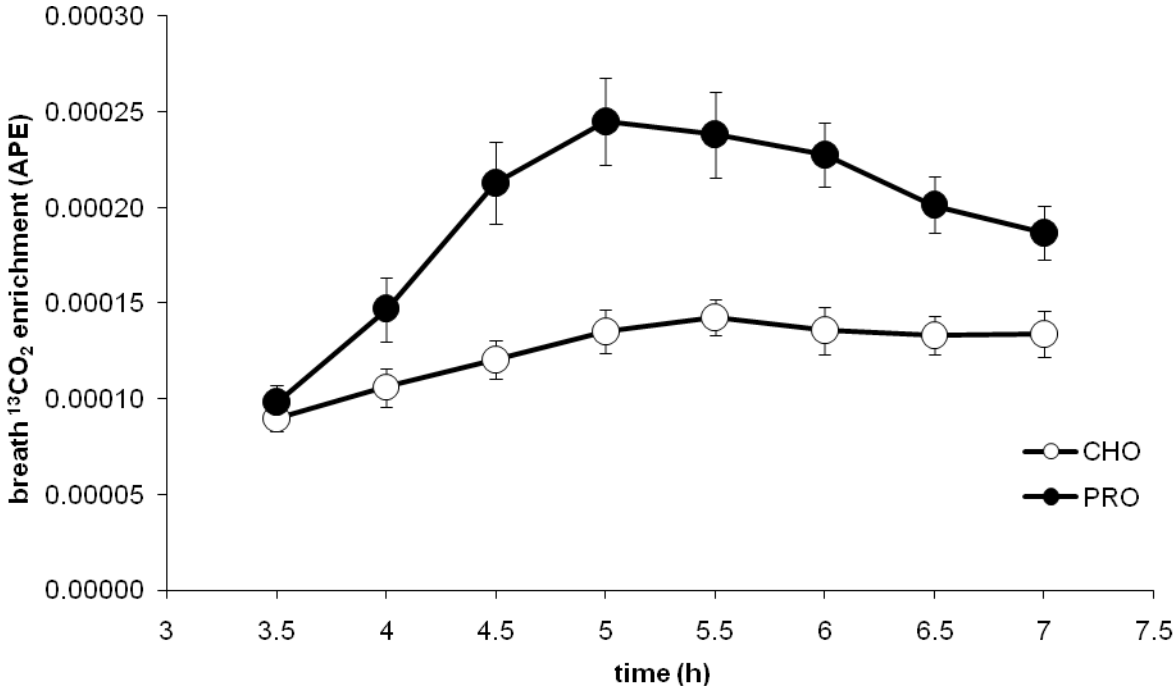


Figure 4- 12 Breath $^{13}CO_2$ enrichment for the final 3.5 hours of infusion for both conditions

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Expired air was collected in 60 L Douglas bags. Breath enrichment was higher in the PRO condition than the CHO condition from one to three hours post-exercise. Time is in hours from the start of infusion. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

4.3.6 Whole body protein synthesis was higher with protein-plus-carbohydrate feeding than with carbohydrate-only

The rates of appearance of leucine into plasma over the final two hours of infusion are shown in Figure 4-13. The R_a for leucine was consistently higher in the PRO condition than in the CHO condition from two through four hours post-exercise. Furthermore, the R_a decreased steadily in the PRO condition throughout this time period such that over the last half an hour of post-exercise recovery the R_a in the two conditions was similar.

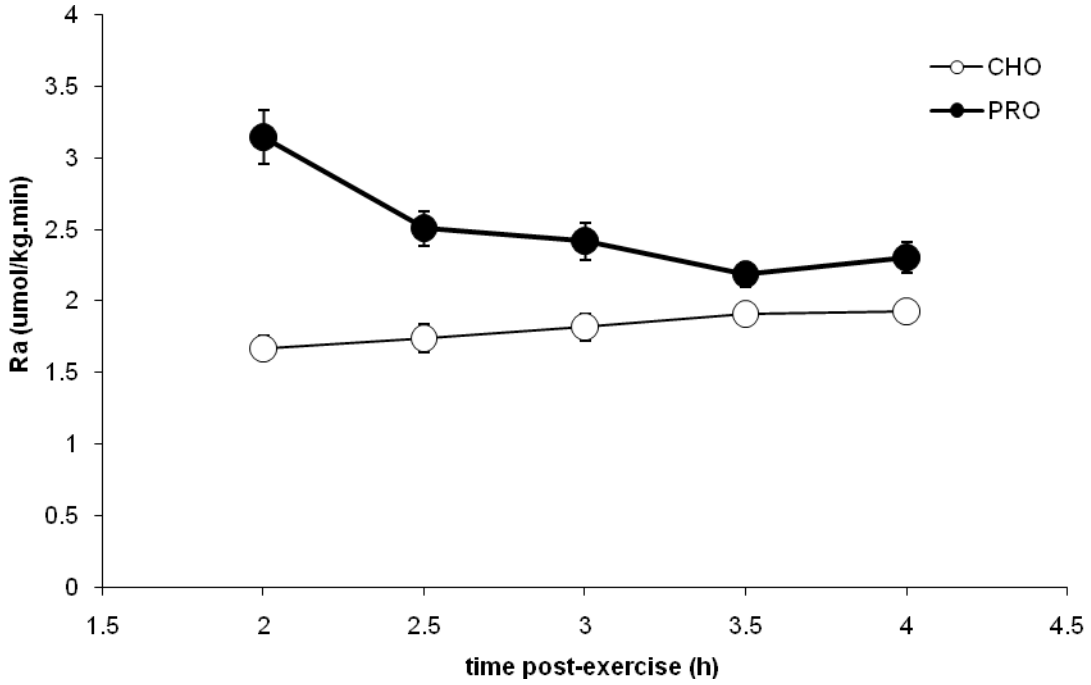


Figure 4-13 Rates of appearance of leucine into plasma for the final two hours of infusion for both conditions

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Blood was collected from an antecubital vein. The R_a of leucine was higher in the PRO condition than the CHO condition throughout the time period shown. Time is in hours from the end of exercise. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

Rates of leucine oxidation for the final two hours of infusion are shown in Figure 4-14. Leucine oxidation for the PRO condition was higher than that for the CHO condition from two through to four hours post-exercise ($p < 0.001$).

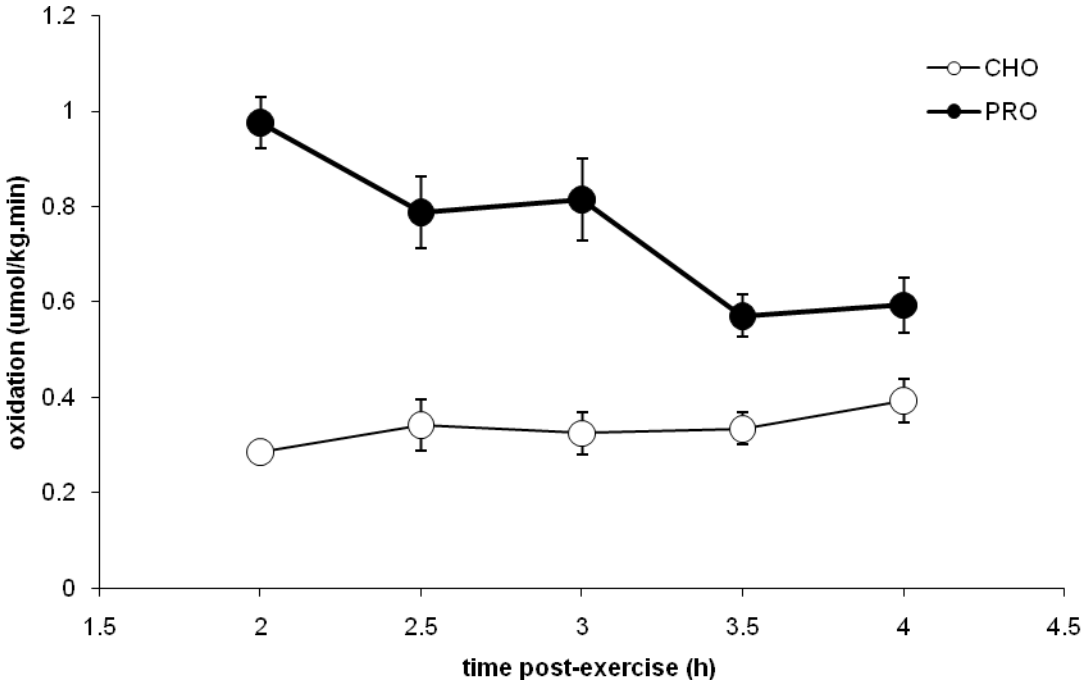


Figure 4-14 Rate of leucine oxidation for the final two hours of infusion for both conditions

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Blood was collected from an antecubital vein and expired air in 60 L Douglas bags. The rate of leucine oxidation was higher in the PRO condition than the CHO condition throughout the time period shown. Time is in hours from the end of exercise. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

Rates of NOLD (a proxy for WBPS) for the final two hours of infusion are shown in Figure 4-15. The PRO drink resulted in greater rates of NOLD from two through to four hours post-exercise than the CHO drink ($p = 0.001$) with some evidence of a time effect ($p = 0.08$).

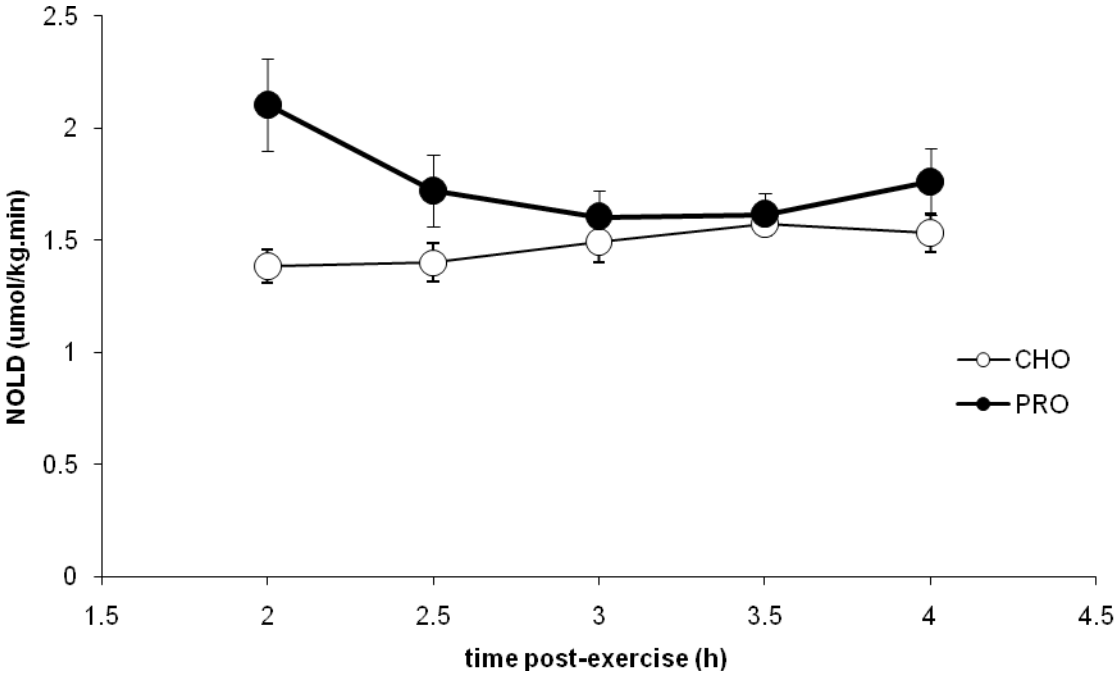


Figure 4-15 Rate of non-oxidative leucine disposal (NOLD) for the final two hours of infusion for both conditions

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. The rate of NOLD was higher in the PRO condition from two to four hours post-exercise. Time is in hours from the end of exercise. Group means ± standard errors are shown; n = 10 (CHO) and 12 (PRO).

4.4 Discussion

This is the first study to date to report the effects of nutrition provided after a bout of aerobic exercise on rates of whole body protein kinetics in older individuals. The main finding in this portion of the study was that the provision of a protein-plus-carbohydrate drink immediately after a bout of aerobic exercise resulted in a 20% higher rate of NOLD, a proxy for WBPS, from the second through to the fourth hour post-exercise than a carbohydrate-only drink. The rate of whole body leucine oxidation was also higher in the PRO condition throughout this time period. The higher ^{13}C enrichment of expired CO_2 in the PRO condition indicates that, in this

condition, a greater proportion of the infused [1-^{13}C]leucine was oxidised instead of being used for protein synthesis.

4.4.1 Amino acids up-regulate translation

The nutrients present in the PRO condition would have caused a greater rate of WBPS by up-regulating both translation initiation and elongation. Leucine signals directly to mTOR, thus up-regulating protein translation via mTOR's downstream effectors 4E-BP1 and p70^{S6K1} (Figure 2-4,). The increase in plasma leucine concentration in the PRO condition (Figure 4-9) suggests that an early response of WBPS to the drink would have been phosphorylation and activation of mTOR and a subsequent rise in the rate of translation initiation. The greater rise in all plasma amino acids seen in the PRO condition in the hour after nutrient ingestion (Table 4-5) would have provided a greater supply of amino acids to the body's free pool, thus increasing the available substrate for the translation elongation step of protein synthesis .

4.4.2 Feeding protein-plus-carbohydrate after aerobic exercise increases the rate of WBPS more than feeding carbohydrate-only

This study's main finding differs from that of Koopman et al. , who found no significant difference between similar CHO and PRO conditions in the rate of WBPS during post-aerobic exercise recovery when using a [1-^{13}C]leucine tracer. Koopman et al.'s study differs from this one however, in that the drink was provided during a five-hour bout of aerobic exercise rather than after a one-hour bout, and that it used endurance-trained young adult subjects. This study's findings are in agreement with those of both Koopman et al. and Roy et al. , who found that subjects receiving carbohydrate-only drinks after a bout of resistance exercise had lower rates of WBPS compared to those receiving a drink containing added protein. As Koopman et al. and Roy et al. used a resistance exercise intervention, the specific proteins stimulated in their studies would likely have been different to those stimulated in the study being reported here. Similarly, Howarth et al. found that subjects receiving a protein-plus-carbohydrate drink

after a two-hour bout of cycling had a more positive whole body protein balance than those receiving an isocaloric, carbohydrate-only drink. Howarth et al. used protein and carbohydrate doses nearly double those used in the study reported here, suggesting that further study of the dose-response relationship of protein ingestion and WBPS is warranted.

It is interesting to note the findings of Koopman et al. , who compared the effects of post-exercise protein-plus-carbohydrate drinks to protein alone on whole body protein kinetics after a bout of resistance exercise. The authors found no difference between the drinks in measures of WBPB, WBPS, and whole body protein balance, suggesting that the presence of the carbohydrate in the PRO drink in their study may have been superfluous. Bennet et al.'s findings, examining the effects of nutrition provided in the rested state support this assertion. The authors found the same increase in NOLD and the same decrease in WBPB whether amino acids were infused alone or in combination with carbohydrate. Regardless of the redundancy of the carbohydrate, it was important to have an isocaloric control in this study so that the effect of energy intake alone could be eliminated.

4.4.3 Subjects exercised at 50% VO_{2max} in both trials

Heart rate and gas analysis data indicate that subjects did exercise at the same intensity in each session in this study, making it likely that the same WBPT response was elicited in each condition. Subjects exercised at roughly 50% of VO_{2max} which was lower than the intended 70%. It is possible that this lower intensity elicited a different response than that which would have occurred had they exercised at the intended intensity. Availability of a functional direct gas analyser at the time of experimentation would have allowed for subjects to exercise at an intensity corresponding to a percentage of their VO_{2max} rather than their W_{max}.

4.4.4 Circulating insulin is permissive for protein synthesis in humans

Both drinks elicited an increase in circulating insulin concentration. The fact that the PRO drink elicited a faster and greater response indicates that amino acid ingestion stimulates insulin release as much as carbohydrate ingestion . This differing insulin effect between the drinks is unlikely to have had an effect on calculated values for whole body protein kinetics. In both conditions, the concentration of circulating insulin increased to well above 30 μU/mL, a concentration that is well over the threshold value of 5 μU/mL required to facilitate protein synthesis in humans .

4.4.5 Possible sources of uncontrolled variability in the results

The lack of temporal variation in ^{13}C-KIC plasma enrichment in the hour before the exercise bout indicates that the steady state assumption was met prior to the start of exercise. The enrichment was higher in the CHO condition at all time points after drink consumption, most likely due to the leucine in the PRO drink not having a ^{13}C label. The presence of more ingested, unlabelled leucine in the PRO condition would have decreased the enrichment by dilution. The fact that enrichment was consistently higher in the CHO than the PRO condition, both before and after exercise, has implications for the calculated values of whole body protein kinetics. Plasma enrichment appears in the denominator of the equations to calculate both R_a and leucine oxidation, therefore a higher plasma enrichment would result in lower calculated values for both R_a and whole body leucine oxidation. As both the latter values were found to be lower in the CHO condition, some of this difference must be attributed to the higher plasma enrichment value and not to the drink itself.

Methodological issues with the drinks also necessitate caution in interpreting the results. Results from bomb calorimetry indicate that the CHO drink provided 938 KJ (223 KCal) while the PRO drink provided 1058 KJ (252 KCal). As protein turnover is an energy-consuming process , the presence of 13% more available energy in the PRO condition could have had a

confounding effect on calculated rates of NOLD. As it was the PRO drink that resulted in a higher rate of NOLD, some small part of this difference may be attributed to its higher energy content and not to the presence of the protein.

Furthermore, the increase in plasma leucine concentration in the PRO condition indicates that the leucine in this drink did reach the circulation where it could have an effect on enrichment values and whole body protein kinetics. The rate of ingestion of leucine could not, therefore, be assumed to be zero in making the calculation for R_a and the leucine in the PRO drink did not have a ^{13}C label to correct for this fact. While the total leucine content of the PRO drink was small (2.165 mg) there is some chance, albeit minimal, that this factor would lend unreliability to the calculations of whole body protein kinetics. If the R_a was higher than calculated, then there is an underestimate in the value for NOLD. The presence of unlabelled leucine in the drink would also have had a dilution effect on ^{13}C-KIC plasma enrichment which would have further underestimated NOLD. It was not possible to calculate the contribution that the leucine in the drink might have made to leucine R_a due to variable rates of splanchnic oxidation in the fed and fasted states and between populations .

A limitation of the whole body method is that it assumes each tissue contributes to R_a in proportion to its rate of breakdown, but this is not in fact the case. Insulin, which was released in response to both drinks, has different effects on the rate of breakdown of different tissues . Therefore it is inappropriate to speculate on the effects of this intervention on muscle protein kinetics or any other sub-fraction of whole body protein. The calculated value for oxidation is not determined precisely either, as it also relies on plasma enrichment. The errors in these calculated values of breakdown and oxidation are then carried over into the calculated value for NOLD. Although there was undoubtedly error introduced into the results by the

methodological issues in this paragraph, none of these would affect the comparison between the drinks because the error would presumably have been consistent between the conditions.

Because this study used both male and female subjects, it would have been helpful to normalise the rate of [1-^{13}C]leucine infusion and the size of the priming boluses to the individual body mass or FFM of each subject. Despite a 40 kg variation in body weight, all subjects received the same dose of tracer. Due to the relatively inaccurate method of determining body fat in this study, the option of normalising to FFM was not available. As the isotope doses were mixed by an outside lab (Liggins Institute, University of Auckland, NZ), such precision in doses was impractical. This issue may have resulted in slightly skewed group means for leucine oxidation as this rate is lower in women than men , possibly due to differences in body size.

Finally, this study may not present a true picture of the ageing population due to the inclusion/exclusion criteria and also because the study was so invasive that the faint-hearted probably did not apply in the first instance. Furthermore, the prospect of an hour of cycling may not have appealed to the more sedentary volunteer. Keeping these factors in mind, it is likely that the subjects in this study were slightly fitter, and consequently healthier, than the average person of their age, as indicated by their mean maximal rate of oxygen consumption falling into the 'very good' range rather than 'average' .

4.4.6 Conclusion

Regular exercise has been found to combat sarcopenia, and increase both muscular endurance and the ability to perform activities of daily living . This study provides evidence that providing appropriate nutrition after each bout in a training programme could further enhance these effects. Although this study only involved a single exercise bout, it has been shown that an aerobic exercise training programme increases the rate of WBPT and attenuates muscle wasting . Chronic adaptations are the result of an accumulation of responses after each bout of

exercise. Chronic endurance exercise could therefore be used to slow the age-related decline in functional capacity. Epidemiological evidence lends credence to such a hypothesis as masters athletes have been found to experience postponement of disability and protection against early mortality as well as maintenance of a VO_{2max} commensurate with a much younger individual . In conclusion, an intervention involving aerobic exercise and protein-plus-carbohydrate post-exercise nutrition could increase physical capacity in older individuals by maintaining protein content and increasing protein quality in tissues throughout the body through increased rates of WBPT.

5 Influence of post-aerobic exercise nutrient composition on skeletal muscle mitochondrial biogenesis in older individuals

5.1 Introduction

Whole body oxidative capacity, measured as VO_{2max}, declines with age to the point at which it can be exceeded by the aerobic demand of activities of daily living . The age-related decline persists following adjustment for remaining FFM, indicating a decline in specific muscle functional capacity . The action of mitochondrial oxidative enzymes determines muscle oxidative capacity, a crucial determinant of resistance to fatigue during exertion . Oxidative enzyme action, strongly correlated with endurance capacity , is therefore dependent on mitochondria number and function . A change in mitochondrial protein content is one factor leading to changes in VO_{2max}, while increases in endurance capacity can be attributed mostly to mitochondrial biogenesis . From youth to middle age the rate of mitochondrial protein synthesis, and corresponding enzyme activity, decreases by 40%, with no further decline in older age . It seems relevant, therefore, to search for ways to increase the rate of mitochondrial protein synthesis in middle-aged individuals whose rates have already slowed, or to slow this decrease in its early progression.

As discussed in 2.1, increasing the rate of muscle protein turnover is desirable in terms of maintaining the quality and function of its constituent proteins, one of which is the mitochondria. The rate of MPB is increased during aerobic exercise while the rate of MPS is increased both postprandially and after exercise . An acute bout of aerobic exercise has been shown to stimulate the rate of MPS . Post-aerobic exercise stimulation of MPS is specific to proteins of the aerobic respiratory chain . Increased synthesis of oxidative enzymes leads to an increase in muscle oxidative capacity . The stimulatory effect of aerobic exercise on oxidative enzyme activity persists in older subjects , albeit at a reduced level when compared to younger

subjects . Ingestion of amino acids, either in isolated form or in protein , inhibits the rate of MPB and stimulates the rate of synthesis while a combination of insulin, released after carbohydrate consumption, and amino acids enhances the rates of both MPS and mitochondrial biogenesis . An intervention involving aerobic exercise and appropriate nutrition may therefore improve skeletal muscle mitochondrial quantity and functional quality.

The FSR method is used to elicit information regarding the turnover of a portion of the whole body protein pool, such as muscle or a muscle sub-fraction. Fractional rates of protein synthesis determine the percentage of total body protein added per hour . To calculate FSR, the enrichment of both the tissue itself (the product) and a suitable precursor pool must be measured . Any amino acid not yet incorporated into a protein chain is only potentially a precursor so the only true precursor pool is, in fact, charged tRNA. Due to the low concentration of aminoacyl-tRNA in muscle tissue and its short half-life , the quantity of muscle required to obtain measurable values of aminoacyl-tRNA, and the technical demands of measuring it, make sampling this pool impractical in human subjects . Alternative precursor pools are muscle intracellular fluid and plasma. The muscle intracellular compartment is, itself, not completely homogenous, nor is it in perfect equilibrium with plasma . Therefore the sampling of intracellular leucine as the precursor pool is preferable to that of plasma when conducting measurements at the level of the muscle . Intracellular fluid is also a more reliable precursor pool than plasma because the ratio of enrichment between intracellular fluid and tRNA remains constant in the fasted, and a variety of fed, conditions .

Recent research in the area of exercise and/or nutrition on muscle protein kinetics has focused mainly on measures at the level of mixed muscle protein or the myofibrillar sub-fraction . There exists very little evidence of the influence of diet and/or exercise on the rate of protein synthesis in mitochondrial protein in human skeletal muscle. One recently published study has

reported an increase in the tracer-measured mitochondrial protein FSR after an acute bout of either aerobic or resistance exercise. To date the concomitant effects of exercise and nutrition on mitochondrial protein synthesis have not been examined, nor has an older population been targeted.

Therefore the aim of this part of the first study was to investigate whether feeding protein immediately after an acute bout of moderate-intensity aerobic exercise would have an additive effect over carbohydrate alone, on the rate of skeletal muscle mitochondria protein synthesis in older individuals. The hypothesis tested was that a protein-plus-carbohydrate (PRO) drink will increase the rate of skeletal muscle mitochondrial protein synthesis during the first four hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (CHO).

5.2 Methods

During the study described in 4.2.2.2 participants received a percutaneous needle muscle biopsy from the *m. vastus lateralis* immediately following exercise, and four hours later from the contralateral leg (Appendix B6). The first leg to be sampled each day was alternated within and between subjects, as was the choice of a proximal or distal site for subsequent incisions. A new incision was made each time as repeated sampling from the same site can cause an increase in muscle protein turnover due to stimulation of remodelling , which could have masked the treatment effects being measured. The skin and fascia incisions were made under local anaesthetic (1% lignocaine, AstraZeneca Ltd., Auckland, NZ) according to the method of Bergstrom . The biopsy samples were separated from any obvious adipose tissue, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

5.2.1 Muscle processing and analysis

To determine the rate of mitochondrial protein synthesis, the intracellular fluid and bound mitochondrial protein sub-fractions were isolated from the muscle samples. Relevant mass

spectrometric methods were developed and used to determine the [1-^{13}C]leucine enrichment of these two sub-fractions.

5.2.1.1 Intracellular fluid and mitochondrial isolation

Free amino acids from the intracellular fluid and those bound in mitochondrial protein were isolated from the muscle samples using adaptations of previously described methods (Figure 5-1). Using a hand-operated Potter-Elvehjem-type glass homogeniser, the weighed tissue sample was homogenised in ice-cold *solution 1* (210 mM sucrose, 2 mM EGTA, 40 mM NaCl, 30 mM HEPES, pH 7.4) and the subsarcolemmal mitochondria (SSM) fraction isolated by spinning the homogenate at low speed (600 g) for ten minutes at 4 °C (5415R, eppendorf). The supernatant from the low-speed spin was then decanted and centrifuged at high speed (10 000 g) for 30 minutes at 4 °C to pellet the mitochondria. The pellet was washed twice by re-suspending in 200 µL of *solution 2* (1 mM EDTA, 10 mM Tris, pH 7.4) and repeating the high-speed spin. The resulting pellet was re-suspended in 100 µL *solution 3* (0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4) and stored at 4 °C until tissue protein hydrolysis.

The supernatant from the first high-speed spin was saved and the amino acids liberated from sarcolemmal structures by homogenising in three volumes of ice-cold 8% perchloric acid for two hours. The solution was then centrifuged at high speed (10 000 g) at 4 °C for 30 minutes to isolate the amino acid fraction, and the supernatant then decanted and neutralised with 5 N KOH to pH 7.5-8.0. The neutralised supernatant was again centrifuged at high speed (10 000 g) for 30 minutes at 4 °C to remove the potassium perchlorate precipitate and isolate the intracellular fluid amino acids.

The mitochondrial protein was precipitated overnight with 300 µL ice cold 10% TCA, then centrifuged at medium speed (3 500 g) at 4 °C for 20 minutes. The resulting pellet was rinsed three times with 2 mL petroleum ether to remove the TCA, re-suspended in 500 µL 0.8 M

NaOH and then incubated at 60 °C for 90 minutes. The solution was then centrifuged at medium speed (3 000 g) at room temperature for 30 minutes and the supernatant decanted and hydrolysed in 2.5 mL of 6 N HCl for 18 hours at 110 °C to liberate the bound amino acids.

An attempt was made to analyse separately the SSM and intermyofibrillar mitochondria (IMM) fractions of the mitochondrial reticulum by following a previously described method . The pellet from the first low-speed spin was re-suspended in a ten-fold dilution of *solution 4* (100 mM KCl, 40 mM Tris-HCl, 10 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.5) and homogenised using an electric homogeniser (Ultra-Turrax, Tekmarco, Cincinnati OH, USA). The resulting suspension was subject to a series of centrifugations and re-suspensions, as shown in Figure 5-1, using s*olution 5* (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, 1.5% BSA, pH 7.4), *solution 6* (100 mM KCl, 10 mM Tris-HCL, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, pH 7.4), and *solution 7* (220 mM sucrose, 70 mM mannitol, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The final re-suspension was subjected to the same precipitation and hydrolysis as described in the preceding paragraph. As Western blotting procedures detected no immunoreactive mitochondrial proteins in the IMM fraction, it was decided to proceed with isolation of the intracellular fluid and SSM fraction only. As the SSM fraction is much more responsive to exercise than the IMM, the decision to analyse only the former was reasonable .

Both the intracellular fluid and mitochondrial protein fractions were partially dried in a lyophiliser (The VirTis Company, Gardiner NY, USA) and then completely dried under a stream of nitrogen gas. The drying process was interrupted due to a malfunction in the lyophiliser leading to a partial defrosting, and some loss, of the samples.

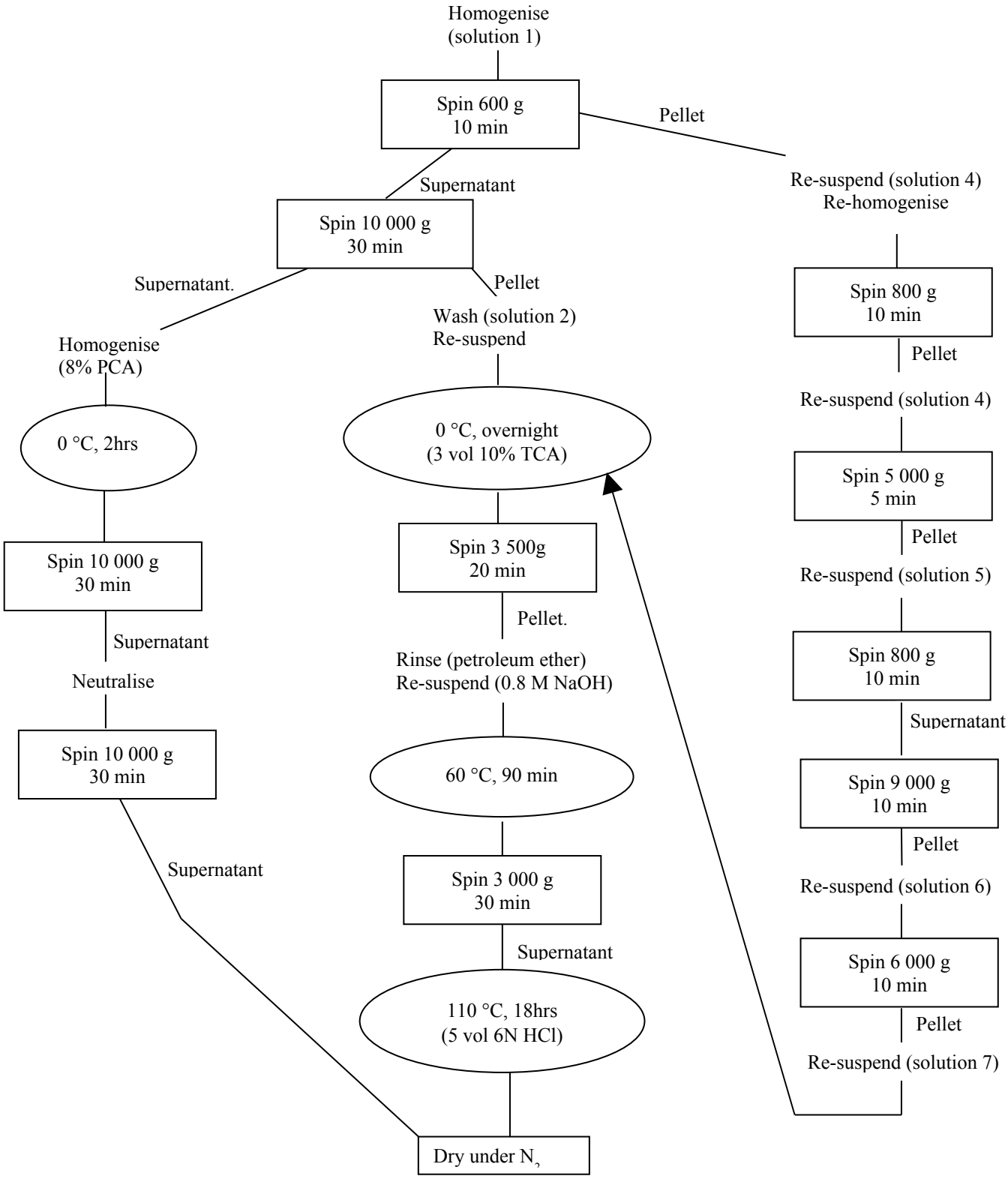


Figure 5-1 Steps taken to isolate intracellular fluid and bound mitochondrial fractions from skeletal muscle samples

Muscle samples were obtained by biopsy of the *m. vastus lateralis*. Left hand pathway isolates intracellular fluid, centre pathway isolates subsarcolemmal mitochondria (SSM), right hand pathway isolates intermyofibrillar mitochondria (IMM). See text for details of method and composition of solutions. PCA, perchloric acid. Adapted from .

5.2.1.2 Western blotting

To determine the efficacy of the isolation and extraction process five pieces of human *biceps brachii* (70-142 mg) from a cadaver sample were obtained through an organ donation programme. Western blotting was performed on the re-suspended pellet and the supernatant from the first high-speed spin and the hydrolysed mitochondria and tissue fluid fractions. Homogenates of rat *tibialis anterior* mitochondrial and cytosolic fractions and rat brain were used as positive controls. The protein concentration of each fraction was determined using the bicinchoninic acid method (Appendix B7). The appropriate volume of each fraction necessary to load the same total protein per lane PAGE was taken (Appendix B9). Samples were diluted with NuPAGE lithium dodecyl sulphate sample buffer, heated for ten minutes at 70 °C, and then loaded onto a 10-12% bis-Tris-polyacrylamide gel (Invitrogen, Carlsbad CA, USA). MagicMark Western Protein Standard (Invitrogen, Carlsbad CA, USA) was used as the molecular weight marker. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, the membrane blocked with 10% milk in TTBS (1% Tween-20 in 500 mM Tris, 1.5 M NaCl, pH 7.5), and then probed with appropriate monoclonal IgG primary and horseradish peroxidase-linked donkey anti-rabbit or sheep anti-mouse IgG secondary antibodies (Amersham Biosciences, UK, Appendix B9). Rabbit anti-VDAC (voltage-dependent anion channel) and mouse anti-cytochrome c oxidase (COX) were used as mitochondrial markers (AbCam, Cambridge MA, USA and Invitrogen, Carlsbad CA, USA), mouse anti-human and rabbit anti-rat antibodies against sodium potassium ATPase (Na^+/K^+ ATPase) as sarcolemmal markers (Upstate, Charlottesville VA, USA), and mouse anti-LDH (lactate dehydrogenase) as a cytosolic marker (Sigma, St. Louis MI, USA). Mouse anti-GAPDH was used as a loading control (Imgenex, San Diego CA, USA), as GAPDH is present in both the cytosol and mitochondria. Antibody binding was detected using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

5.2.1.3 Mass spectrometry

The liberated leucine from the bound protein samples was derivatised as its N-acetyl-n-propyl ester and analysed by capillary GC-C-IRMS by Iso-trace (Dunedin, NZ). The methods specified by this commercial company are described in this and the following paragraph. Derivatisation of the carboxylic acid portion of the leucine molecules began with the addition of 400 µL propyl acetate and 200 µL 14% propanol/boron trifluoride (BF_3) to the dried sample. This solution was then incubated in a crimped autosampler vial at 110 °C for 30 minutes, allowed to cool, and the solvent and reagent then removed by drying under a stream of N_2 at 60 °C. The residue was re-dissolved and then dried twice more in 500 and 300 µL propanol/BF_3 respectively. To derivatise the amine part of the leucine molecule, the resulting amino acid propylates were dissolved in 200 µL acetonitrile, 100 µL 1,4-dioxane, 150 µL triethylamine, and 90 µL dry, freshly distilled acetic anhydride. This solution was then incubated in a crimped autosampler vial at 60 °C for 15 minutes, allowed to cool, and transferred into a conical Eppendorf cap. 250 µL $CHCl_3$ and 2 x 300 µL 0.001 M $NaHCO_3$ were added and the aqueous layer discarded. The remaining organic phase was dried under a stream of N_2 at 60 °C and then re-dissolved in ethyl acetate in preparation for GC-C-IRMS.

Samples were separated on a trace GC (Thermo, Bremen, Germany) before online combustion to CO_2 in a GC/C Interface III (Thermo, Bremen, Germany). Each sample was injected only once. CO_2 was transferred to a Delta^{plus}XP (Thermo, Bremen, Germany) where $\delta^{13}C$ ratios were measured. The trace GC was configured with a split/splitless injector in splitless mode at 250 ºC. To achieve baseline separation of leucine from other compounds in the mixtures, the temperature in a BP20 (30 m, 0.25 mm i.d., 0.25 µm; SGE, Melbourne, Australia) GC column was increased using a ramp function, from 60 °C to 230 °C at a rate of 20 °C/min. The column was held at the final temperature for two minutes. The carrier gas was helium and flowed at

2.0 ml/min. The $\delta^{13}C$ ratio for leucine in each sample was calculated by linear regression of the sample against three individual leucine compounds prepared in duplicate. One leucine sample was also analysed every ten samples to check for instrument drift. In addition, an internal reference gas was injected in duplicate at the beginning and end of each sample.

Mitochondrial enrichment was calculated as an atom percent excess (greater than reference gas), a value returned by the IRMS instrument, determined according to the equation;

$$\frac{100}{1/[(\delta C_{VPDB}/1000 + 1)*VPDB] + 1}$$

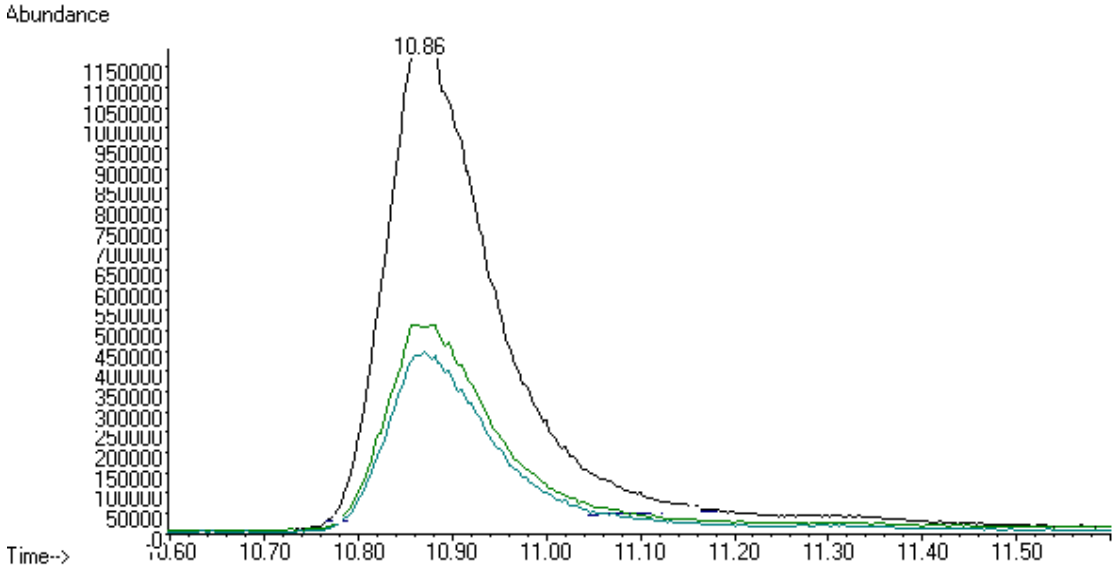
The atom percent of the reference gas was also returned by the instrument, determined according to the equation;

$$\frac{100}{1/0.011237 + 1}$$

Standard solutions of ^{12}C and ^{13}C leucine were prepared from ^{12}C leucine stock powder and a stock solution of 111 mg in 20 mL (0.9% NaCl) [1-^{13}C]leucine and, from these, a series dilution of solutions containing 0, 2.5, 5, 7.5, and 10 MPE was prepared. The standards were derivatised according to previously published methods with modifications . Ice-cold ethanol (1 mL) was added to 50 µL of standard and incubated at 4 °C for 20 minutes, then 500 µL of this solution was taken and the ethanol evaporated under a stream of nitrogen gas at 90 °C. 500 µL 0.5 M HCl and 2 mL ethyl acetate were added and the upper layer discarded. The ethyl acetate was evaporated under nitrogen gas at 90 °C, 50 µL each of MTBSTFA (N-Methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide) and pyridine added, and the capped tubes incubated at 90 °C for 90 minutes. Samples were then transferred to GC-MS vials for analysis.The whole mass spectrum was scanned using electron ionisation in order to detect the fingerprint of the leucine molecule under the same conditions as for α-KIC (section 4.2.3). The only variations from the α-KIC GC-MS parameters were that the inlet pressure was set at 18.60 psi and the GC oven final temperature was 260 °C held for one minute. When a 40 µg/mL solution of

[^{12}C]leucine and a 10 μg/mL solution of [1-^{13}C]leucine were run, peaks were detected at 200, 274, 302 and 303 *m/z* with an elution time of 10.9 minutes (Figures 5-2, 5-3), so these ions were targeted for determination of enrichment by selected ion monitoring at 302 and 303 *m/z*. The peak was reasonably wide (roughly 25 seconds) so the flow rate through the column was increased to 1.6 mL/min, to attempt to tighten the peak, and the initial oven temperature increased to 120 °C, to shorten the run time. Under these new conditions the elution time was 6.8 minutes (Figure 5-4).

A



B

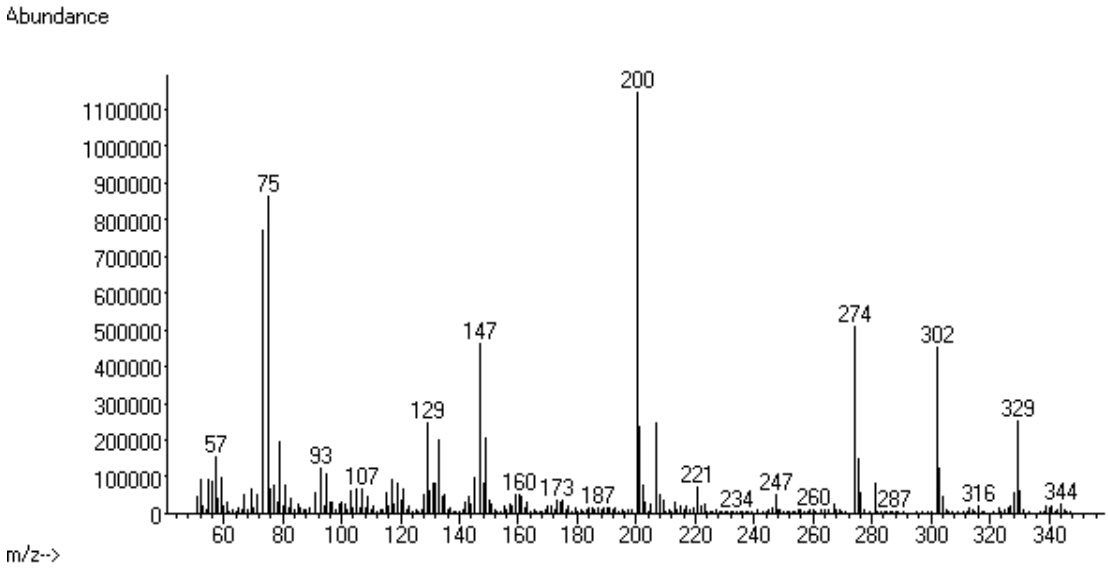
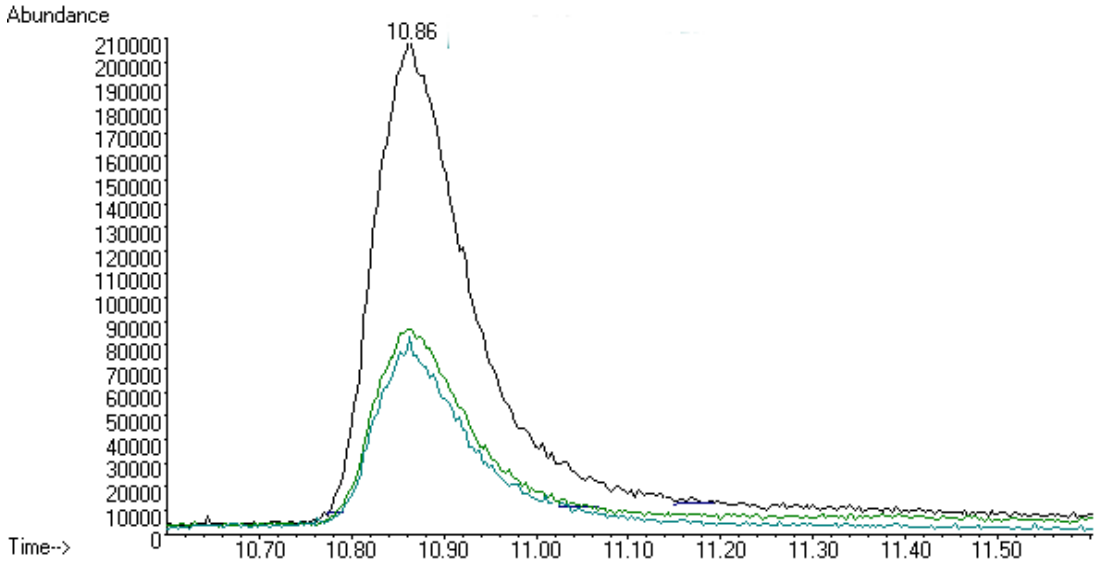


Figure 5-2 Representative GC-MS trace for 40 µg/mL solution of [^{12}C]leucine

Samples of diluted stock solution were subjected to derivatisation with MTBSTFA prior to loading onto GC column. Initial oven temperature was 80 °C, increased 10 °/min. *A)* absorbance spectrum from substances eluting between 10.6 and 11.6 minutes shown as separate extracted ion chromatograms. Elution time of leucine is 10.86 min. The highest peak is the absorbance of the 200 *m/z* ion, the middle peak 274 *m/z*, and the smallest peak 302 *m/z* as indicated by spectrum in B. *B)* mass spectrum showing abundant fragments at 200, 274, and 302 *m/z*. 302 *m/z* is the fragment of interest. MTBSTFA, N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide.

A



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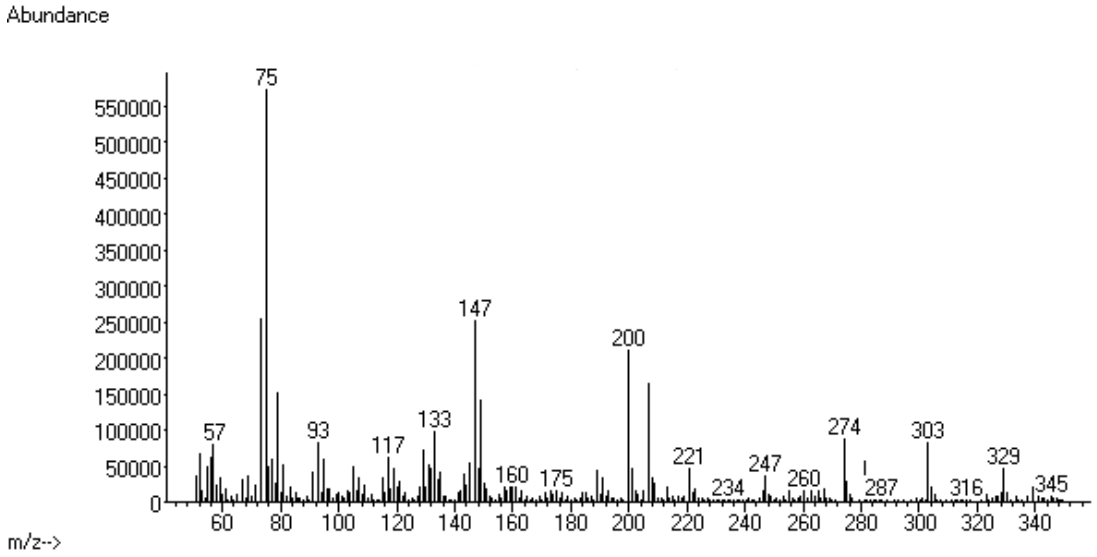
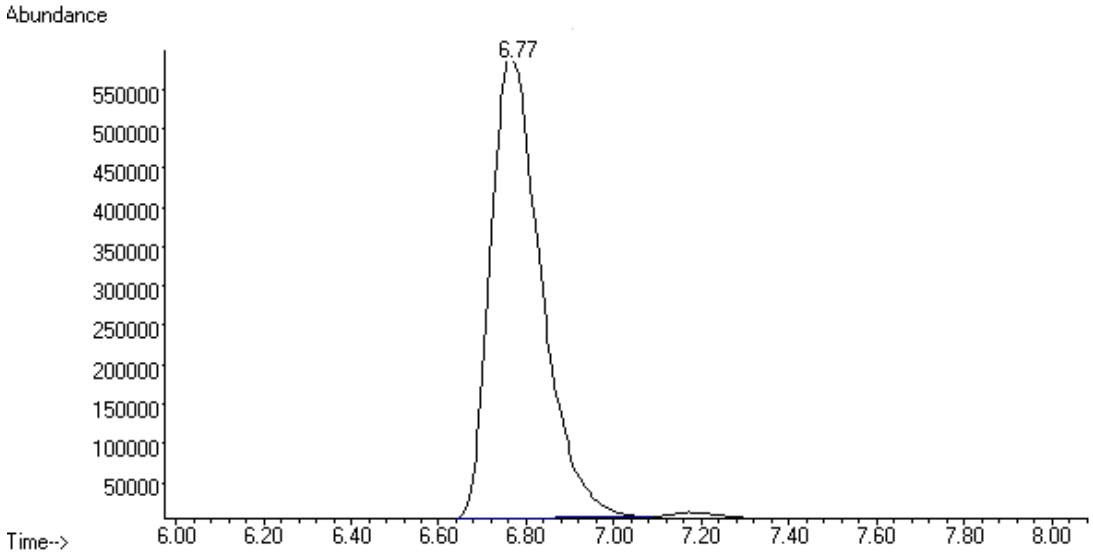


Figure 5-3 Representative GC-MS trace for 10 µg/mL solution of [1-^{13}C]leucine

Samples of diluted stock solution were subjected to derivatisation with MTBSTFA prior to loading onto GC column. Initial oven temperature was 80 °C, increased 10 °/min. *A)* absorbance spectrum from substances eluting between 10.6 and 11.6 minutes. Elution time of leucine is 10.86 min. The highest peak is the absorbance of the 200 *m/z* ion, the middle peak 274 *m/z*, and the smallest peak 302 *m/z* as indicated by spectrum in B. *B)* mass spectrum showing abundant fragments at 200, 274, and 303 *m/z*. 303 *m/z* is the fragment of interest. MTBSTFA, N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide.

A



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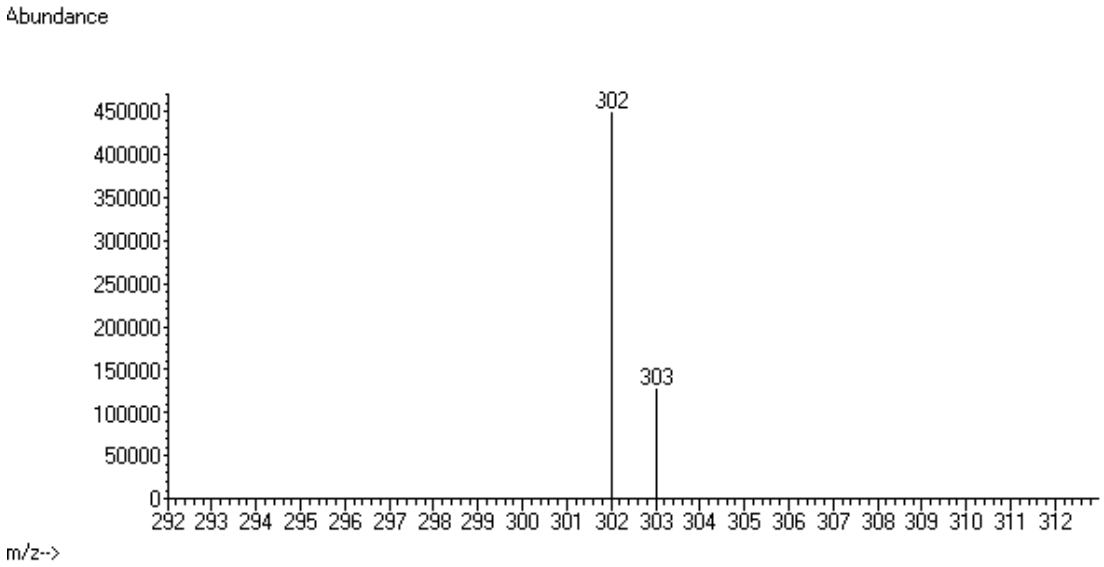


Figure 5-4 Representative GC-MS trace for 40 µg/mL solution of [^{12}C]leucine under new oven conditions

Samples of diluted stock solution were subjected to derivatisation with MTBSTFA prior to loading onto GC column. Initial oven temperature was 120 °C, increased 10 °/min. *A)* absorbance spectrum from substances eluting between 6 and 8 minutes. Elution time of leucine is 6.77 min. *B)* mass spectrum showing results of selected ion monitoring at 302 and 303 *m/z*. MTBSTFA, N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide.

Once the GC-MS parameters were established the whole standard curve was run (as explained in 4.2.3) with the graph of the expected MPE versus M+1 meeting quality control standards (R^2 = 0.9957, m = 1.4008). Once the standard curve was developed the intracellular fluid samples were analysed for tracer to tracee ratio. Samples were reconstituted in 1 mL ice-cold ethanol and then 500 µL of each of five samples was derivatised in the same way as the standards. A

small amount of leucine was detected in one sample and none in any of the others. To determine whether there actually was leucine present in the intracellular fluid, four samples were run through HPLC using pre-column derivatisation with AQC (430) by technical staff at The Liggins Institute (Auckland, NZ). Two of these samples had been reconstituted with ethanol and two were reconstituted with 800 µL 0.04 M H_2SO_4 and 200 µL ddH_2O. An internal standard was added to the latter. Leucine was indeed present but at very low concentrations (Table 5-1), so it was decided to derivatise the whole 1 mL of each intracellular fluid sample to maximise the chances of detection by the GC-MS.

	sample 1	sample 2	sample 3	sample 4
Essential				
Leucine	32	27	13.0	21.2
Valine			16.9	24.6
Isoleucine			5.5	8.9
Phenylalanine			4.8	7.0
Lysine			40.3	72.6
Histidine			18.2	28.6
Threonine			107.3	105.6
Tyrosine			6.8	9.7
Nonessential				
Asparagine			13.2	18.4
Serine			33.5	35.8
Glutamine	350		840.7	1160.4
Arginine			95.8	26.7
Alanine			80.1	139.0
Citrulline			25.3	42.0
Glutamate	300		132.6	183.6
Glycine	200		95.9	135.4
Ornithine			7.1	12.8
Proline			361.7	334.8
Taurine	800	80	518.2	743.8
Aspartate			24.8	37.6
Hydroxyproline			4.2	6.7

Table 5-1 Intracellular fluid amino acid concentration (µmol/L) for four representative samples

Intracellular fluid was isolated from muscle biopsy samples before being subjected to HPLC. Samples 1 and 2 were reconstituted in ethanol and samples 3 and 4 in H_2SO_4 and ddH_2O. Samples 3 and 4 had an internal standard added prior to analysis. Amounts for samples 1 and 2 are estimates based on peak areas compared with a standard curve. Sample 2 was low in every amino acid, with concentrations roughly $1/10^{th}$ those in sample 1.

To further increase the chances of successful detection, it was decided to purify the samples by strong cation exchange . The cation exchange resin (AG 50W . 8 resin, 50-100 mesh) was suspended in ddH_2O and 2.75 mL pipetted into each polystyrene column. The columns were washed with 6 mL 1 M HCl, neutralised with 6 mL ddH_2O, washed with 6 mL 2M NH_4OH, neutralised again with 6 mL ddH_2O and then primed with a further 6 mL 1 M HCl. The reconstituted samples were then added and the columns washed with 3 mL 1 M HCl and then 6 mL ddH_2O. 1 mL 2 M NH_4OH was then added and the eluant discarded before a further 4 mL was added and the eluant collected. The NH_4OH was dried under a stream of nitrogen gas, and the isolated leucine then re-suspended in 500 μL ice-cold ethanol and derivatised and analysed in the same way as the standards.

The decision to collect the second through to the fifth eluted mL from the cation exchange was made based on the elution profile of a standard 2 μg/mL [^{12}C]leucine sample. Each mL of eluant was collected separately and these derivatised and run through GC-MS. The results (Table 5-2) showed that leucine began to elute in the second mL, with 97% of the leucine eluted by the fifth mL. This decision was affirmed by running an intracellular fluid sample through the same protocol and finding the same results.

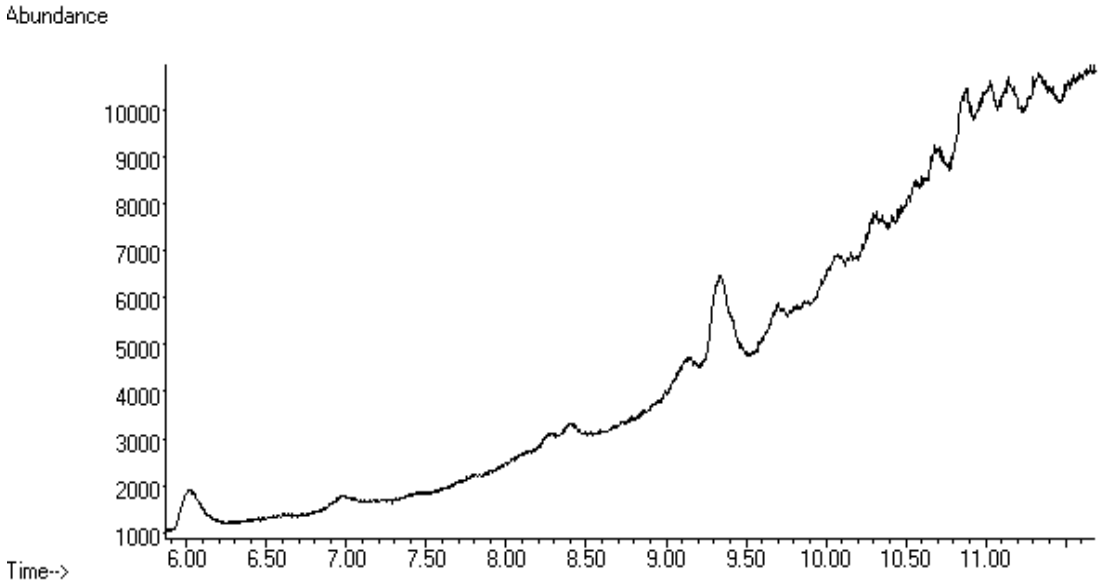
mL	% of total leucine present
1	0
2	66
3	21
4	7
5	2.4

Table 5-2 Percentage of total eluted leucine present in each 1 mL of collected eluant
Eluant collected from strong cation exchange of reconstituted intracellular fluid samples. 97% of all leucine in a practice sample eluted from the column in the first 5 mL.

Once the cation exchange methodology was established, half of the remaining intracellular fluid samples were analysed for tracer to tracee ratio. No useable traces were obtained from the

GC-MS; all were simply noise, even the standards which contained known amounts of leucine (Figure 5-5). As MTBSTFA derivatives are very sensitive to oxygen and water in the air , it was decided that the remaining derivatised samples would be kept under a stream of nitrogen gas until the moment their GC-MS vials were capped as gassing with nitrogen flushes out room air . A further problem was encountered with the injection syringe getting stuck during the sample run. This was corrected by changing the pre-injection wash solvent from ethyl acetate to methanol, the post-injection wash solvent to dichloromethane, and increasing the number of washes from three to six. The remaining samples were run successfully (Figure 5-6).

A



B

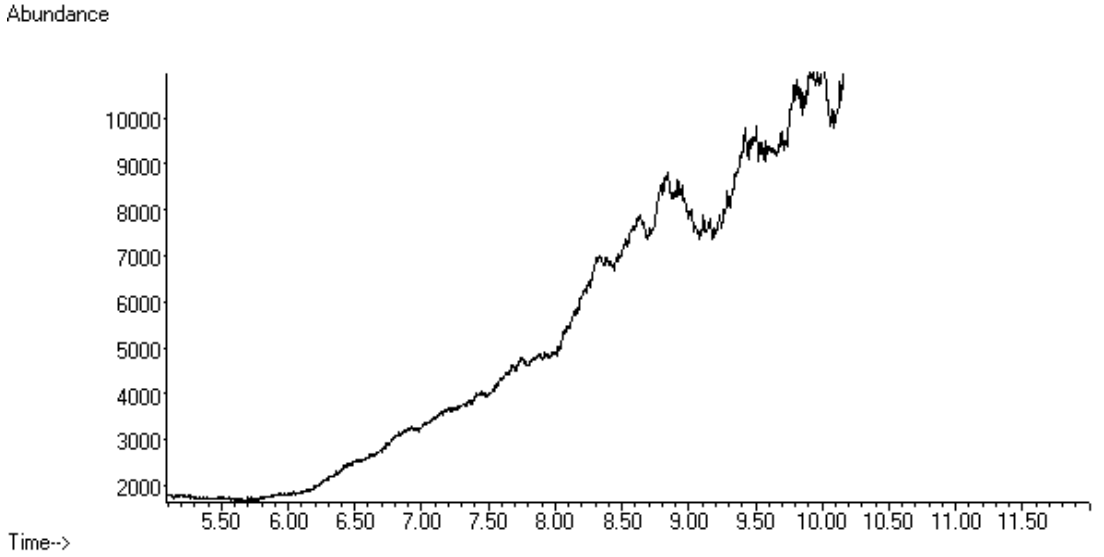
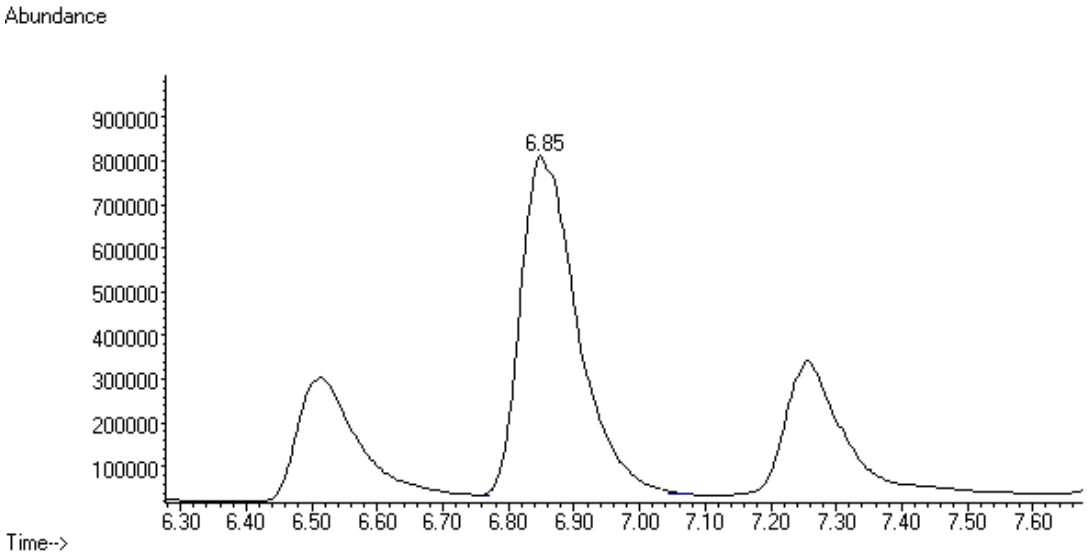


Figure 5-5 Representative GC-MS traces for 5% MPE leucine standard and intracellular fluid sample

Intracellular fluid was isolated from a skeletal muscle biopsy sample and subjected to strong cation exchange. A diluted stock solution of [1-^{13}C]leucine and the intracellular fluid sample were subjects to derivatisation with MTBSTFA before being loaded onto the GC column. *A)* absorbance spectrum for 5% MPE leucine standard from substances eluting between 6.0 and 11.6 minutes showing no discernable peaks that could be analysed. *B)* absorbance spectrum for intracellular fluid sample from substances eluting between 6.0 and 11.6 minutes showing no discernable peaks that could be analysed. MTBSTFA, N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide.

A



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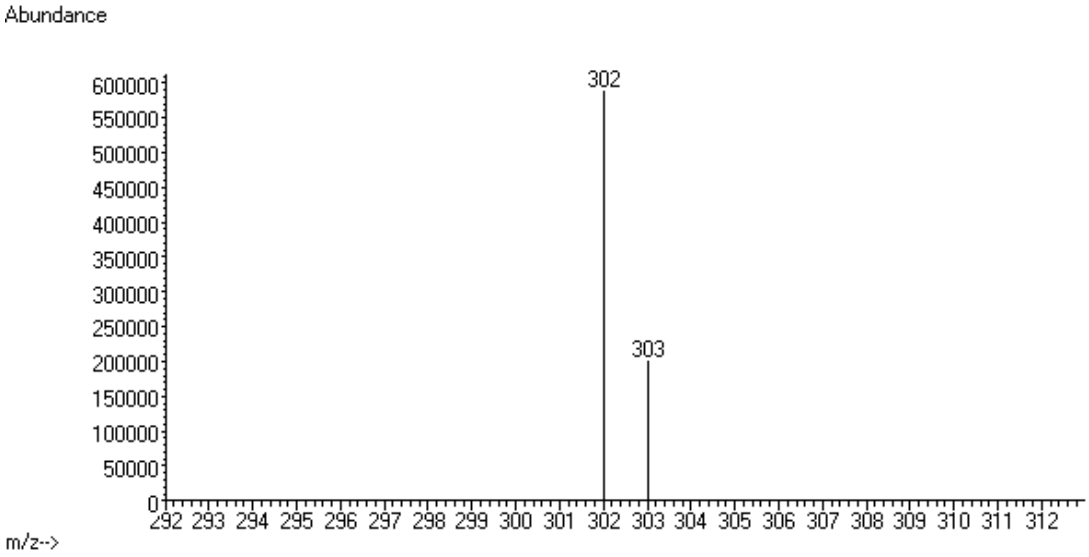


Figure 5-6 Representative GC-MS trace for intracellular fluid sample

Intracellular fluid was isolated from a skeletal muscle biopsy sample and subjected to strong cation exchange, then derivatised with MTBSTFA before being loaded onto the GC column. *A)* absorbance spectrum from substances eluting between 6.3 and 7.6 minutes. Elution time of leucine is 6.85 min. Peak at 6.5 minutes is valine and peak at 7.3 minutes is isoleucine. *B)* mass spectrum showing results of selected ion monitoring at 302 and 303 *m/z*. MTBSTFA, N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide.

5.2.2 Calculations and statistics

FSR was calculated according to the equation;

$$\frac{(E_{Bt2} - E_{Bt1})}{E_{PREt12}*(t_2-t_1)} * 100\%$$

where E_B is the enrichment of the bound protein fraction at times 2 and 1 respectively, E_{PRE} is the enrichment of the precursor pool, and t is the time from the start of the infusion to when the muscle sample was taken, in hours.

All of the assumptions described in Chapter 4 regarding the use of stable isotopes are inherent in performing the above calculation. There are, additionally, assumptions specific to calculations of FSR. The first is that mitochondrial protein is synthesised at a constant rate, thus ensuring a constant rate of incorporation of [^{12}C]leucine . It is realistic to assume such a physiological steady state over the four hours between muscle biopsies as this is a relatively short time frame for muscle protein turnover. Because the difference in enrichment between the two biopsy time points is taken, it is assumed that this difference represents the average change in enrichment over this interval. The second FSR-specific assumption is that amino acids are immediately incorporated into bound protein. In reality there is a time delay of usually unknown duration while free amino acids are bound to tRNA before the tRNAs are assembled into bound proteins. The time taken at the beginning of the experiment while waiting for isotopic equilibrium is generally suitable to account for the time taken in translation

.

Due to the limited number of successfully analysed intracellular fluid samples, it was not possible to use intracellular leucine exclusively for the precursor pool. Therefore the average enrichment from the plasma samples obtained just before (9-20 minutes) each biopsy was used as the value for precursor enrichment to calculate FSR. Where a value for intracellular fluid

enrichment was available, this was used to calculate FSR and a comparison made with the value calculated using the plasma enrichment. Where intracellular fluid was used as the precursor, an average value for the two injections was taken, the enrichment was calculated using the standard curve method, and then an average value was taken of the two enrichments. It was hoped that there might be a standard offset between FSR values calculated using the two different precursor pools, so that estimates could be made for the missing intracellular fluid values, but this was not the case (Figure 5-7).

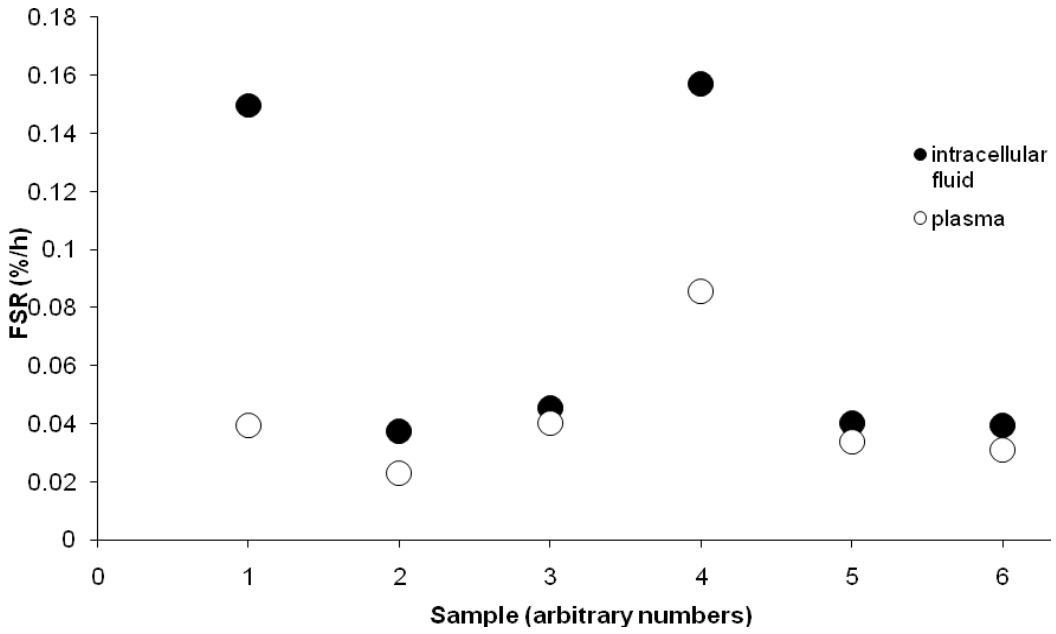


Figure 5-7 Mitochondrial protein FSR values obtained for subjects/conditions, showing offset between values obtained using intracellular fluid as the precursor pool and those obtained using plasma
There is no consistent offset although most tissue fluid values are higher than plasma values.

To determine whether there was a difference between conditions, paired, one-tailed *t*-tests were performed and significance was set at $\alpha < 0.05$.

5.3 Results

The wet weight of each biopsy sample prior to homogenisation is shown in table 5-3.

	CHO		PRO	
subject	sample 1	sample 2	sample 1	sample 2
1	52	85	88	112
2	n/a	n/a	42	119
3	109	57	42	75
4	87	74	92	55
5	93	96	43	88
6	75	123	31	33
7	148	18	63	24
8	n/a	n/a	31	92
9	136	119	152	124
10	84	127	54	109
11	90	46	141	82
12	95	n/a	156	160

Table 5-3 Wet weight (mg) of muscle samples collected in the first study

Subjects cycled at roughly 50% VO_{2max} for one hour before receiving a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* immediately, and four hours after cessation of exercise.

5.3.1 Western analysis confirmed isolation procedures

There was some cross-contamination of the bound mitochondrial protein and intracellular fluid fractions (Figure 5-8). Immunoreactive Na^+/K^+ ATPase-α, a sarcolemmal marker, was detected in both the intracellular fluid and subsarcolemmal mitochondrial fractions, thus indicating the presence of some sarcolemmal contamination of the protein analysed as mitochondria. Prior to hydrolysis, the intracellular fluid would be expected to contain some sarcolemmal and cytosolic proteins but the mitochondrial fraction should not. It is reassuring to note in Figure 5-8 that there is far more immunoreactive Na^+/K^+ ATPase in the intracellular fluid than in the mitochondria. The mitochondrial marker VDAC was abundant in the mitochondrial fraction of both the human and rat muscle samples but also in the intracellular fluid in three of the five human samples tested. Presence of a mitochondrial marker in the intracellular fluid indicates that some subsarcolemmal mitochondria were still attached to the sarcolemma in these samples. Again it is reassuring to note that the presence of immunoreactive VDAC is far greater in the

mitochondrial fraction. LDH, usually used as a cytosolic marker, is also present in mitochondria . LDH was present in all intracellular fluid fractions tested as well as one of the five mitochondrial fractions (Figure 5-8). Due to the cross-contamination noted above it is not possible to determine whether the detected LDH was mitochondrial or cytosolic.

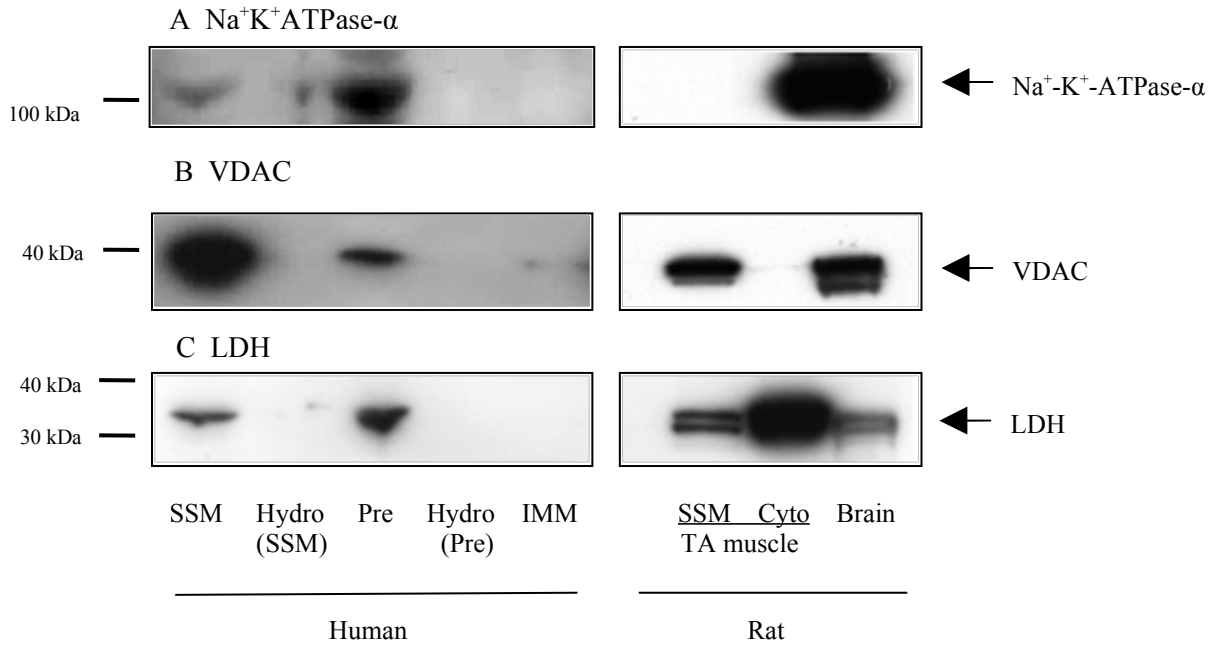


Figure 5-8 Representative Western blots of sarcolemmal, mitochondrial, and cytosolic markers in intracellular fluid and mitochondrial protein fractions

Expressions of *A)* cell membrane Na^+-K^+-ATPase-α (human and rat reactive), *B)* outer mitochondrial membrane marker VDAC, and *C)* inner mitochondrial membrane/cytosolic marker LDH of bound SSM, SSM followed by hydrolysing step (Hydro (SSM)), supernatant (sarcolemma and cytosol) before the hydrolyzing step (Pre), intracellular fluid (Hydro (Pre)) and IMM fractions from human skeletal muscle and of SSM and cytosolic (Cyto) fractions from rat TA muscle and of rat brain are shown. At least 17 µg total protein was loaded onto a 10 - 12% poly-acrylamide gradient gel and subjected to electrophoresis. The gel was transferred to PVDF membrane, probed with relevant primary and secondary antibodies, and exposed to x-ray film. The molecular weight of Na^+-K^+-ATPase-α is 112 kDa, of VDAC is 31 kDa, and of LDH is 35 kDa;. VDAC, voltage-dependent anion channel; LDH, lactate dehydrogenase; SSM, subsarcolemmal mitochondria; IMM, intermyofibrillar mitochondria; TA, *tibialis anterior*.

In the hydrolysing steps all proteins were precipitated so that no bands were detected in either fraction after hydrolysis. The IMM fractions showed no detectable bands, probably due to the lower abundance of this portion of the reticulum. It is likely that the mitochondrial fraction analysed in this study contained both SSM and IMM. The loading control GAPDH was present in roughly equal amounts in both the subsarcolemmal mitochondria and intracellular fluid fractions prior to hydrolysis in four of the five human samples tested (Figure 5-9).

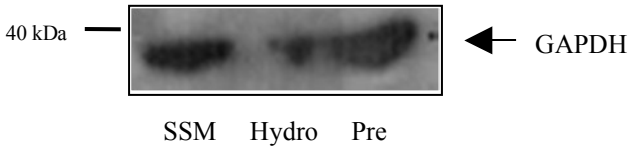


Figure 5-9 Representative Western blots of loading control in intracellular fluid and subsarcolemmal mitochondrial protein fractions

Expression of loading control GAPDH in SSM, SSM followed by hydrolysing step (Hydro (SSM)), and supernatant (sarcolemma and cytosol) before the hydrolyzing step (Pre). At least 17 µg total protein was loaded onto a 10 - 12% poly-acrylamide gradient gel and subjected to electrophoresis. The gel was transferred to PVDF membrane, probed with primary and secondary antibodies, and exposed to x-ray film. The molecular weight of GAPDH is 37 kDa. Blots are of a similar size in all three lanes. SSM, subsarcolemmal mitochondria.

5.3.2 No difference between feeding conditions or sampling times in mitochondrial protein and intracellular fluid enrichment

The [1-^{13}C]leucine enrichment of the intracellular fluid from each sample is shown in Figure 5-10. There was no significant difference in enrichment either between the conditions or from the first to the second sample.

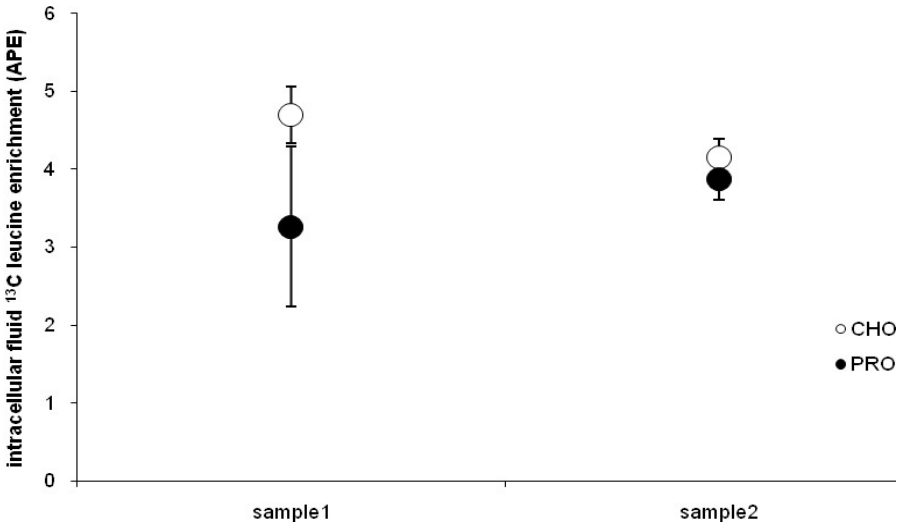


Figure 5-10 Intracellular fluid [1-^{13}C]leucine enrichment for each muscle sample for both conditions

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Intracellular fluid was isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Group means ± standard errors for all subjects with useable data are shown. APE, atom percent excess; CHO, carbohydrate-only; PRO, protein-plus-carbohydrate.

The [1-^{13}C]leucine enrichment of the bound mitochondrial protein from each sample is shown in Figure 5-11. Eight samples were initially "not detected" by Iso-trace; these samples were re-

injected and three remained "not detected" so no data point is available for these. There was no significant difference in enrichment either between the conditions or from the first to the second sample.

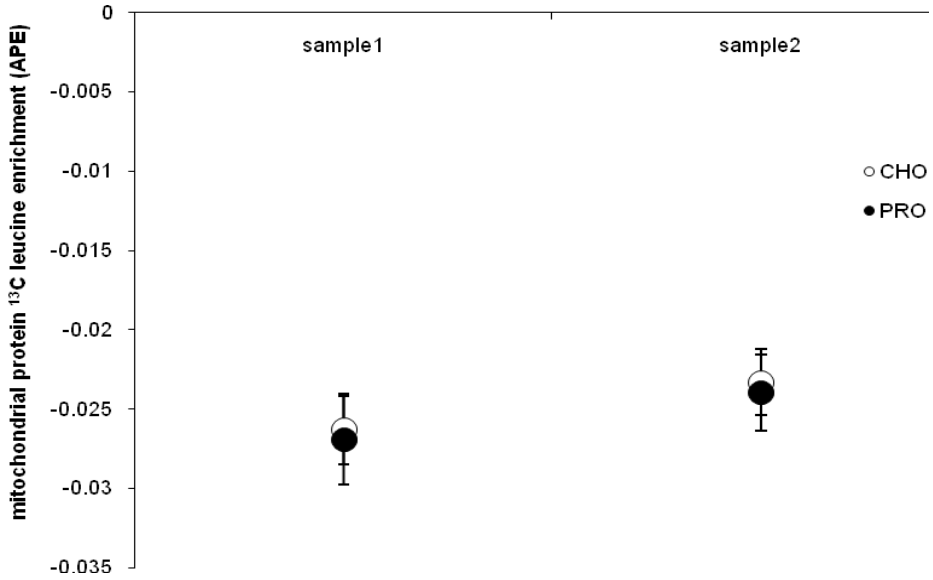


Figure 5-11 Mitochondrial protein [1-^{13}C]leucine enrichment for each muscle sample for both conditions
Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Mitochondrial protein was isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Values for atom percent excess (APE) are the difference between the atom percent of the sample and that of a reference gas determined by GC-C-IRMS. Group means ± standard errors are shown. APE, atom percent excess.

5.3.3 No difference between feeding protein-plus-carbohydrate or carbohydrate-only after a bout of aerobic exercise on the rate of mitochondrial protein synthesis

There was no difference in mitochondrial protein FSR between conditions regardless of whether plasma ($p = 0.3$) or intracellular fluid ($p = 0.2$) was used as the precursor pool (Figures 5-12, 5-13).

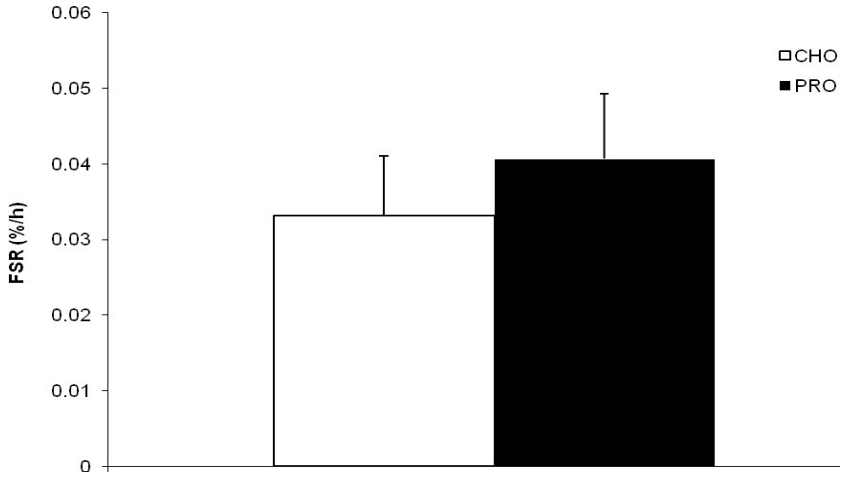


Figure 5-12 Mitochondria protein FSR using plasma as precursor pool

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Plasma was collected from an antecubital vein and mitochondrial protein was isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Mitochondrial protein enrichment was determined as atom percent excess (APE), the difference between the atom percent of the sample and that of a reference gas determined by GC-C-IRMS. There was no significant difference between the conditions. Group means ± standard errors for all subjects returning useable data are shown (n = 5 in CHO, 7 in PRO). FSR, fractional synthesis rate.

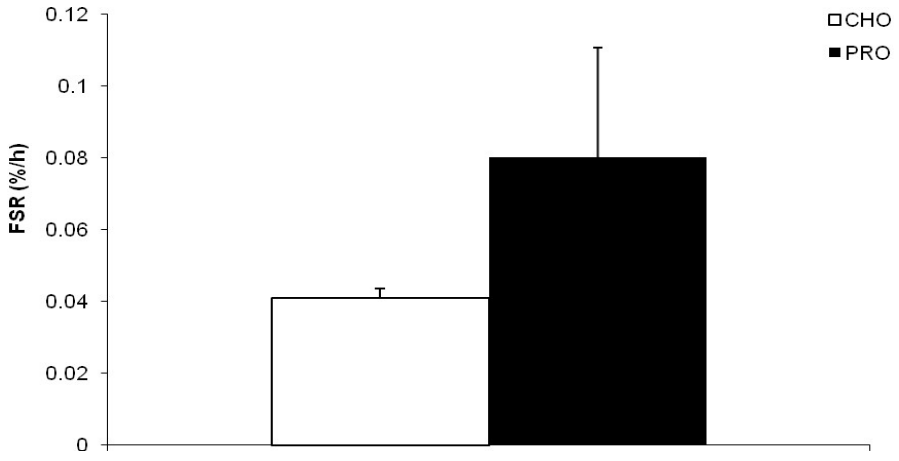


Figure 5-13 Mitochondria protein FSR using intracellular fluid as precursor pool

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Intracellular fluid and mitochondrial protein were isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Mitochondrial protein enrichment was determined as atom percent excess (APE), the difference between the atom percent of the sample and that of a reference gas determined by GC-C-IRMS. There was no significant difference between the conditions. Group means ± standard errors for all subjects returning useable data are shown (n = 3 in CHO, 5 in PRO). FSR, fractional synthesis rate.

The values returned by Iso-trace for bound protein enrichment did not always make physiological sense. Figure 5-14 shows the [1-^{13}C]leucine enrichment of the mitochondria isolated from each muscle sample where data were available for both biopsies on a given testing day. Samples are identified by two numbers. The first number in the series is the subject and the second number is the testing day. In several cases, the enrichment of the second sample was lower than that of the first sample.

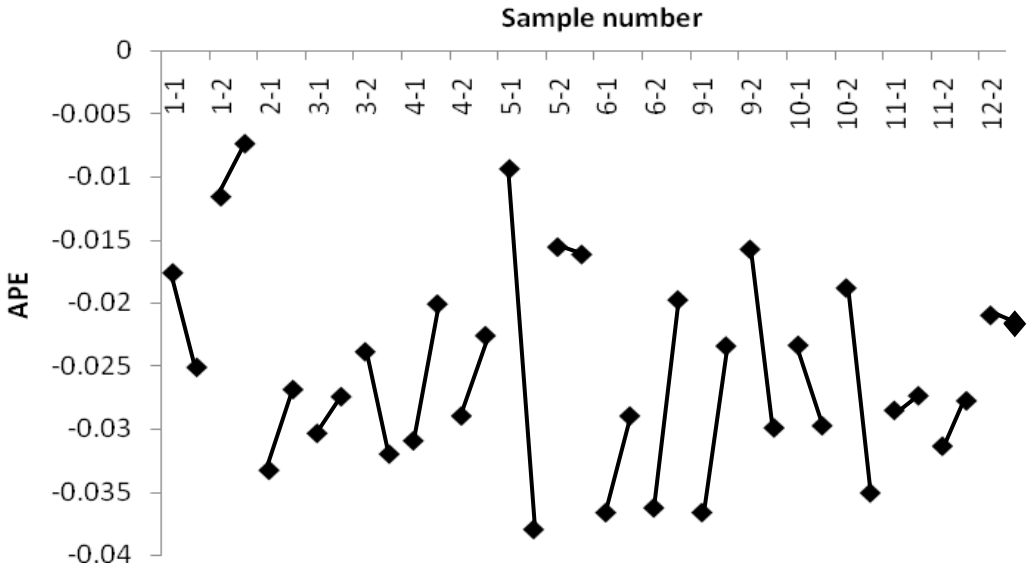


Figure 5-14 ^{13}C enrichment of bound mitochondrial protein (APE) in each muscle sample

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}. Mitochondrial protein was isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Value for atom percent excess (APE) were determined as the difference between the atom percent of the sample and that of a reference gas, by GC-C-IRMS. Samples 1-1, 3-2, 5-1, 5-2, 9-2, 10-1, 10-2, and 12-2 all have a lower enrichment at the second time point than at the first. APE, atom percent excess.

When considering only subjects for whom a complete, sensible data set was obtained (n = 4), that is enrichment values for bound protein were available for both time points and both conditions, and in which enrichment was found to increase over the infusion period, and calculating FSR using plasma as the precursor pool, there was no pattern in the difference between conditions. Two subjects experienced a higher rate of mitochondrial protein FSR in

the PRO condition and two subjects experienced a higher rate in the CHO condition (Figure 5-14).

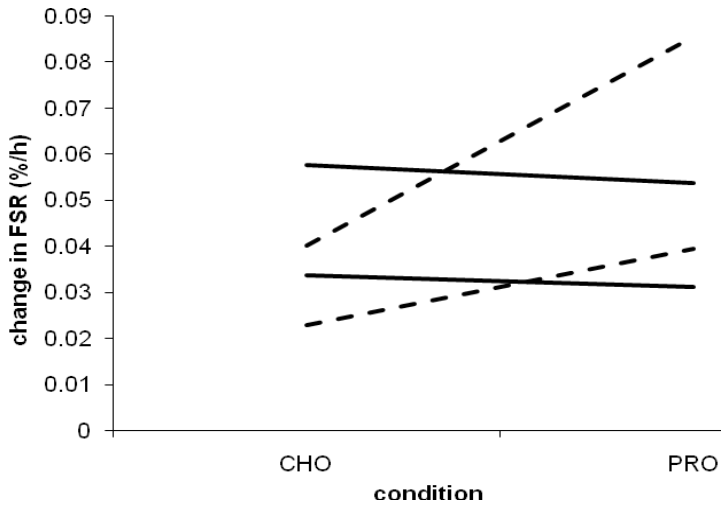


Figure 5-15 Within-subject change in mitochondrial protein FSR from CHO condition to PRO condition using plasma as the precursor pool

Subjects received a primed-continuous infusion of [1-^{13}C]leucine at a dose of 60 mg for the prime and 75 mg/h for the continuous infusion. After two hours of infusion, subjects completed one hour of cycling at roughly 50% VO_{2max}, then received a 240 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Plasma was obtained from an antecubital vein and mitochondrial protein was isolated from muscle biopsy samples obtained immediately, and four hours following the cessation of exercise. Mitochondrial protein enrichment was determined as atom percent excess (APE), the difference between the atom percent of the sample and that of a reference gas determined by GC-C-IRMS. Two subjects experienced a higher mitochondrial protein FSR during post-exercise recovery in the PRO condition than the CHO condition, while two subjects experienced the opposite. FSR, fractional synthesis rate.

The enrichment determined for the sample analysed in triplicate for reliability was 6.66 ± 0.54 with a %CV of 8.05. These values correspond to a calculated value for FSR of 0.062 ± 0.005%/h with a %CV of 7.61. A similar study using resistance exercise and feeding detected a 34% difference in FSR between a carbohydrate-only and a carbohydrate-plus-protein feeding so a roughly 8% variation would not mask the difference we might have expected to detect.

5.4 Discussion

The most interesting finding in this portion of the study is that there is considerable variability in calculated values for FSR depending on whether plasma α-KIC or intracellular leucine is used as the precursor pool (Figure 5-7). In keeping with previous findings , the use of plasma α-KIC resulted in consistently lower values of FSR than the use of intracellular leucine. Furthermore, the variability was itself variable, in that there was not a standard offset between FSR values using one or the other precursor pool. Ljungqvist et al. found that the ratio of tRNA to intracellular leucine enrichment holds constant in the fed state while that of tRNA to plasma α-KIC does not. Similarly, Bennet et al. found that intracellular leucine and plasma α-KIC are not in equilibrium with one another, the former's enrichment increasing to a higher percentage of the latter's during amino acid infusion. As transmembrane transport of amino acids from plasma into intracellular fluid is affected by amino acid transporters, the action of which is influenced by insulin, this variable ratio is hardly surprising . The findings in this study and those of others cited in this paragraph all highlight the importance of sampling the intracellular fluid when studying FSRs.

5.4.1 Plasma α-KIC is a reliable precursor pool for calculations of mitochondrial protein FSR

As plasma α-KIC enrichment does not vary systematically relative to that of tRNA, it seems it cannot be used reliably as the precursor pool. Nevertheless, plasma α-KIC is often chosen in studies of MPS, perhaps because it is produced intracellularly and so reflects the enrichment of the precursor pools for both MPS and oxidation, making it the best plasma surrogate measure. Furthermore, plasma α-KIC is a much easier pool to sample than intracellular fluid leucine. In addition, the work of Baumann et al. suggests that the variability introduced by inter-subject differences and experimental error could outweigh the effect of having to use a less than ideal

precursor pool. The authors found that, regardless of differences in enrichment, there was very little difference in calculated FSRs using these two different precursor pools.

5.4.2 Technical issues rendered some data un-useable

Unfortunately, the poor quality of the data returned by Iso-trace makes it impossible to draw any further conclusions from the data in this portion of the study. If some samples were "not detected" by Iso-trace, then several more were probably at the limit of accurate detection. It is questionable whether even the data used to construct Figure 5-14 have been interpreted appropriately as there may be so much variability in the data set that the existence of this 'sensible' subset is simply based on chance rather than on accurate findings. Iso-trace's use of only single injections does not provide much confidence in the reliability of their numbers. In one instance, a sample was injected once on two separate occasions and the values returned had a %CV of 22 indicating poor repeatability. Similar work by other researchers (Miller et al., unpublished data) suggests that repeated measurements should be within about 0.0002 APE rather than the difference of 0.005 APE reported by Iso-trace for the repeat injection of this sample. The same unpublished data also indicate that baseline levels of bound enrichment tend to be about -0.030 APE, with some slight diet-induced variation considered acceptable . The data in Figure 5-14 show a much larger amount of scatter, with baseline values ranging from -0.009 to -0.037 APE. Furthermore, as already noted, some data pairs show increases and some decreases from baseline. All of the above points indicate a lack of control in the analysis process. Higher than expected baseline enrichment could have occurred via contamination with enriched leucine, while those samples that are lower than expected could possibly have had a non-leucine source of CO_2 introduced. It is also possible that at some stage of the processing the sample labels were accidentally switched. The source of variation could also lie in the isolation protocol in that the isolated mitochondria could have been contaminated with

such cellular components as endoplasmic reticulum and lysosomes. There may also have been some cross-contamination of the samples when the lyophiliser defrosted.

Other methodological issues may have introduced error into the data. The recommended best practice of cleaning muscle biopsy samples of blood using a saline wash prior to freezing was not followed. Furthermore, while visible connective tissue was removed, this was not done under a dissection microscope as suggested by Wagenmakers . Both the above procedures would have resulted in increased accuracy in the determination of intracellular enrichment. Variability may also have been introduced due to the heterogeneous distribution of fibre type in the *m. vastus lateralis* as different fibre types exhibit different protein metabolic responses to exercise .

5.4.3 Aerobic exercise increases mitochondrial protein FSR

Wilkinson et al. measured a 154% increase from rest in the rate of mitochondrial protein synthesis after a 45-minute bout of cycling exercise at 75% VO_{2peak} so it seems likely that the exercise bout used in this study would have had a stimulatory effect on mitochondrial protein FSR. Anthony et al. provided rats with a carbohydrate-only or a carbohydrate-plus-whey protein meal following two hours of exercise at 75% VO_{2max} and found that the mixed muscle protein FSR in the rats receiving the protein-plus-carbohydrate meal was 0.1%/h, or a 43% greater rate than in the rats receiving the carbohydrate-only meal. If the mitochondrial fraction of muscle protein is affected by exercise and nutrition in the same way as all the mixed muscle proteins, then it might have been reasonable to expect a difference of this magnitude. A repeat of this portion of the study with more reliable mass spectrometry instruments and technician help is certainly desirable.

5.4.4 Conclusion

In conclusion, it is possible that an effective nutritional intervention could increase aerobic capacity in older individuals by both maintaining mitochondrial protein content and increasing mitochondrial protein quality through increased rates of turnover. Due to methodological problems, it is not possible to make any conclusions from this portion of the study.

6 Influence of post-aerobic exercise nutrient composition on signalling for skeletal muscle mitochondrial biogenesis

6.1 Introduction

Skeletal muscle plasticity allows for adaptation to stresses imposed by exercise while adequate nutrition allows for a maximal adaptive response to exercise. As revealed in Chapter 4, provision of a protein-plus-carbohydrate drink after a bout of aerobic exercise resulted in a greater increase in the rate of WBPS in older adults than a carbohydrate-only drink. Whether or not this stimulation included mitochondrial proteins was examined but not conclusively determined in Chapter 5. Whilst the higher steady state level of mitochondrial protein that would result from regular aerobic training is not measurable until a period of weeks of training has elapsed, signalling events begin from the first bout of exercise . Therefore, activation of the intracellular signalling pathway potentially leading to synthesis of new mitochondrial protein was examined.

As reviewed in Chapter 2, a bout of exercise induces such changes within muscle cells as calcium influx from the sarcoplasmic reticulum, and a change in the ATP/ADP ratio, the latter being a cause of AMPK phosphorylation and activation . Various upstream events lead to up-regulation of PGC-1α, the master regulator of mitochondrial biogenesis, which affects such downstream targets as the PPARs and TFam. There is a strong correlation between PPARα mRNA expression and the mRNA levels of many of the genes of oxidative metabolism . PPARδ is the predominant isoform in skeletal muscle and its targeted expression has been shown to increase mitochondrial enzyme activity and the rate of mitochondrial biogenesis in transgenic mice . TFam initiates transcription of genes encoded in the mitochondria, one of which is COXI. Other mitochondrial genes, such as COXIV, are encoded in the nucleus. Evidence from isolated animal muscle preparations indicates that stimulation of nuclear and

mitochondrial transcription may occur on different time scales . Increased expression of PGC-1α, PPARα and δ, and TFam mRNA has been previously found in human skeletal muscle within the first six hours of post-exercise recovery, indicating their responsiveness over this time scale . While the sequence of events in this signalling pathway is well established (Figure 2-3), the combined effects of aerobic exercise and nutrition on it have not been studied comprehensively. Furthermore, PGC-1α-induced increases in the mRNA expression of genes encoding mitochondrial protein have yet to be confirmed in human skeletal muscle.

Previous work has focused on the effect of carbohydrate provision before, during, or after a bout of aerobic exercise . It has been suggested that a glycogen-depleted state, such as would be achieved with aerobic exercise in the absence of sufficient carbohydrate availability, increases AMPK signalling, which amplifies the cellular signalling cascade leading to mitochondrial biogenesis . Only one study, using a sustained, high-intensity bout of cycling, has found evidence to support this assertion with carbohydrate deprivation leading to a more prolonged, but not more marked, up-regulation of the pathway .

An increased circulating level of insulin has been shown to increase mRNA expression of genes encoding mitochondrial proteins in resting human skeletal muscle . In Chapter 4 it was shown that ingestion of carbohydrate and/or protein increases circulating insulin. It has been shown that, at rest, the provision of amino acids also increases the rate of mitochondrial biogenesis in human skeletal muscle . It seems likely that an exogenous source of amino acids, the building blocks of all body proteins, would have a positive effect on the formation of new mitochondrial protein. The possibility of insulin or individual amino acids acting as specific signalling molecules to enhance activity in the PGC-1α pathway after a bout of aerobic exercise has not been previously investigated.

Therefore the aim of this part of the second study was to investigate whether feeding protein in combination with carbohydrate immediately after an acute bout of sustained, high-intensity aerobic exercise has an additive effect over carbohydrate alone on signalling for mitochondrial biogenesis in skeletal muscle. The hypothesis tested was that a protein-plus-carbohydrate drink (PRO) would increase the mRNA expression of selected genes in the PGC-1α signalling pathway during the first six hours of post-exercise recovery more than an isocaloric, carbohydrate-only drink (CHO).

6.2 Methods

6.2.1 Subjects

Healthy volunteers aged between 18 and 34 were recruited by printed advertisements (Appendix D1) posted in the Departments of Health and Exercise Science and Nutrition, and the Student Recreation Centre on the Colorado State University Campus. Eligibility required non-smokers with freedom from hypertension, obesity, and cancer, and from cardiac, kidney, pulmonary, and autoimmune diseases. Those on any metabolically active medication were also excluded. Six women and ten men met these criteria; one woman withdrew due to lack of time before testing started. During the course of the study one man dropped out of his own accord, one was excluded as his VO_{2max} was too high, and two women withdrew due to adverse reactions to the biopsy. Participants were informed of the experimental procedures both verbally and in writing, and provided written, informed consent before any tests were performed (Appendix D3). The study complied with the principles of the Declaration of Helsinki and received approval from Colorado State University's Office of Regulatory Compliance (07-039H) and the University of Auckland Human Participants Ethics Committee (2007/173).

6.2.2 Study design

6.2.2.1 Screening and preliminary testing

At initial contact, subjects were asked a series of screening questions to determine their potential eligibility for the study (Appendix D4). Those meeting these initial criteria were asked to complete a medical and exercise questionnaire (Appendix D4). Subjects who had no more than one risk factor for coronary artery disease, who did not meet any other exclusion criteria as described in 6.2.1, and who were deemed recreationally active returned for further testing. Recreationally active was defined as participating in more than two dedicated aerobic exercise sessions per week but not participating in a regular training programme. This last condition was imposed because it was expected that people with an average level of fitness rather than an exceptional one, would show the most robust response, while those with very poor fitness would not respond in a consistent manner .

The participants' height and weight were measured to determine BMI, and body fat percentage was determined by dual-energy x-ray absorptiometry (DEXA) scanning (DPX-IQ Imaging Densitometer using DPX-IQ X-Ray Bone Densitometer with Smart Scan™ software version 4.7e, Lunar Corp., Madison WI, USA, Appendix B11). VO_{2max} was determined during an incremental exercise test on an electrically-braked cycle ergometer (Excalibur with Workload Programmer v1.52, Lode, Groningen, Netherland, Appendix B3). Direct gas analysis was performed throughout the protocol in order to measure VO_2 (TrueOne 2400, Parvo Medics, Sandy UT, USA). Men began the test at a power output of 150 Watts and power output was increased using a ramp function at a rate of 30 Watts every two minutes. Women began the test at a power output of 100 Watts which was increased at a rate of 25 Watts every two minutes. Subjects cycled until volitional fatigue and the maximum values for oxygen consumption and power output (W_{max}) were recorded. One male subject with a VO_{2max} higher than 55mL/kg/min was excluded as being too fit.

Included subjects returned overnight-fasted at least three days later to provide a resting muscle sample. Samples were obtained by percutaneous needle biopsy as described in 5.2. For the two days prior to this sample being taken, subjects maintained a food and exercise diary (Appendix D4). They were asked to maintain normal eating and exercise habits during those two days, but to refrain from any strenuous aerobic exercise for the 24 hours prior to providing the biopsy sample.

6.2.2.2 Testing procedure

Participants were studied after two separate acute bouts of exercise, each exercise test session being separated by a minimum period of one week. Female subjects were tested in the early follicular stage of their menstrual cycle, defined as the first ten days taken from the first day of menses . Participants reported to the laboratory overnight-fasted and the order of the tests was randomly determined. Each test session consisted of one hour of steady state exercise at 75% VO_{2max} (a moderately high intensity) preceded, and followed, by rest periods during which subjects reclined on a bed or sat at a desk (Figure 6-1). In one session the subjects received the PRO drink PRO immediately after their exercise bout, while in the other they received the isocaloric CHO drink.

Subjects used their food and exercise diary from before the resting muscle biopsy to replicate their intake and expenditure for the two days prior to each testing day. They completed a new diary each time to ensure compliance with this requirement. Review of the diaries indicated that subjects complied well with this request. Where specific foods were not available for the two days preceding the second day, subjects chose replacement foods with very similar macronutrient composition. Upon arrival at the laboratory subjects were given a standardised breakfast of cornflakes, 2% milk, and apple juice (7.7 KCal/kg FFM). The exercise bout started 90 minutes after the breakfast was finished. Muscle samples were obtained by

percutaneous needle biopsy three and six hours following cessation of exercise, with the same randomisation protocol as specified in 5.2. Muscle samples were divided into two portions and stored at -85 °C until analysis.

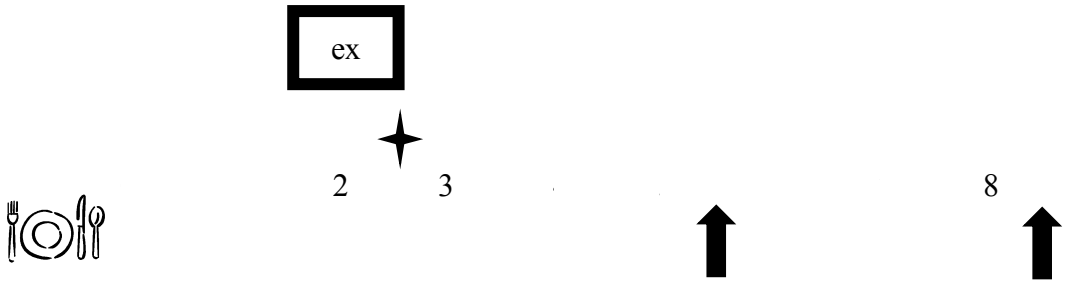


Figure 6-1 Schematic representation of the experimental protocol for the second study

Time is in hours from the time subjects finished eating breakfast ( ). The drink ( ) was given immediately following the exercise bout (ex). Biopsies ( ) were taken three and six hours later.

6.2.2.3 Drinks

Subjects received either 75 g carbohydrate (maltodextrin, CHO) or 55 g carbohydrate and 20 g milk protein (whey and casein, PRO). Drink powders were designed by a sports supplement company (The Gatorade Company, Richardson TX, USA) and 77 g of each powder was mixed in 400 mL water to provide the post-exercise drink. The energy content of the drink powders was 17.2 kJ/g for both the CHO and PRO drinks, indicating that the drinks were isocaloric.

6.2.3 Muscle processing and analysis

6.2.3.1 RNA extraction

Total RNA was extracted from half of each muscle sample by homogenising using a hand-held electric homogeniser (Kema Keur Pro 200, Pro Scientific, Oxford CT) in 1 mL TRIzol reagent (Invitrogen New Zealand Limited, Auckland, NZ) for 2 x 45 seconds. The homogeniser probe was rinsed sequentially in diethyl pyrocarbonate (DEPC)-treated water, RNase ZAP, and 75% ethanol before and after each homogenisation. When not in use the probe was soaked in a 1%

SDS solution. The RNA extraction process then proceeded according to the TRIzol manufacturer's instructions with some modifications (Figure 6-2). The variability in the first incubation time, to allow nucleoprotein complex dissociation, was to allow for more than one sample to be homogenised in a given run. Variation in incubation time had no effect on RNA quality (data not shown). The absorbance of the re-suspended pellet was determined at 260 and 280 nm (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington DE, USA) to ascertain RNA quality and quantity. An absorbance ratio of 1.9-2.0 was considered acceptable . In instances where the extracted RNA was of poor quality or insufficient quantity for RT (2.5 μg required), the second half of the sample was homogenised and the RNA extracted. Extracted RNA was stored at -85 °C until further analysis.

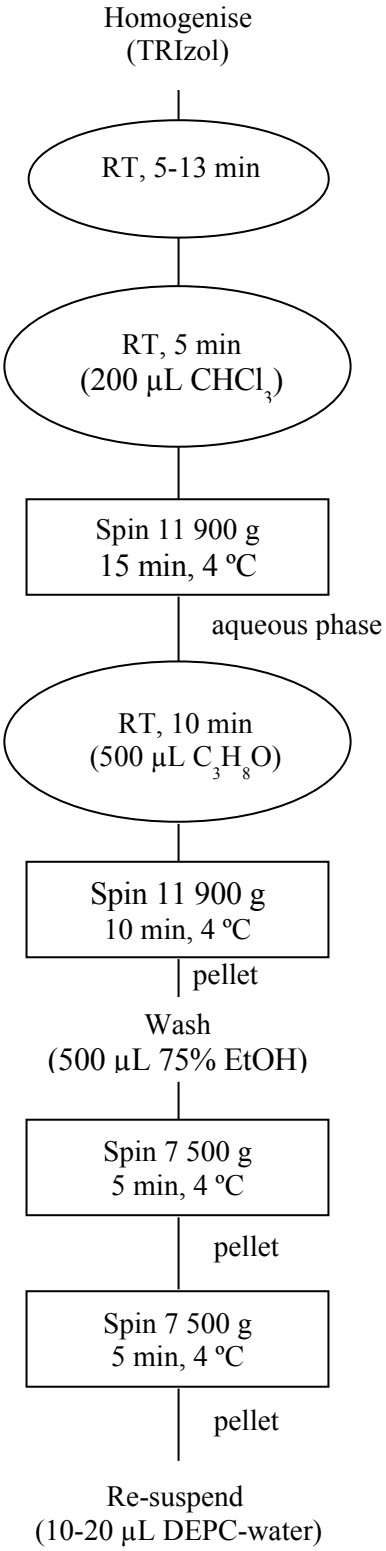


Figure 6-2 Steps taken in extraction of RNA

RNA was extracted from muscle biopsy samples using TRIzol according to the manufacturer's instructions with some modifications (see text for further detail). RT, room temperature; DEPC, diethyl pyrocarbonate; min, minutes.

6.2.3.2 Reverse transcription-quantitative polymerase chain reaction

RT-qPCR began with DNase treatment of extracted RNA to digest any DNA remaining in the samples. The RNA was then reverse transcribed to synthesise cDNA. The resulting cDNA was then amplified using real time qPCR to quantitate the amount of mRNA present in the original sample. Each step in this process is explained in more detail below and in Appendix B12.

DNase from two different manufacturers (Invitrogen, Auckland, NZ and Applied Biosystems, Foster City CA, USA) was trialled to determine which provided the best results in subsequent amplification of cDNA and which best minimised experiment time. Tubes were prepared for RT as described in Table 6-1. All tubes contained RNA extracted from the same muscle sample. All seven tubes were analysed in triplicate for expression of COXI, and the housekeeping gene GAPDH. The threshold cycle (C_T) values for the first six tubes were all quite similar (29.5 ± 0.3), while that for the seventh tube was a clear outlier at 16.25. As there were no differences between the first six tubes, it was decided to use the less costly Applied Biosystems product in a 1 : 1.2 ratio of DNase : DNase buffer mix with the shorter incubation time.

tube	product	DNase : DNase buffer	incubation
1	Invitrogen	1 : 1	25 ºC, 15 min
2	Invitrogen	1 : 1	37 ºC, 30 min
3	Applied Biosystems	1 : 1.2	25 ºC, 15 min
4	Applied Biosystems	1 : 1.2	37 ºC, 30 min
5	Applied Biosystems	0.5 : 1.2	25 ºC, 15 min
6	Applied Biosystems	0.5 : 1.2	37 ºC, 30 min
7	Applied Biosystems	n/a	37 ºC, 30 min

Table 6-1 Methodology for comparison of Invitrogen and Applied Biosystems DNase products
The two products were used in a variety of combinations of reagents and incubation conditions. The seventh tube was prepared by adding 2.5 µL DNase to the aqueous phase during RNA extraction (see 6.2.3.1). All tubes contained RNA extracted from the same muscle sample. min, minutes; n/a, not applicable.

To digest any remaining DNA in the samples, 2.5 µg re-suspended RNA (or all extracted RNA in cases where less than 2.5 µg was extracted – see Appendix G1) was incubated with 2.2 µL DNase/buffer (Applied Biosystems, Foster City CA, USA) at 25 °C for 15 minutes. 3 µL 25 mM EDTA was then added and samples incubated at 65 °C for ten minutes to inactivate the DNase and stop RNA hydrolysis. See Appendix B12 for further detail.

Extracted RNA was reverse transcribed to synthesise first-strand cDNA using the SuperScript III RNase H-system (Invitrogen, Auckland, NZ) according to the manufacturer's instructions but at half volumes (Table 6-2). For use in PCR, the resulting cDNA was diluted with DEPC-treated water to a concentration of 1.45 ng/µL. Negative controls were prepared by diluting DNase-treated RNA samples with DEPC-treated water at a nearly eight-fold greater concentration to maximise the chance of detecting any remaining contaminating genomic DNA. See Appendix B12 for further detail.

	tube contents	incubation
denature RNA	2 µL 1 : 1 : 1 : 1 mix (10 mM dNTPs, 50 µM oligo(dT)$_{20}$, 50 ng/µL random hexamers, DEPC-water) + 3 µL DNase-treated RNA	25 ºC, 50 min
synthesise cDNA	as above + 5 µL 2:4:2:1:1 mix (10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U/µL RNase OUT, 200 U/µL SuperScript III)	65 ºC, 5 min
stop reaction	as above	85 ºC, 5 min
remove RNA	as above + 0.5 µL 2 U/µL RNase H	37 ºC, 20 min

Table 6-2 Steps taken in reverse transcription of extracted RNA

dNTPs, deoxyribonucleotide triphosphate; oligo(dT)$_{20}$, oligonucleotide; DEPC, diethyl pyrocarbonate; min, minutes.

Taqman primer/probe mixes for PGC-1α, TFam, PPARα, PPARδ, COXI, COXIV, β2m (beta 2-microglobulin), and GAPDH were purchased from Applied Biosystems (Foster City CA, USA, Appendix C2). β2m and GAPDH were used as housekeeping genes based on findings from other groups indicating that the mRNA expression of these two genes is minimally affected by an acute bout of aerobic exercise . Products were chosen based on their primer sequences being the same as those used successfully by other groups using both *Taqman* and

SYBR technologies , their having melting temperatures around 60 ºC and 30-80% cytosine and/ or guanine content, and that they spanned an exon-exon junction so as not to detect genomic DNA. Forward primer sequences are listed in Table 6-3. All primer/probe mixes were supplied by the manufacturer at concentrations of 18 μM for the unlabelled primers and 5 μM for the minor groove binding (MGB) probes, and FAM™ dye-labelled.

Gene	Forward primer sequence
PGC-1α	CACTTACAAGCCAAACCAACAACTT
TFam	ATTCACCGCAGGAAAAGCTGAAGAC
PPARα	GCTCGGTCATCACGGACACGCTTTC
PPARδ	TTCTGTGTGGAGACCGGCCAGGCCT
COXI	CCAGTCCTAGCTGCTGGCATCACTA
COXIV	GGCGGGCAGTGGCGGCAGAATGTTG
β2m	AGTGGGATCGAGACATGTAAGCAGC
GAPDH	TTGGGCGCCTGGTCACCAGGGCTGC

Table 6-3 Forward primer sequences of primer/probe mixes used in polymerase chain reaction
PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; TFam, mitochondrial transcription factor A; PPAR, peroxisome proliferation-activated receptor; COX, cytochrome c oxidase; β2m, beta 2-microglobulin.

PCR reaction mix for each plate was prepared in master tubes for each gene according to the following recipe: 5.628 μL DEPC-treated water, 0.720 μL 50 mM $MgCl_2$, 1.200 μL 10X PCR buffer minus Mg (Invitrogen New Zealand Limited, Auckland, NZ), 0.240 μL 10 mM dNTPs (Roche, Penzberg, Germany), 0.240 μL 6-carboxyl-X-rhodamine (ROX) passive reference dye (Invitrogen New Zealand Limited, Auckland, NZ), 0.072 μL Platinum *Taq* DNA Polymerase (Invitrogen New Zealand Limited, Auckland, NZ) and 0.600 μL 20X primer/probe mix per sample. The quantity required for all plate wells was calculated, and then amounts rounded to two decimal places for preparing the mix. Plates were prepared according to Table 6-4. All samples for two subjects were analysed for mRNA expression of all six genes on a single day. Plates were set up with triplicates adjacent to one another and a row of non-template controls (NTC) separating the two subjects' samples. Negative controls were analysed in duplicate on a

second plate in the same run. Once prepared, the PCR plates were spun at 1000 g for two minutes to ensure mixing of well contents (Sigma 2-5, Quantum Scientific, Germany). Plates were incubated at 50 °C for two minutes and 95 °C for two minutes to denature the cDNA and activate the Platinum *Taq*. 45 cycles of real-time PCR amplification were performed (7900HT, Applied Biosystems, Victoria, Australia) with the denaturing step set at 95 °C for 15 seconds, and the annealing/extension step at 60°C for one minute. See Appendix B13 for further detail.

	well contents	number of replicates
sample	reaction mix + 3.3 µL cDNA	triplicate
negative control	reaction mix + DNase-treated RNA	duplicate
non-template control	reaction mix + DEPC-water	triplicate

Table 6-4 Contents of wells for PCR analysis
DEPC, diethyl pyrocarbonate.

After the first plate was run, two samples were chosen which provided a range of C_T values. The samples selected were both from subject six; one was the resting sample and the other was the three-hours-post-exercise sample from the CHO condition. These samples were then run in triplicate for each gene in each subsequently analysed plate to assess the day-to-day variation in plate runs. According to the manufacturer's instructions, *Taqman* probes can reliably detect a two-fold change in relative gene expression. The results from these two common samples run in each plate indicate that the day-to-day variation in measured values normalised to one subject was mostly less than two-fold, and therefore within the acceptable limits of accuracy for the reagents used in this study (Table 6-10).

	PGC-1α		TFam		PPARα		PPARδ		COXI		COXIV	
Plate	1	2	1	2	1	2	1	2	1	2	1	2
1	0.59	0.63	0.63	0.64	2.36	2.17	0.73	0.80	0.76	0.97	n/a	n/a
2	0.55	0.60	0.65	0.66	2.14	1.96	0.73	0.82	1.05	1.27	1.13	0.81
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
4	0.55	0.68	0.73	0.67	1.57	1.60	0.64	0.93	0.91	1.30	0.90	0.95
5	0.45	0.49	0.49	0.44	1.92	1.91	0.71	0.92	0.87	1.02	1.28	1.12

Table 6-5 Relative gene expression for each of two samples run on each plate

Extracted RNA from two skeletal muscle biopsy samples was subjected to RT-qPCR to determine relative expression. These two samples were loaded onto a plate in each day's run. Values are normalised to that for the first plate. PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; PPAR, peroxisome proliferation-activated receptor; TFam, mitochondrial transcription factor A; COX, cytochrome c oxidase.

6.2.4 Calculations and statistics

C_T values were obtained using Applied Biosystems' Sequence Detection System software (version 2.2.2). Thresholds and background amplification were set as described in Table 6-5. The threshold value is set at approximately halfway within the exponential phase of the amplification curve. The amplification cycle at which the curve crosses the threshold is the C_T. The background cycles are those in which amplification has not yet started and are not considered in analysis (Appendix F3). Where one of three triplicate values varied from the other three by more than half a cycle, that value was omitted. Where the variation between all three triplicate values was greater than half a cycle the sample was rerun.

Gene	Threshold	Background (cycles)
PGC-1α	0.3	3 - 15
TFam	0.2	3 - 18
PPARα	0.1	3 - 17
PPARδ	0.2	3 - 18
COXI	0.2	3 - 7
COXIV	0.25	3 - 16
β2m	0.2	3 - 15
GAPDH	0.15	3 - 11

Table 6-6 Thresholds and background amplification for each gene

PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; TFam, mitochondrial transcription factor A; PPAR, peroxisome proliferation-activated receptor; COX, cytochrome c oxidase; β2m, beta 2-microglobulin.

Each raw C_T was normalised to the expression of the two housekeeping genes (β2m, GAPDH) for that sample, by subtracting the mean of the housekeeping genes' expression from the raw C_T value. The quantity of mRNA was determined according to the equation;

$$\text{quantity} = 2^{-\text{mean normalised Ct}}$$

The mRNA quantity for each of the post-exercise samples was then divided by the value obtained for the resting sample, in order to determine relative gene expression for each individual. Within-subject changes were then averaged to provide group means. The amplification efficiency for all primer/probe mixes provided by Applied Biosystems was 2.0.

To determine whether there was a difference between conditions, and between baseline and later time points, paired, one-tailed *t*-tests were performed and significance was set at $\alpha < 0.05$.

6.3 Results

6.3.1 Physiological characteristics

Subjects' physiological characteristics are shown in Table 6-6.

	Men	Women
Age (y)	25 ± 2	28 ± 2
Height (cm)	183 ± 8	172 ± 8
Weight (kg)	76.3 ± 4.2	64.3 ± 9.0
BMI ($kg/m^{2)}$	22.7 ± 1.3	21.7 ± 1.2
VO_{2max} (mL/kg/min)	47.6 ± 3.1	45.4 ± 2.3
Wmax	293 ± 35	242 ± 38
BF (%)	13 ± 4	22 ± 2
HR_{rest}	56 ± 12	67 ± 12
HR_{max}	180 ± 9	183 ± 7

Table 6-7 Subjects' physiological characteristics

Eight men and three women were studied. BMI, body mass index; VO_2, rate of oxygen uptake; W, power output; BF, body fat; HR, heart rate. Group means ± standard deviations are shown.

The wet weight of four representative subjects' biopsy samples prior to homogenisation are shown in Table 6-7.

		CHO		PRO	
subject	baseline	sample 1	sample 2	sample 1	sample 2
2	77	55	60	73	125
3	74	130	89	101	74
4	90	94	67	50	74
9	131	123	170	64	35

Table 6-8 Wet weight (mg) of muscle samples obtained in the second study

Subjects cycled at roughly 75% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and three and six hours after cessation of exercise.

6.3.2 Exercise parameters

Subjects exercised at the same relative intensity during both trials. Men cycled at 78.0 ± 1.5% of VO_{2max} and women at 75.6 ± 0.7% of VO_{2max}. Maintaining this constant VO_2 required a reduction in power output over the course of the trial as shown in table 6-8, and caused an

increase in heart rate as shown in table 6-9. Heart rate increased to roughly 90% of maximum by the end of each exercise bout with no significant difference between the conditions.

exercise period:	1	2	3	4
Men	211 ± 14	196 ± 16	185 ± 16	184 ± 17
Women	168 ± 9	168 ± 15	165 ± 18	152 ± 16

Table 6-9 Power output (W) during each 15 minute period of the exercise bout in the second study for men and women for both conditions

Subjects cycled at roughly 75% VO_{2max} for one hour. Power output was decreased over the course of the exercise bout in order to maintain a constant VO_2. Group means ± standard errors are shown.

condition:	CHO	PRO
Absolute (bpm)	173 ± 6	160 ± 5
Relative (%)	93 ± 1	88 ± 2

Table 6-10 Heart rate during exercise bout in the second study in men and women for both conditions

Subjects cycled at roughly 75% VO_{2max} for one hour. Heart rate increased to roughly 90% of maximum by the end of each exercise bout. Relative value is expressed as a percentage of HR_{max}. Group means ± standard errors are shown.

6.3.3 mRNA expression

It is helpful at this point to recall the sequence of signalling in the PGC-1α pathway (Figure 6-3). The results in this section are organised according to the sequence of activation downstream of PGC-1α in this pathway.

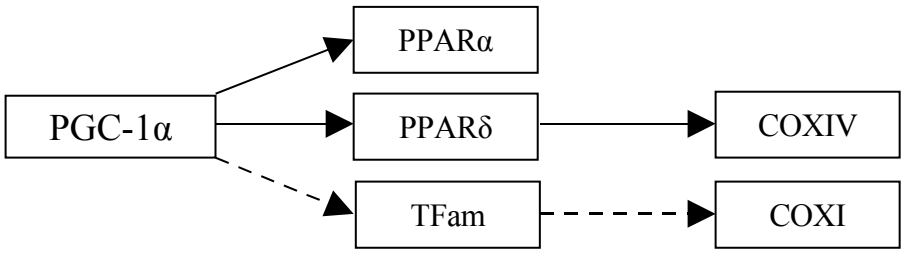


Figure 6-3 Portion of signalling cascade resulting in increased concentrations of mitochondrial protein examined in the second study

See Figure 2-3 for full sequence. → leads directly to the activation/up-regulation of; –▶ leads indirectly to the activation/up-regulation of; PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; PPAR, peroxisome proliferation-activated receptor; TFam, mitochondrial transcription factor A; COX, cytochrome c oxidase.

Prior to experimental intervention, there was considerable variability in subjects' mRNA expression of all six genes tested (Figure 6-4). Most of the higher values for baseline expression were from muscle samples obtained from female subjects.

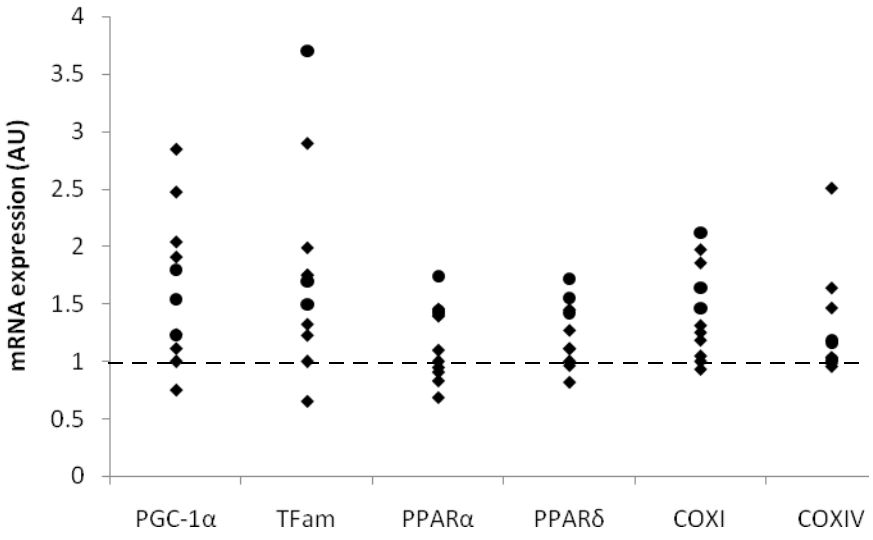


Figure 6-4 Inter-subject variability in relative gene expression in the baseline condition

RNA was extracted from biopsy samples of subjects' *m. vastus lateralis* in the rested and fasted state. RT-qPCR was performed to determine relative expression. Values are normalised to subject one. PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; TFam, mitochondrial transcription factor A; PPAR, peroxisome proliferation-activated receptor; COX, cytochrome c oxidase; AU, arbitrary units; circles represent female subjects; diamonds represent male subjects.

6.3.3.1 Aerobic exercise and nutrition causes an increase in PGC-1α mRNA expression

There was an increase in PGC-1α mRNA expression at both post-exercise time points in both the CHO and PRO conditions (Figure 6-5A, $p < 0.001$). At three hours post-exercise, relative PGC-1α mRNA expression was 9.33 ± 1.09 and 7.72 ± 1.10 times higher than before the exercise bout in the CHO and PRO conditions respectively. At six hours post-exercise, relative expression remained elevated 4.89 ± 0.71 and 4.39 ± 0.66 times higher than before the exercise bout in the CHO and PRO conditions respectively. This was a significant decrease from the three hour time point ($p < 0.001$). There was no significant difference in relative gene expression between the PRO and CHO conditions at either three or six hours after exercise using a one-tailed *t*-test (Figure 6-5A, $p = 0.1$). Interestingly, in eight of the eleven subjects PGC-1α mRNA expression was higher in the CHO than the PRO condition at three hours post-exercise. A two-tailed, non-parametric calculation indicated that this trend came very close to significance (Figure 6-5B, $p = 0.06$).

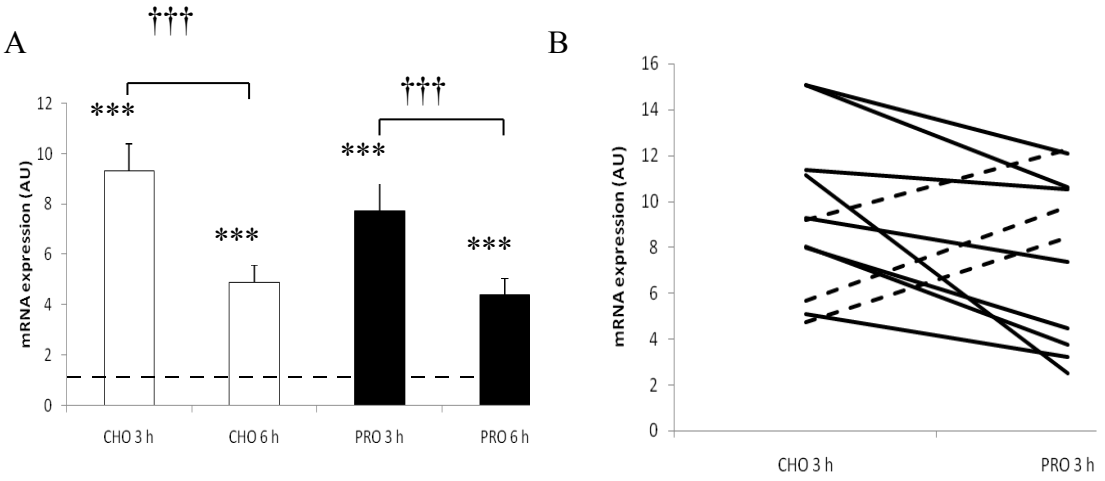


Figure 6-5 Changes in mRNA expression for PGC-1α

A) Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. The combination of a bout of aerobic exercise and post-exercise nutrition caused an increase in gene expression for PGC-1α at both time points in both conditions. *Error bars* SE. *** different from baseline, $p < 0.001$; ††† difference between time points, $p < 0.001$. *B)* Individuals' expression at three hours post-exercise in the CHO and PRO conditions. Solid lines indicate expression was higher in CHO condition; dashed lines indicate expression was higher in the PRO condition. In eight of the eleven subjects expression was higher in the CHO condition at three hours post-exercise. AU, arbitrary units; PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha.

6.3.3.2 No effect of aerobic exercise and nutrition on TFam mRNA expression

There was no effect of a bout of aerobic exercise and post-exercise nutrition on gene expression for TFam (Figure 6-6). There was no difference in relative gene expression between the PRO and CHO conditions at either three or six hours after exercise.

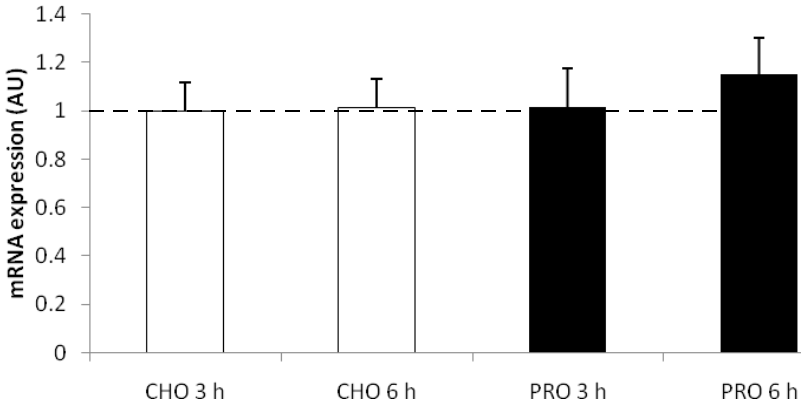


Figure 6-6 Changes in mRNA expression for TFam

Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and at three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. There were no significant changes from baseline or between conditions. *Error bars* SE. TFam, mitochondrial transcription factor A; AU, arbitrary units.

6.3.3.3 Increase in PPARα transcript with nutrition after aerobic exercise

There was no effect of a bout of aerobic exercise and post-exercise nutrition on gene expression for PPARα from baseline to three hours post-exercise (Figure 6-7). However, in both the PRO and CHO conditions there was a significant increase in expression from three to six hours post-exercise ($p < 0.05$). There was no difference in relative gene expression between the PRO and CHO conditions at either three or six hours after exercise.

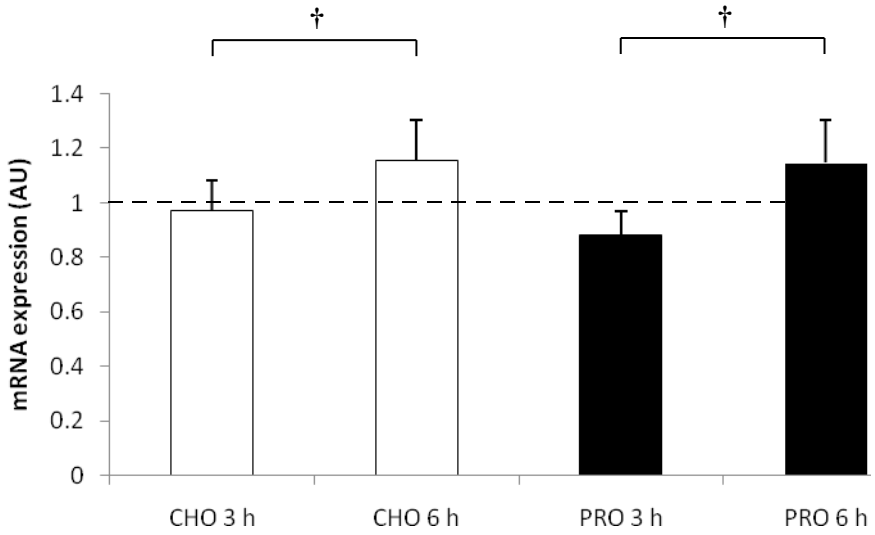


Figure 6-7 Changes in mRNA expression for PPARα

Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and at three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. In the PRO and CHO condition there was a significant increase in expression from three to six hours post-exercise. There were no differences between the conditions. *Error bars* SE. † difference between time points, $p < 0.05$; PPARα, peroxisome proliferation-activated receptor alpha; AU, arbitrary units.

6.3.3.4 Aerobic exercise and nutrition increases PPARδ mRNA expression

The combination of a bout of aerobic exercise and post-exercise nutrition caused an increase in mRNA expression for PPARδ (Figure 6-8A, $p < 0.05$). At three hours post-exercise, relative PPARδ mRNA expression was 1.86 ± 0.28 and 1.44 ± 0.11 times higher than before the exercise bout in the CHO and PRO conditions respectively. At six hours post-exercise, relative expression remained elevated 1.64 ± 0.19 and 1.50 ± 0.21 times higher than before the exercise bout in the CHO and PRO conditions respectively. There was no significant difference in relative gene expression between the PRO and CHO conditions at either three or six hours after exercise using a one-tailed *t*-test (Figure 6-8A, $p = 0.08$). In eight of the eleven subjects expression was higher in the CHO condition at three hours post-exercise, with a two-tailed, non-parametric test revealing that this trend came very close to significance (Figure 6-8B, $p = 0.06$).

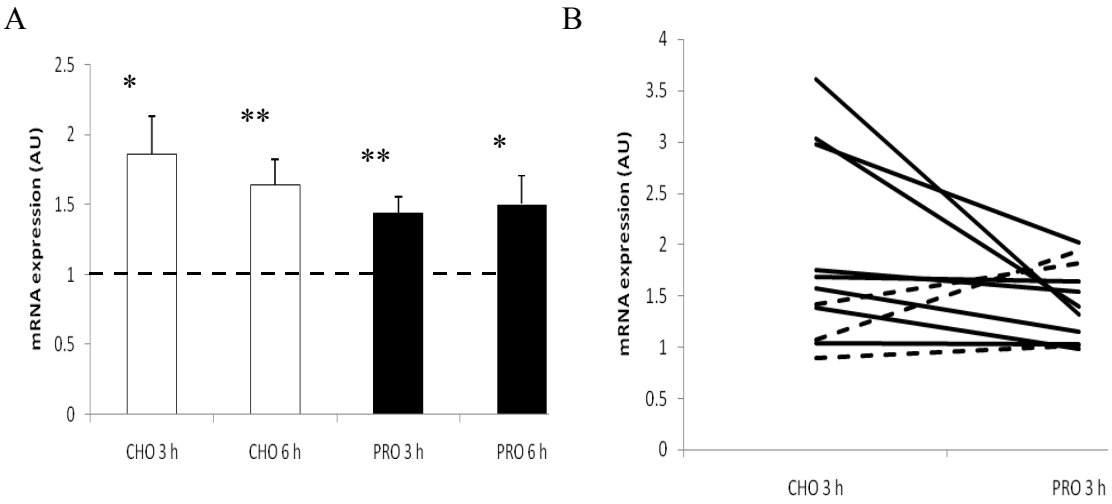


Figure 6-8 Changes in mRNA expression for PPARδ

A) Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and at three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. The combination of a bout of aerobic exercise and post-exercise nutrition caused an increase in gene expression for PPARδ at both time points in both conditions. *Error bars* SE. * value is different from baseline, $p < 0.05$; ** value is different from baseline, $p < 0.01$. *B)* Individuals' expression at three hours post-exercise in the CHO and PRO conditions. Solid lines indicate expression was higher in CHO condition; dashed lines indicate expression was higher in the PRO condition. In eight of the eleven subjects expression was higher in the CHO condition at three hours post-exercise. PPARδ, peroxisome proliferation-activated receptor delta; AU, arbitrary units.

6.3.3.5 Decrease in COXI transcript with protein nutrition after aerobic exercise

The combination of a bout of aerobic exercise and post-exercise nutrition containing protein caused a decrease in mRNA expression for COXI (Figure 6-9A, $p < 0.05$). At three hours post-exercise in the PRO condition, relative COXI mRNA expression was 0.81 ± 0.08 times that at baseline. Expression returned to baseline by six hours post-exercise, with expression at this time point being statistically different to that at three hours post-exercise ($p < 0.05$). There was a significant difference in relative gene expression between the PRO and CHO conditions at three hours after exercise using a one-tailed *t*-test (Figure 6-9A, $p < 0.05$). In nine of the eleven subjects expression was higher in the CHO condition at three hours post-exercise, with a two-tailed, non-parametric calculation revealing that this difference was significant (Figure 6-9B, $p = 0.012$).

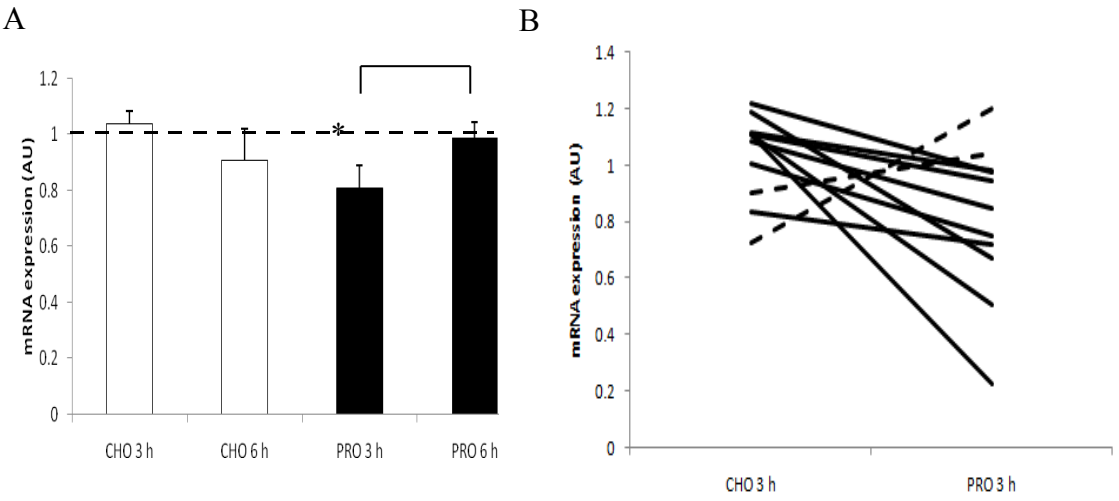


Figure 6-9 Changes in mRNA expression for COXI

A) Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and at three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. There was a decrease in relative COXI expression at three hours post-exercise in the PRO condition. *Error bars* SE. * value is different from baseline, $p < 0.05$; † difference between time points, $p < 0.05$. *B)* Individuals' expression at three hours post-exercise in the CHO and PRO conditions. Solid lines indicate expression was higher in CHO condition; dashed lines indicate expression was higher in the PRO condition. In nine of the eleven subjects expression was higher in the CHO condition at three hours post-exercise. COXI, cytochrome c oxidase subunit I; AU, arbitrary units.

6.3.3.6 No effect of aerobic exercise and nutrition on COXIV mRNA expression

There was no effect of a bout of aerobic exercise and post-exercise nutrition on gene expression for COXIV (Figure 6-10). There was no difference in relative gene expression between the PRO and CHO conditions at either three or six hours after exercise.

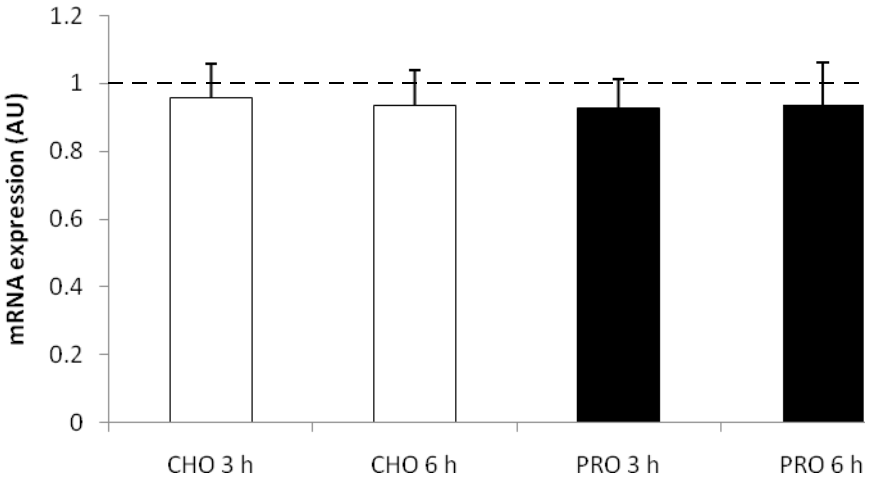


Figure 6-10 Changes in mRNA expression for COXIV

Expression is normalised to a baseline value of 1 for each individual; group mean changes from baseline are shown. Subjects cycled at roughly 70% VO_{2max} for one hour before receiving a 300 KCal drink. CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition. Muscle biopsies were obtained from the *m. vastus lateralis* at rest, and at three and six hours after cessation of exercise. Extracted RNA was subjected to RT-qPCR to determine relative expression. There were no significant changes from baseline or between conditions. *Error bars* SE. COXIV, cytochrome c oxidase subunit IV; AU, arbitrary units.

6.4 Discussion

This is the first study to investigate the effects of post-aerobic exercise protein feeding on signalling for mitochondrial biogenesis. The combination of aerobic exercise followed by nutrition led to an increase in PGC-1α mRNA expression in the PRO condition and a greater increase at three hours post-exercise in the CHO condition. There was a downstream increase in PPARα and δ mRNA expression in both conditions with the increase in PPARδ expression at three hours post-exercise being greater in the CHO condition. There was no change in TFam mRNA expression in either condition, nor was there a change in COXIV expression. There was a decrease in COXI mRNA expression at three hours post-exercise which was greater in

the PRO condition. In summary, and contrary to the hypothesis, providing a carbohydrate-only drink after a bout of high-intensity aerobic exercise tended to have a greater stimulatory effect on mRNA expression in the PGC-1α signalling pathway than an isocaloric protein-plus-carbohydrate drink.

6.4.1 Carbohydrate on its own stimulates mitochondrial biogenesis more than when provided in combination with amino acids

The notion that carbohydrate, on its own, may have a greater stimulatory effect on mitochondrial biogenesis, than when available in combination with amino acids, is supported by others' work in pig muscle . Boirie et al. infused saline, insulin alone, or insulin-plus-amino acids into pigs. Both insulin conditions increased mitochondrial FSR with no significant difference between the conditions, although there was a tendency for FSR to be higher in the insulin-only condition. The response of mitochondrial FSR was different to those of sarcoplasmic and myosin heavy chain proteins, indicating a possibly unique reaction of mitochondrial protein FSR to insulin. Recall that, while in Chapter 4 it was shown that providing a protein-plus-carbohydrate drink increased circulating insulin more than a carbohydrate-only drink, human skeletal muscle protein synthesis does not show a dose-dependent response to insulin . Therefore, the stimulation due to insulin would have been the same in both the CHO and PRO conditions in this study, with the presence of the amino acids in the PRO condition possibly blunting this response.

6.4.2 VO_{2max} and feeding status affect the response of PGC-1α mRNA expression to a bout of aerobic exercise

The eight- to nine-fold increase in PGC-1α mRNA expression at three hours post-exercise is commensurate with previous findings using a similar exercise protocol without post-exercise feeding . In comparison with previous work using post-exercise feeding protocols, the increase in this study is considerably higher with Pilegaard et al. and Cluberton et al. both reporting

only two- to three-fold increases at similar post-exercise time points after high-intensity exercise bouts. As both Pilegaard et al. and Cluberton et al. reported the same degree of change in both fed and unfed conditions, the source of the difference in findings between their studies and this one cannot be attributed to the feeding protocol. The subjects in Pilegaard et al. and Cluberton et al.'s studies had a similar mean VO_{2max} to those in this study, indicating a similar degree of fitness, so a similar response to exercise perturbation would be expected based on this variable. In contrast, Coffey et al. used subjects who were considerably less fit (VO_{2max} = 36.9 ± 2.8 mL/kg/min) so the exercise stimulus probably caused them a greater perturbation, hence the greater post-exercise increase in PGC-1α mRNA expression compared to the other two studies. Fitness level may, therefore, explain the difference among previously reported findings , and between these findings and those presented in this thesis. The magnitude of the increase in PGC-1α mRNA expression in this study was more in line with the findings by Russell et al. and Mathai et al. , who reported seven- to ten-fold increases in PGC-1α mRNA expression after two hours of cycling at a moderate intensity. While Pilegaard et al., Coffey et al., and Cluberton et al. all used a similar exercise protocol to that used in this study, they also all used overnight-fasted subjects, while the subjects in this study received a breakfast after their overnight fast. Similarly, both Russell et al. and Mathai et al. fed their subjects a breakfast-type meal one or two hours before the start of the exercise bout, so this may be the critical factor in maximising the response of PGC-1α mRNA expression to exercise and post-exercise nutrition.

The only previous study to measure a difference in PGC-1α mRNA expression between two post-exercise nutritional interventions found a more prolonged, but not higher, up-regulation in expression from five through eight hours post-exercise in a low-carbohydrate (36 g) condition compared to a high-carbohydrate (365 g) condition. There is a significant negative correlation between muscle glycogen content and PGC-1α protein abundance . In keeping with this

correlation, Pilegaard et al. found that muscle glycogen content was returned to its pre-exercise level by five hours post-exercise in the high-carbohydrate group, but not until 24 hours post-exercise in the low-carbohydrate group. Although glycogen content was not analysed in the study reported here, perhaps the ideal situation for maximising signalling is to train in a glycogen-replete state, as would be created by 24 hours of rest followed by provision of a breakfast to top up liver glycogen, then recover in a glycogen-depleted one (see for a review on this subject). There was only a 20 g (26%) difference in carbohydrate ingestion between the two conditions in this study (CHO provided 75 g carbohydrate while PRO provided 55 g), which is minimal compared to the ten-fold difference between the conditions in Pilegaard et al.'s study, so it is unlikely that there was much difference in glycogen status between the groups. Due to the low amount of carbohydrate provided in both conditions, subjects would have been glycogen-depleted in both trials, and therefore would have experienced a maximal level of AMPK signalling, which stimulates mitochondrial biogenesis (Figure 2-3). Therefore glycogen status can probably not be held accountable for the observed differences in mRNA expression between the CHO and PRO conditions in this study.

Protein synthesis consumes a large amount of energy, so in times of cellular energy stress, energy is diverted away from those processes not essential for cell survival. Following a bout of exhaustive aerobic exercise without post-exercise feeding, the rate of post-exercise protein synthesis would not increase as much as if food was provided. While both drinks provided the same total number of Calories, the carbohydrate-only drink would have provided more readily available energy as the protein in the protein-plus-carbohydrate drink would have had to be metabolised via gluconeogenesis to be used as fuel. The small difference in carbohydrates between the PRO and CHO conditions might not have been enough to affect glycogen replenishment, but perhaps just enough to supply more readily available energy to protein synthesis.

6.4.3 A bout of aerobic exercise followed by feeding stimulates PGC-1α and PPARδ mRNA expression at three hours post-exercise

As well as considering the differences in expression between the CHO and PRO conditions, it is interesting to analyse the differences in mRNA expression of each gene at three and six hours post-exercise. The time course of the post-exercise change in PGC-1α mRNA expression with values peaking at three hours of recovery and remaining elevated, but at lower levels, by six hours, is consistent with others' findings . While Pilegaard et al found an increase in TFam mRNA at six hours post-exercise and the study presented here did not, Pilegaard et al.'s samples were taken after three hours of exercise compared to the one hour of exercise in the study being discussed here. It is possible that changes in signalling begin from the onset of exercise, and that sampling at eight hours post-exercise in this study may have identified a measurable change in TFam mRNA expression. Others , using gene microarray technology validated by SYBR green RT-qPCR, have reported a 1.7-fold increase in PPARα mRNA expression, after 75 minutes of high-intensity cycling, whereas no increase was found in the study presented here. As the precision of qPCR is in the range of a two-fold increase, the significance of Mahoney et al.'s findings with regards to PPARα is questionable. A difference in stimulation of only 1.7-fold would fall within the margin of error for the technology used in these experiments. The 1.43 ± 0.11 to 1.86 ± 0.28 fold increases in PPARδ mRNA expression at three hours post-exercise is similar in magnitude to that found by Mahoney et al., but lower than the three-fold increases reported by others .

6.4.4 No significant changes in COX mRNA expression after post-aerobic exercise feeding

The COX isoforms in this study were selected as genes encoding mitochondrial proteins. Any increase in mRNA expression of COXI and COXIV may have indicated subsequent increases in mitochondrial protein expression. Although increases in mRNA expression of these genes have not been reported previously, following the analysis of two COX genes might have yielded interesting information seeing as one is encoded in mtDNA and the other in nuclear DNA. Recall that the expression of mitochondrial protein requires co-ordinated up-regulation by PGC-1α of genes encoded in both the nucleus and the mitochondria . Unfortunately, no changes were observed. The lack of a measurable change in mRNA expression of the COX isoforms may indicate that these proteins are too far downstream in the signalling pathway to be significantly up-regulated during the time course of this experiment. Evidence from isolated animal muscle preparations suggests that elevated expression in these downstream genes may not be apparent for several days .

6.4.5 Changes in mRNA expression suggest later changes in protein expression

It has been shown previously that downstream changes in the genes encoding mitochondrial proteins occur as a consequence of increased PGC-1α mRNA expression, and several hours before changes in the amount of PGC-1α protein. Wright et al. found that the expression of some mitochondrial proteins increases before that of PGC-1α. It is worthwhile to remember that PGC-1α is a regulator of mitochondrial protein synthesis and not actually a mitochondrial protein, so information regarding its protein expression is not crucial. PGC-1α can exert its role as master regulator through changes in phosphorylation and mRNA abundance without actually increasing its level of protein expression . Other studies using cell culture and rat skeletal muscle preparations indicate that an increase in PGC-1α mRNA, or even simply its transcriptional activation, is responsible for the signalling cascade resulting in mitochondrial

biogenesis . It seems likely then that the increase in PGC-1α mRNA, and its downstream effector PPARδ, observed during the time course of this experiment would support longer-term accumulation of mitochondrial protein.

It is likely that the observed changes in mRNA expression will drive changes in protein content as mRNA supports the synthesis of new protein. Correspondingly, the direction of change of mRNA is usually the same as that of protein during the post-exercise adaptive period, or during adaptation to a new steady state during a training programme . Studies in rat skeletal muscle have indicated the veracity of this statement with mRNA levels of cytochrome c, citrate synthase, and COX III and VI all closely correlated with both protein content and enzyme activity . Genes with higher mRNA expression may be selectively favoured over those with lower expression, for translation into protein by factors which up-regulate this process such as circulating insulin . Changes in mRNA and protein expression may not be proportional in magnitude however , and the only way to answer this question with certainty is to conduct a longer-term study in which mitochondrial protein content is analysed over a period of days to weeks.

The cumulative effect of many bouts of exercise such as the one in this study, i.e. a period of aerobic exercise training, will likely be an increased basal level of PGC-1α mRNA expression , and consequently, enhanced mitochondrial biogenesis. An increase in mitochondrial protein content will result in enhanced oxidative capacity and fatigue resistance. Over-expression of PPARδ in transgenic mice has also been shown to enhance endurance capacity .

6.4.6 Possible sources of uncontrolled variability in the results

The high degree of inter-subject variability in gene expression, even in the rested and fasted state, indicates the importance of analysing within-subject change using the relative expression method, rather than considering absolute values of post-exercise expression within the group.

By measuring within-subject change, rather than absolute values of expression, any inter-subject variability in insulin response to the meals or prior glycogen status did not affect the results in this study.

Subjects' training status may have had an effect on the variability in the results. While an untrained person would experience a greater stimulus from the bout of exercise presented here than a trained athlete, previous studies have found that untrained muscle displays such variability in response to exercise that it is difficult to find a statistically significant result . Thus the choice was made to use recreationally active, rather than sedentary, subjects. While a maximum VO_{2max} was imposed during screening to limit the participation of athletes who perhaps would not have experienced sufficient perturbation to show a measurable change in mRNA expression, it may have been wise also to impose a minimum. The range in VO_{2max} of subjects in this study was 8.2 mL/kg/min which may have introduced enough variability to prevent some of the findings from being statistically significant. Alternatively, perhaps a tighter, but lower, range of VO_{2max} should have been selected as Pilegaard et al. found measurable changes in TFam and PPARα mRNA levels after six hours of post-exercise recovery in untrained muscle only. Training history would also have had an effect on AMPK phosphorylation, as a trained subject would experience less perturbation to cellular energy status and so less downstream signalling .

All subjects reported extreme feelings of hunger by the end of the post-exercise recovery period. This situation could be likened to fasting in which plasma free fatty acid levels are elevated. Such an elevation could stimulate mRNA expression of PGC-1α and the PPARs , which may have affected the findings in this study. However, as other authors have found no differences in the mRNA expression of these genes in between their fasted and fed subjects during three hours of post-exercise recovery, this possibility seems unlikely.

6.4.7 Conclusion

To conclude, a high-intensity bout of aerobic exercise followed by nutrition promotes transcription of genes in the mitochondrial biogenesis signalling pathway. A carbohydrate-only compared to a carbohydrate-plus-protein drink tended to have a greater effect on mRNA expression of PGC-1α, PPARδ, and COXI at three hours post-exercise. These findings suggest that energy in the form of carbohydrate after aerobic exercise may be more important than amino acid availability in stimulating mitochondrial biogenesis in human skeletal muscle. A successful investigation into the expression of mitochondrial proteins after the exercise and nutrition intervention described here is needed in order to test this hypothesis. It is likely that, because protein synthesis and aerobic exercise are both energy-consuming processes, the drink that provided more readily available energy (CHO) would allow the diversion of more cellular ATP from functions vital to cell survival to the anabolic process of protein synthesis.

This research has implications beyond the sporting arena and the desire to optimise exercise adaptations, as mitochondrial ATP production is required to fuel muscular contraction . As reviewed in 2.3.3, mitochondria have been implicated in the ageing process as oxidative damage leads to mitochondrial dysfunction . An increased rate of mitochondrial biogenesis would replace these damaged proteins faster, allowing for maintenance of functional capacity with ageing. Information regarding signalling in the PGC-1α pathway can also provide for the development of targeted therapy for mitochondrial diseases, such as drugs that up-regulate key elements of the pathway, or a prescribed aerobic exercise programme accompanied by appropriate nutrition.

7 Influence of post-aerobic exercise nutrient composition on signalling for protein translation – the mTOR pathway

7.1 Introduction

In Chapter 6, the change in mRNA expression of genes regulating mitochondrial biogenesis, in response to a bout of aerobic exercise followed by feeding was examined. Changes in the activation state of signalling proteins also play an important role the signalling response to exercise and nutrition. While an increase in mRNA expression is usually followed by an increase in protein expression for the same gene, increased protein expression may also occur without a change in mRNA due to changes in translation . The mRNA response occurs with some delay after a bout of exercise, while the latter occurs much earlier. A change in the rate of mixed muscle MPS has been measured well before a change in mRNA expression of genes encoding constituent proteins, due to the early post-transcriptional response of MPS to nutrition or exercise . Nutrient availability will have an effect on the immediate response, in that amino acids might increase the rate of translation elongation by providing more building blocks to the process . Post-translational changes, such as alterations in phosphorylation status may also occur before increases in mRNA expression . How exercise and nutrition affect this early response is not well understood.

Many changes in the rates of protein synthesis are mediated by factors in the mTOR pathway regulating translation, so the response of the mTOR pathway to the combination of aerobic exercise and nutrition was investigated. This chapter considers the phosphorylation of mTOR and 4E-BP1 following post-aerobic exercise feeding, as well as protein expression of cytochrome c, a downstream responder.

As reviewed in 2.2.2.1, the mTOR pathway integrates information regarding energy availability for protein synthesis, as well as the presence of sufficient raw materials . mTOR is a

convergence point for a variety of signals coming from insulin, the BCAAs, and the effects of exercise (Figure 2-4). The phosphorylation and activation of mTOR is influenced by signals from all three of these stimuli and is, therefore, a key point of interest. mTOR has been shown to exert a specific effect on mitochondrial protein synthesis through its regulation of the transcription factor YY1, which interacts with PGC-1α to up-regulate the expression of genes encoding mitochondrial protein . Furthermore, cellular fractionation has revealed the presence of mTOR in the mitochondrial fraction of skeletal muscle . All of these factors makes mTOR an interesting target in this study of mitochondrial protein synthesis.

Downstream of mTOR lie proteins involved in the regulation of translation initiation and elongation, one of which is 4E-BP1 . As reviewed in 2.2.2.1, active 4E-BP1 binds to eIF4E, thus inhibiting the formation of an active translation initiation complex. Phosphorylation of 4E-BP1 causes its dissociation from eIF4E, allowing the latter to bind with eIF4G, and translation initiation to proceed.

Previous studies have revealed that insulin acts post-transcriptionally to phosphorylate 4E-BP1 and up-regulate the expression of mitochondrial genes in miniature swine , and that the ingestion of essential amino acids also increases 4E-BP1 phosphorylation in humans . The combination of resistance exercise and carbohydrate-plus-protein feeding has been shown to result in a roughly two-fold increase in 4E-BP1 phosphorylation in human skeletal muscle .

Measuring the phosphorylation of mTOR and its downstream effector 4E-BP1 may also provide a reasonable proxy for information about mitochondrial protein synthesis not gained in Chapter 5. Phosphorylation in the mTOR pathway has been found to be closely correlated with FSR at one and two hours of post-resistance exercise recovery . On the other hand, translation of many proteins is mediated by the mTOR pathway, so information regarding the phosphorylation of two components in this pathway does not necessarily indicate specific

regulation of mitochondrial protein synthesis. As it is known that stimulation of MPS by aerobic exercise is specific to proteins of the aerobic respiratory chain , it is most likely that a significant portion of the up-regulation of protein translation inferred from mTOR pathway phosphorylation will be mitochondrial. Furthermore, concomitant increases in mTOR signalling and mitochondrial protein synthesis have been measured recently after a bout of aerobic exercise . The up-regulation of PGC-1α and PPARδ mRNAs measured in Chapter 6 also supports this suggestion as these genes are known regulators of mitochondrial biogenesis. As cytochrome c has a half life of eight days, it is possible that its expression may increase by a measurable amount over the six-hour time course in this study .

At the time of planning and inception of this portion of the second study, there was no published evidence of the effect of nutrition provided after a bout of aerobic exercise on signalling in the mTOR pathway. Therefore the aim of this part of the second study was to investigate whether feeding protein in combination with carbohydrate immediately after an acute bout of sustained, high-intensity aerobic exercise would have an additive effect over carbohydrate alone on signalling for protein translation. The hypothesis tested was that a protein-plus-carbohydrate drink (PRO) would increase the phosphorylation of mTOR and 4E-BP1, and the total protein content of cytochrome c, during the first six hours of post-exercise recovery, more than an isocaloric, carbohydrate-only drink (CHO).

7.2 Methods

These experiments were conducted using the muscle samples from the second study, collected as described in Chapter 6. Half of each sample was intended for use in analysis of mRNA expression and the other half for the protein work described in this chapter. As the quality and quantity controls for RNA extraction (described in 6.2.3.1) required the use of both halves of several samples, only four subjects had a complete set of five samples on which to perform an

analysis of protein expression. In light of the lack of information regarding the rate of mitochondrial protein synthesis gained in Chapter 5, to add to the information gained in Chapter 4 about protein synthesis after post-aerobic exercise nutrition, the analysis of protein expression and phosphorylation in these samples was performed.

7.2.1 Muscle processing and analysis

7.2.1.1 Protein extraction

Muscle samples (27-85 mg) were homogenised in 500 μL ice-cold buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 5 mM EGTA; 10 mM $MgCl_2$, 50 mM β-glycerophosphate; 1 mM Na_3VO_4, 2 mM DTT; 1% Triton X-100; 20 μg/mL leupeptin; 50 μg/mL aprotinin; 40 μg/mL PMSF) using a hand-held electric homogeniser (KemaKeur PRO 200, Pro Scientific Inc., Oxford CT, USA) for two periods of 30 seconds each. Homogenates were centrifuged at 20 800 g for 30 minutes at 4 ºC and the pellet discarded. Protein concentration was determined in 1 : 2, 1 : 4, and 1 : 10 dilutions of the supernatant using a BSA detergent-compatible assay (reagents and Ultramark microplate imaging system, Bio-Rad Laboratories, Hercules CA, USA). Where the R^2 value for the standard curve run with each microplate was less than 0.99, or the concentration values across the series dilution were not consistent, the assay was repeated in 1 : 4, 1 : 8, and 1 : 16 dilutions of the supernatant.

Preliminary experiments with two muscle biopsy samples confirmed that there was enough protein to conduct the planned analysis of protein expression. Protein concentration ranged from 2.1 – 4.3 μg/μL in lysates from the two samples weighing approximately 25 and 50 mg respectively. Both samples yielded approximately 400 μL of lysate which was sufficient to load at least 20 gel lanes for Western blot analysis.

7.2.1.2 Protein expression by Western blot analysis

Ten-lane gels were poured with a 4% stacking layer (Appendix B8). The left-most lane was used for the molecular weight marker (PrecisionPlus All Blue, Bio-Rad Laboratories, Hercules CA, USA), and the remaining nine lanes were loaded with triplicate samples alternating between the baseline sample, the sample from the CHO condition, and the sample from the PRO condition. The samples from three hours post-exercise for a given subject were run on one gel, and the samples from six hours post-exercise for the same subject were run simultaneously on another gel. In this way, all the samples for a given subject for one protein were run on the same day in order to minimise the effect that day-to-day variations in laboratory conditions and experimental handling may have had on the results.

Aliquots of muscle lysate (30-60 μg total protein) were mixed with 4X SDS reducing load buffer (0.25 M Tris HCl, pH 6.8; 8% SDS; 40% glycerol; 20% β-mercaptoethanol, 0.004% bromophenol blue) and the volume made up to 20 μL with Milli-Q water (Millipore, Billerica MA, USA). Samples were heated for three minutes at 95 ºC to denature the proteins before loading onto 7% (mTOR), 12% (4E-BP1), or 15% (cytochrome c) Tris-glycine buffered acrylamide gels for electrophoresis (Hoefer Mighty Small II SE 250/SE 260, Amersham Bio, San Francisco CA, USA). Following electrophoresis, proteins were transferred (Bio-Rad Mini trans-blot cell, Bio-Rad Laboratories, Hercules CA, USA) to a PVDF (mTOR) or nitrocellulose (4E-BP1, cytochrome c) membrane at 30 V overnight in a cold room at 4 ºC (mTOR) or 100 V for 75 minutes on ice (4E-BP1, cytochrome c). For mTOR, the transfer buffer (25 mM Tris HCl, pH 8.3; 192 mM glycine; 20% methanol) contained 0.02% SDS to enhance transfer of this high molecular weight protein (Chris McMahon, personal communication). To confirm protein transfer, the membranes were stained with Ponceau S. Following de-staining, membranes were blocked with 5% non-fat dry milk (NFDM) in TTBS for one hour at room temperature. The blocked membranes were probed overnight at 4 ºC with constant agitation with appropriate primary phospho-specific polyclonal antibodies; mTOR at Ser^{2448}, 4E-BP1 at

Thr$^{37/46}$, or for total cytochrome c (Cell Signalling Technology Inc., Boston MA, USA; lot nos. 10/2007, 02/2008 and Abcam, supplied by Sapphire Bioscience, Redfern NSW, Aus; lot no. 170477 respectively). Antibodies were diluted 1 :

1 000 in TTBS containing 1% (4E-BP1) or 5% (mTOR and cytochrome c) NFDM.

As previous publications report a range of NFDM content in TTBS, from 0 to 5%, for antibody incubation and subsequent washing when probing for 4E-BP1, a comparison was made of various combinations (Table 7-1) in order to determine which would provide the clearest bands for analysis. The membrane incubated and washed in TTBS containing 1% NFDM provided a band that was both sharp and visible, while the TTBS containing 5% NFDM produced a band that, while clearly visible, was quite diffuse thereby complicating analysis (data not shown). Therefore, TTBS containing 1% NFDM was used in incubations and washes when probing for 4E-BP1.

primary antibody dilution	secondary antibody dilution	washes
TTBS containing 5% NFDM	TTBS containing 5% NFDM	TTBS containing 5% NFDM
TTBS containing 1% NFDM	TTBS containing 1% NFDM	TTBS containing 1% NFDM
TTBS containing 1% NFDM	TTBS	TTBS containing 1% NFDM

Table 7-1 Different antibody dilution and wash conditions trialled for membranes probed for 4E-BP1

Nitrocellulose membranes were probed with primary antibody, then washed four times before probing with secondary antibody. A second series of washes was performed prior to exposure by chemiluminescence. TTBS containing 1% milk was chosen for use in all incubations and washes as these conditions provided the clearest bands for analysis. TTBS, Tween-20 in Tris-buffered saline; NFDM, non-fat dry milk; 4E-BP1, eukaryotic initiation factor binding protein.

Following primary antibody incubation, the membranes were washed four times for eight minutes in TTBS (mTOR) or TTBS containing 1% (4E-BP1) or 5% (cytochrome c) NFDM. The membranes were then incubated for two hours at room temperature with constant agitation with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove PA, USA) diluted 1 : 5 000 in TTBS containing 1% NFDM, and re-washed as before. The final wash of the PVDF membranes lasted 30 minutes.

Immunoreactive bands were detected using enhanced chemiluminescence (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology, Rockford IL, USA), and quantified by densiometric analysis (Multi Gauge ImageReader software version 2.2) of images collected using a digital camera system (LAS-3000, Fujifilm NZ Ltd., Auckland, NZ).

Following exposure of membranes probed with phospho-specific antibodies, the membranes were incubated in stripping buffer (100 mM β-mercaptoethanol; 2% SDS; 65.5 mM Tris HCl, pH 6.7) for 30 minutes at 50 ºC, then cut at 50 (mTOR) or 25 kDa (4E-BP1, cytochrome c). The cut membranes were re-blocked, and the relevant portion re-probed with appropriate polyclonal antibodies to detect total expression levels of either mTOR or 4E-BP1 (Cell Signalling Technology Inc., Boston MA, USA; lot nos. 01/2008, 01/2008). The effectiveness of the stripping buffer was confirmed by re-incubating the stripped blot with secondary antibody and then chemiluminescent reagent. No chemiluminescence was observed in the stripped membrane (data not shown).

After stripping and cutting, the relevant section of membrane was probed for GAPDH at 37 kDa. Analysis of GAPDH was performed as a loading control as its expression is minimally affected by an acute bout of aerobic exercise . The primary antibody (Abcam, supplied by Sapphire Bioscience, Redfern NSW, Australia) was diluted 1 : 33 333 in low-sodium TTBS (LS-TTBS) containing 1% NFDM, washes done in LS-TTBS, and the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; Jackson ImmunoResearch, West Grove PA, USA) diluted 1 : 5 000 in LS-TTBS containing 1% NFDM. Immunoreactive bands were detected using enhanced chemiluminescence (Roche Lumi-Light Western Blotting Substrate, Roche, Penzberg, Germany). In four of the eight 7% gels, the 37 kDa band did not resolve from the dye front. This lack of loading control information was resolved as described in 7.2.2.

Many publications (e.g.) recommend boiling samples at 100 ºC for ten minutes, centrifuging, and then using the supernatant for 4E-BP1 analysis. As this is a heat-stable protein, such treatment could potentially provide for a much cleaner membrane in Western blotting analysis. However, no difference was found in the quality of results obtained from heat-treated and untreated samples (data not shown). Therefore, it was decided not to include this heating step as it could potentially denature the GAPDH in the lysate and so make the loading control information unavailable.

7.2.2 Calculations and Statistics

Values of phosphorylated or total protein expression were normalised to the intensity of the immunoreactive GAPDH band. In instances where the 37 kDa band did not resolve from the dye front on the gels used for mTOR analysis, the expression of GAPDH from the same samples on the gels used for 4E-BP1 analysis was multiplied by two and used for normalisation. The factor of two was used as the load on the 7% gels (mTOR) was twice that used on the 12.5% gels (4E-BP1). The extent of phosphorylation was determined as the ratio of the intensity of the phosphorylated band to the intensity of the total immunoreactive protein band. Protein expression in the pre-exercise, unfed (baseline) muscle sample was set to one, and all other values were expressed relative to this value for each individual (see Appendix E4 for sample calculation).

To determine whether there was a difference between the CHO and PRO conditions, and between baseline and either of the post-exercise time points, paired, one-tailed *t*-tests were performed and significance was set at $\alpha < 0.05$.

7.3 Results

The results in this section are organised according to the sequence in which signals act in the mTOR pathway (Figure 7-1). Phosphorylation of mTOR on Ser^{2448}, in its carboxy terminal regulatory domain, is considered crucial for its activation and downstream phosphorylation of 4E-BP1 . Inhibition of 4E-BP1 depends on sequential phosphorylation. The phosphorylation sites chosen for this work are the early residues on $Thr^{37/46}$, while subsequent sites are $Thr^{70}$and Ser^{65} . Inhibition of 4E-BP1 relieves its inhibitory effect on initiation of protein translation.

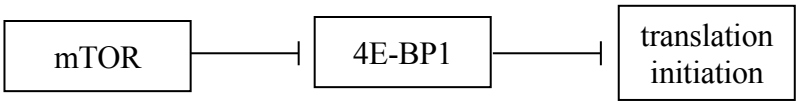


Figure 7-1 Portion of signalling cascade resulting in increased translation initiation of skeletal muscle protein in the second study

See Figure 2-4 for full sequence. Phosphorylation of mTOR causes its activation and downstream phosphorylation of 4E-BP1. Phosphorylation and inhibition of 4E-BP1 relieves its inhibitory effect on initiation of protein translation. ⟝ inhibits; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor binding protein.

7.3.1 No trends in mTOR phosphorylation with aerobic exercise and feeding

Preliminary experiments using standard Western blot transfer conditions revealed no signal for mTOR with up to 33 μg of total protein loaded per lane (data not shown). Increasing the protein load to 60 μg, inclusion of SDS in the transfer buffer to enhance transfer efficiency (Chris McMahon, personal communication), and the use of an overnight transfer resulted in a band of the expected size (289 kDa, Figure 7-2). There were no other significant bands detected, indicating the specificity of the assay.

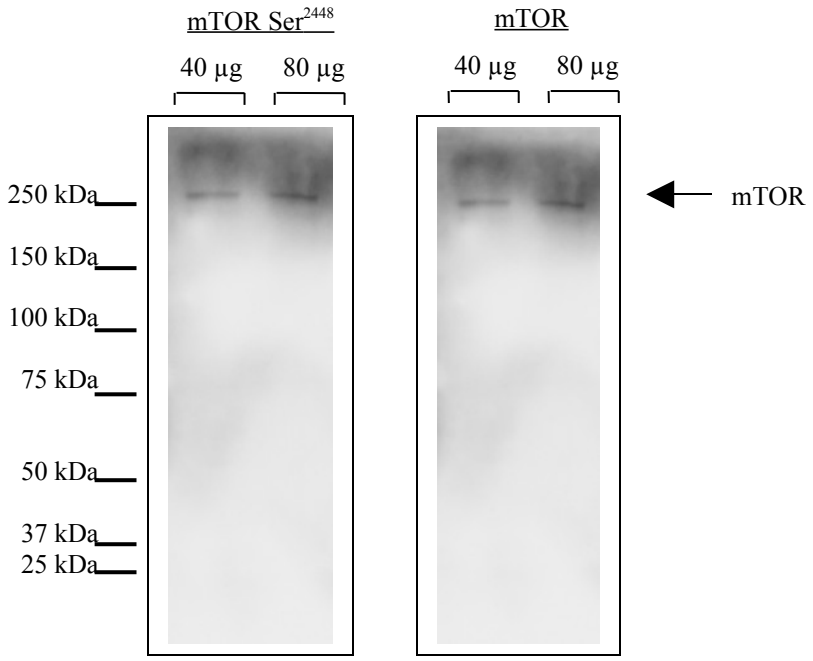


Figure 7-2 Assessment of antibody sensitivity and specificity for phospho-specific (Ser^{2448}) and total immunoreactive mTOR

40 and 80 µg total protein from homogenised muscle samples was loaded onto 7% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to PVDF membrane, probed for phosphorylated and total mTOR, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of mTOR is 289 kDa; clear bands appeared above the 250 kDa molecular weight mark. Exposure time is 3 minutes. mTOR, mammalian target of rapamycin; PVDF, polyvinylidene difluoride.

Western blots for subject 9 are shown in Figures 7-3A, B, and C. For this subject, the extent of mTOR phosphorylation was greater in the CHO condition than the PRO at both three and six hours post-exercise (Figure 7-3A). Two other subjects expressed greater phosphorylation in the PRO condition than the CHO at six hours post-exercise (Figure 7-3D). There were no other differences in mTOR phosphorylation between the conditions at either time point.

In the CHO condition, there was a slight decrease in subject 9's mTOR phosphorylation from 2.8-fold greater than baseline at three hours post-exercise, to 2.1-fold at six hours post-exercise. Only one other subject exhibited this decrease in phosphorylation from three to six hours post-exercise in the CHO condition (Figure 7-3E).

At six hours post-exercise, all four subjects expressed more total immunoreactive mTOR in the CHO condition compared to the PRO condition with this difference approaching significance ($p = 0.1$).

In the CHO condition, one subject expressed more total immunoreactive mTOR at three hours post-exercise than at six hours, while another subject showed the opposite difference (Figure 7-3F). The latter subject also expressed less total mTOR at six hours than three hours in the PRO condition. There were no other trends in the change in expression of total immunoreactive mTOR over time within a condition (Figure 7-3E).

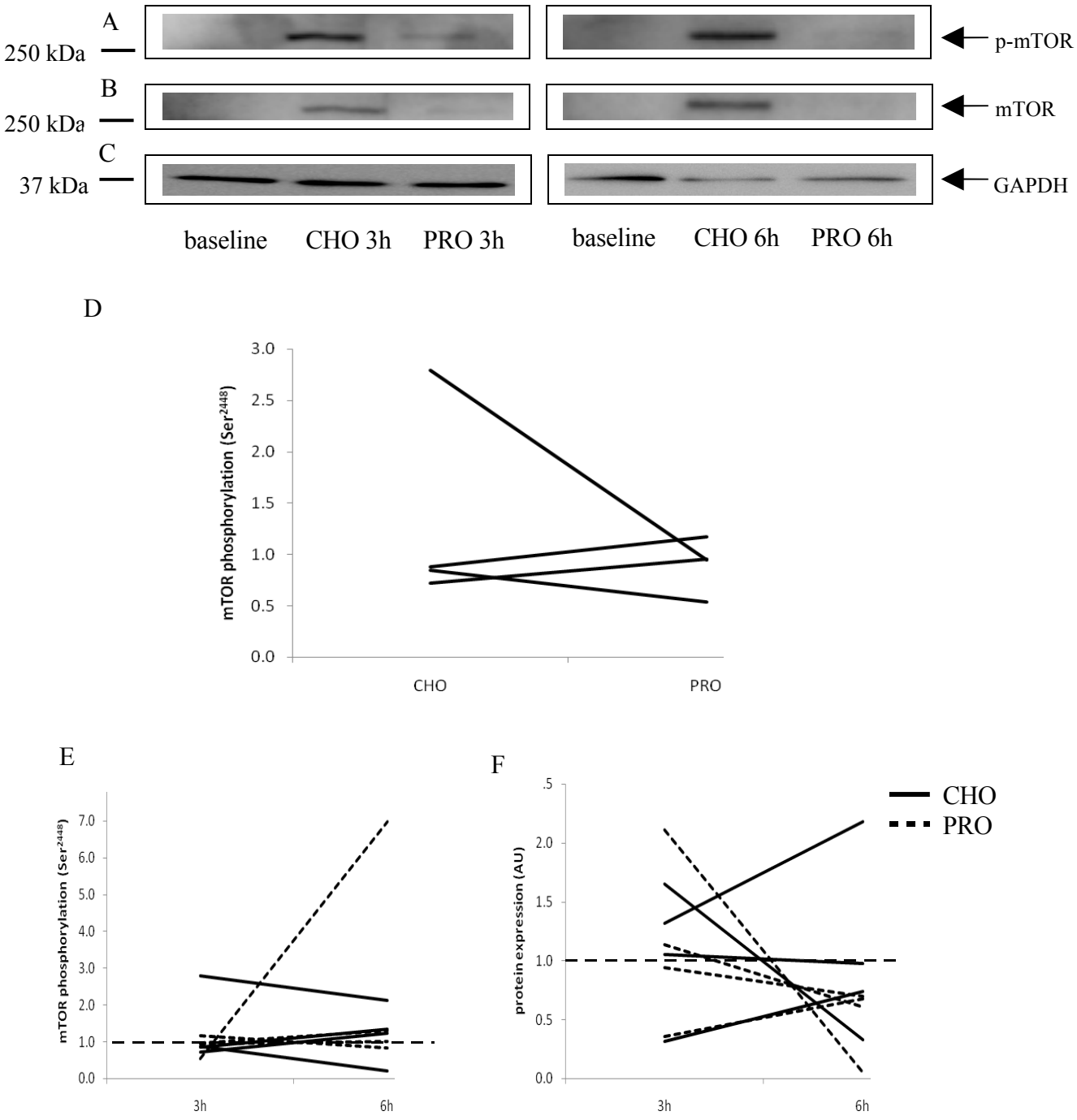


Figure 7-3 Change in expression of phosphorylated (Ser^{2448}) and total immunoreactive mTOR

Western blots from a single subject showing *A)* phosphorylated and *B)* total mTOR and *C)* GAPDH (exposure times were 18, 5, and 4 minutes respectively). 60 µg total protein was loaded onto 7% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to PVDF membrane, probed for phosphorylated and total mTOR, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of mTOR is 289 kDa; clear bands appeared above the 250 kDa molecular weight mark. All individuals' expression of *D)* phosphorylated mTOR at six hours post-exercise and *E)* phosphorylated and *F)* total mTOR at three and six hours post-exercise for both conditions; expression is relative to baseline which is set to 1. Error bars not shown for ease of interpretation. Phosphorylation was determined as the ratio of phosphorylated to total immunoreactive mTOR. Intensity of bands was normalised to expression of GAPDH. mTOR, mammalian target of rapamycin; PVDF, polyvinylidene difluoride; AU, arbitrary units.

7.3.2 Greater 4E-BP1 phosphorylation with protein feeding after aerobic exercise

Preliminary experiments using standard Western blot transfer conditions with 10, 20, and 33 µg of total protein loaded per lane revealed bands of the expected size (15-20 kDa) in all lanes, although some were very faint and therefore not suitable for analysis (Figure 7-4). The multiple bands indicate the different electrophoretic mobilities of the multiple phosphorylation sites. Some non-specific bands were also detected above 50 kDa. For quantitation, 30 µg of total protein was loaded to maximise the signal.

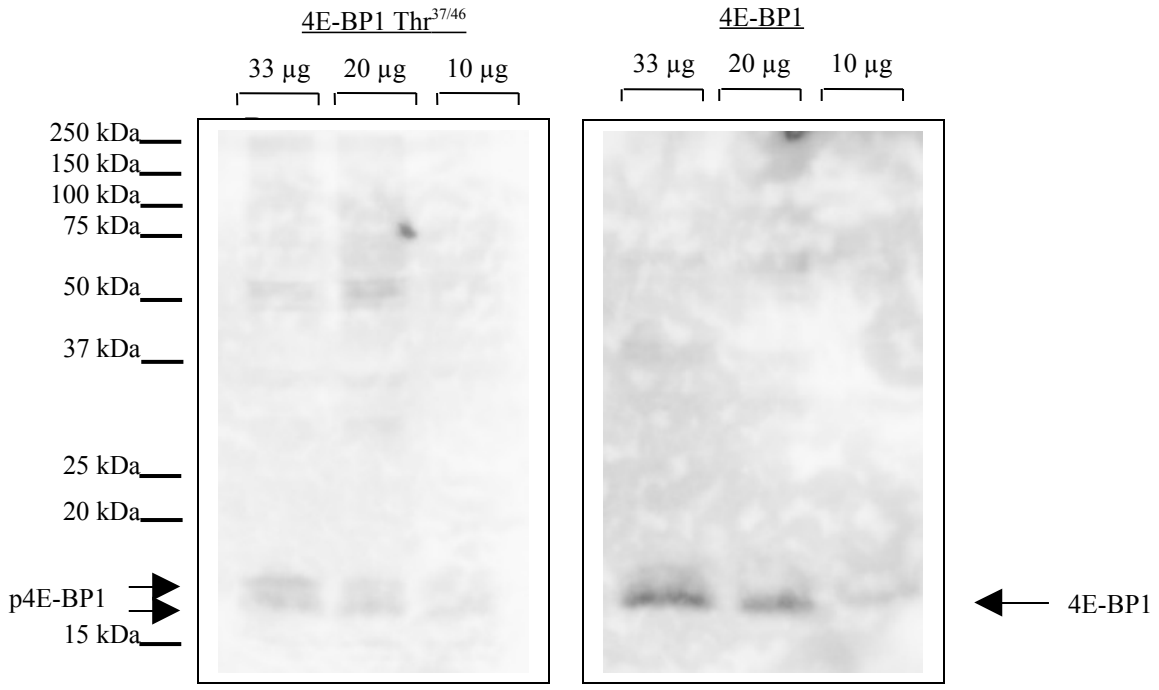


Figure 7-4 Assessment of antibody sensitivity and specificity for phospho-specific (Thr$^{37/46}$) and total immunoreactive 4E-BP1

33, 20, or 10 µg total protein from homogenised muscle samples was loaded onto 12% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to nitrocellulose membrane, probed for phosphorylated and total 4E-BP1, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of 4E-BP1 is 15-20 kDa. Exposure time is 18 minutes. 4E-BP1, eukaryotic initiation factor binding protein.

At three hours post-exercise, there was greater 4E-BP1 phosphorylation in the PRO condition compared to the CHO condition in three of the four subjects with this difference approaching significance ($p = 0.08$, Figure 7-5D). This result for subject 2 is shown in Figure 7-5A. It is possible that the multiple bands are not visible in this figure due to a weak signal. At six hours

post-exercise, there was greater phosphorylation in the CHO condition than the PRO, for one subject. No other subjects expressed a difference in phosphorylated 4E-BP1, between the conditions, at six hours post-exercise.

In the PRO condition, all four subjects displayed a significant decrease ($p < 0.05$) in 4E-BP1 phosphorylation from three to six hours post-exercise, while in the CHO condition only two subjects demonstrated this decrease (Figure 7-5E).

At both three and six hours post-exercise, three out of four subjects expressed more total immunoreactive 4E-BP1 in the CHO condition compared to the PRO condition, with this difference being either significant or very close to significance, at both time points ($p < 0.05$ and $p = 0.05$ respectively). This result is shown for subject 2 in Figure 7-5B.

One subject expressed less total immunoreactive 4E-BP1 at six hours post-exercise than at three hours, in both the CHO and PRO conditions, while another subject expressed the opposite trend. The other two subjects demonstrated these opposing differences in the PRO condition only (Figure 7-5F).

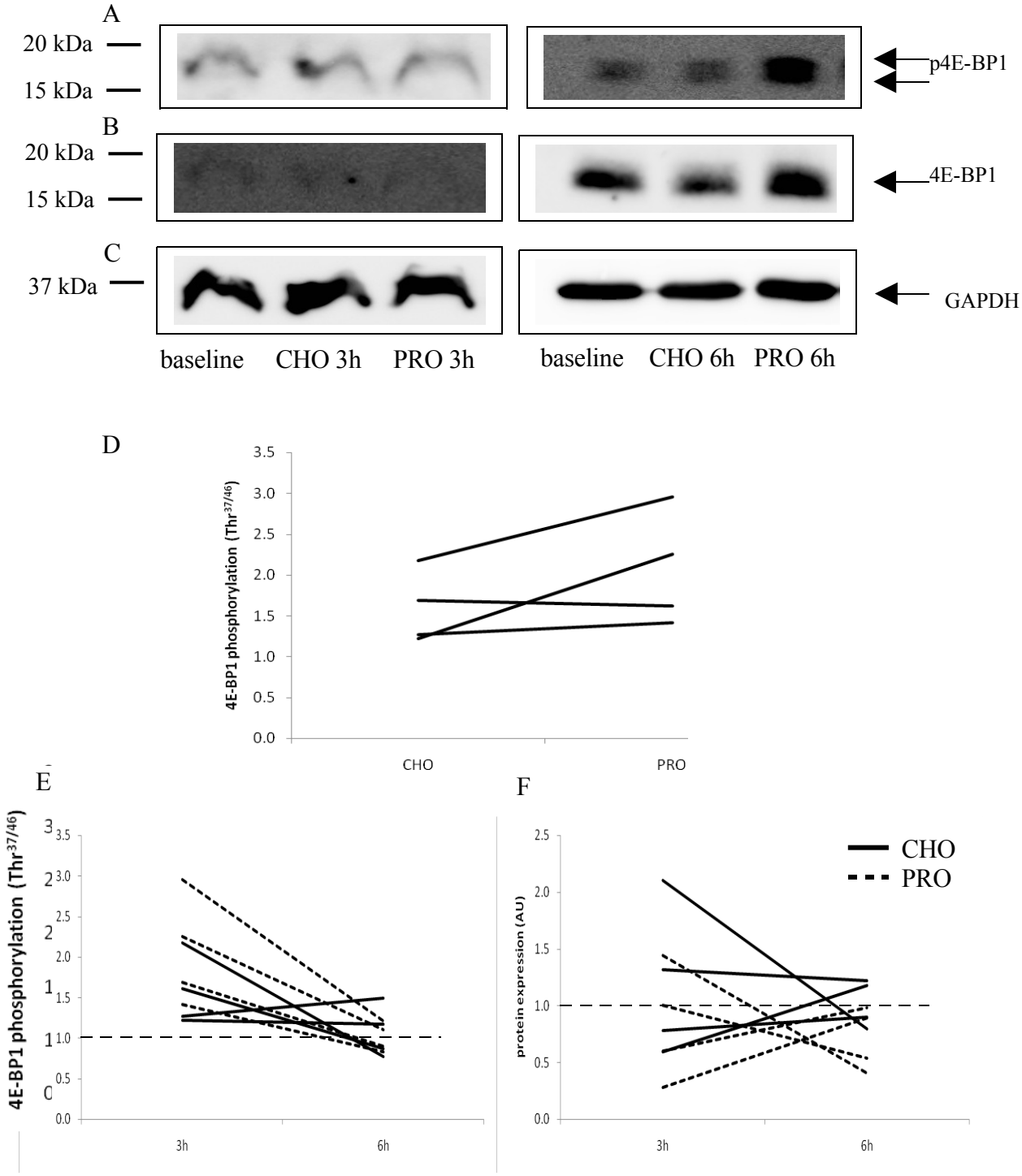


Figure 7-5 Change in expression of phosphorylated ($Thr^{37/46}$) and total immunoreactive 4E-BP1

Western blots for a single subject showing *A)* phosphorylated and *B)* total 4E-BP1 (exposure times were 54 and 2 minutes respectively). *C)* Immunoreactive GAPDH for the same three lanes as in *A* and *B* (exposure time was 1 minute). 30 µg total protein was loaded onto 12% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to nitrocellulose membrane, probed for phosphorylated and total 4E-BP1, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of 4E-BP1 is 15-20 kDa. All individuals' expression of *D)* phosphorylated 4E-BP1 at three hours post-exercise and *E)* phosphorylated and *F)* total 4E-BP1 at three and six hours post-exercise for both conditions; expression is relative to baseline which is set to 1. Error bars not shown for ease of interpretation. Phosphorylation was determined as the ratio of phosphorylated to total immunoreactive mTOR. Intensity of bands was normalised to expression of GAPDH. 4E-BP1, eukaryotic initiation factor binding protein; AU, arbitrary units.

7.3.3 Greater cytochrome c protein expression with protein feeding and aerobic exercise

Preliminary experiments using standard Western blot transfer conditions with 10, 20, and 33 µg of total protein loaded per lane revealed a band of the expected size (15 kDa) in all lanes (Figure 7-6). 35 µg of total protein was loaded per lane to provide a robust signal for quantitative analysis. There were no other significant bands detected, indicating the specificity of the assay.

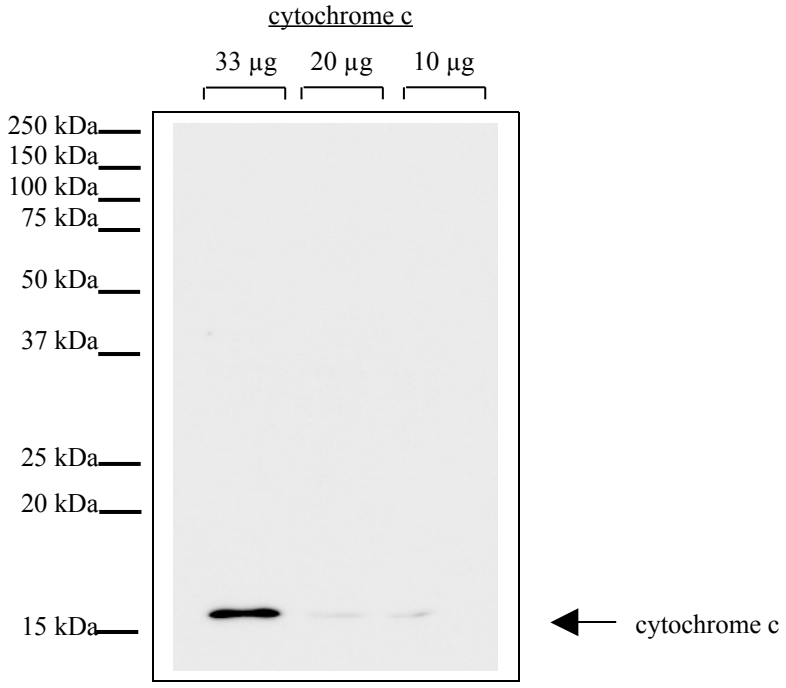


Figure 7-6 Assessment of antibody sensitivity and specificity for total immunoreactive cytochrome c
33, 20, or 10 µg total protein from homogenised muscle samples was loaded onto 15% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to nitrocellulose membrane, probed for cytochrome c, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of cytochrome c is 15 kDa. Exposure time is 15 minutes.

From the rested and fasted state (baseline), to six hours post-exercise and nutrition, all four subjects expressed either an equal amount or more immunoreactive cytochrome c in both the CHO and PRO conditions (Figure 7-7D). Three out of four subjects expressed more immunoreactive cytochrome c at six hours post-exercise in the PRO condition compared to the

CHO condition, although this trend did not reach statistical significance (p = 0.09, Figure 7-7C). These results are shown for subject 2 in Figure 7-7A.

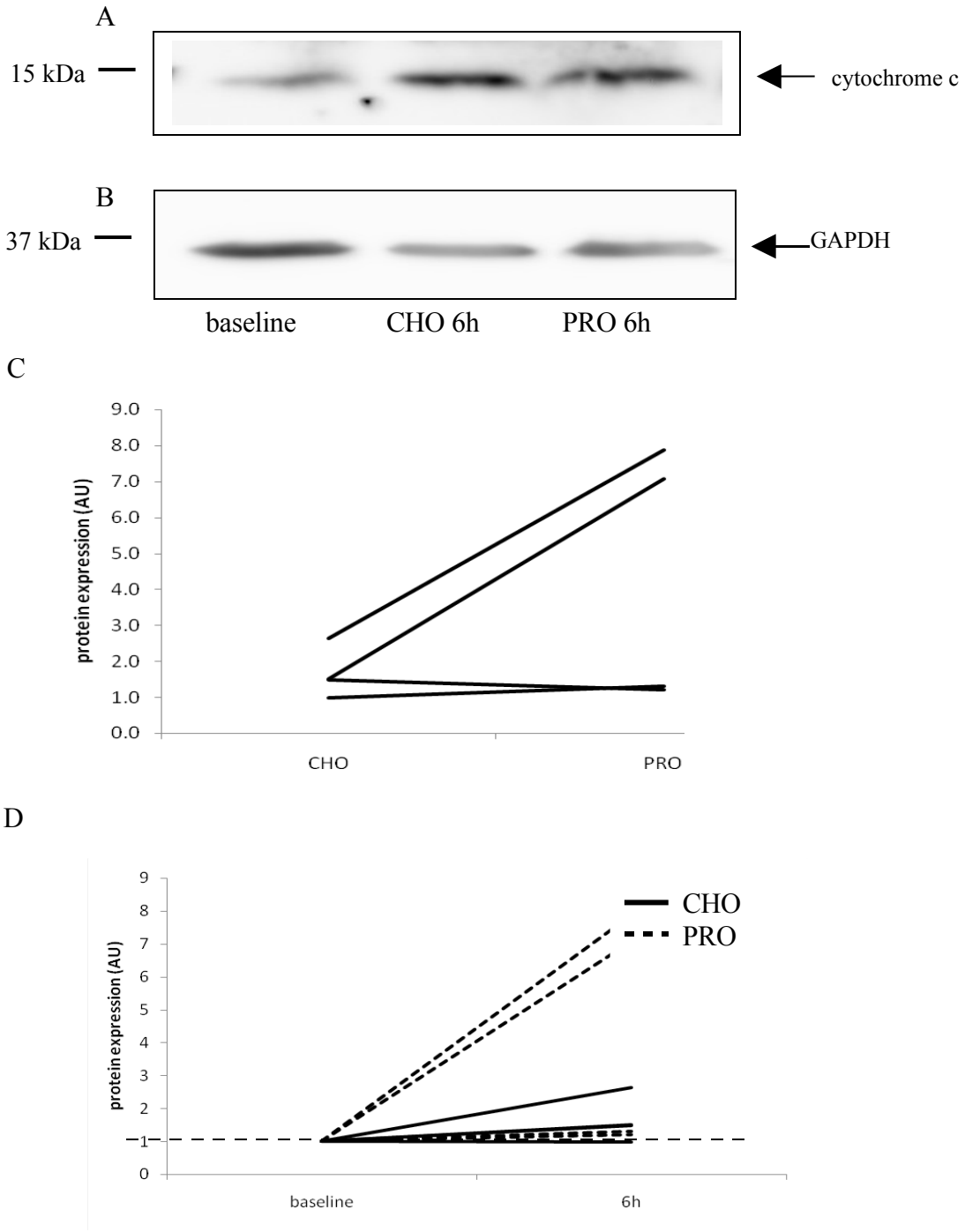


Figure 7-7 Change in expression of immunoreactive cytochrome c

A) Western blot for a single subject showing immunoreactive cytochrome c (exposure time was 4 min); quality was variable, the best results are shown. *B)* Immunoreactive GAPDH for the same three lanes as in *A* (exposure time was 1 minute). 35 µg total protein from homogenised muscle samples was loaded onto 15% Tris-glycine buffered acrylamide gel and subjected to electrophoresis. The gel was transferred to nitrocellulose membrane, probed for cytochrome c, and visualised using a Fuji LAS-3000 digital camera system. The molecular weight of cytochrome c is 15 kDa. All individuals' expression of cytochrome c *C)* at six hours post-exercise and *D)* -fold change from baseline; expression is relative to baseline which is set to 1. Error bars not shown for ease of interpretation. Intensity of bands was normalised to expression of GAPDH. AU, arbitrary units.

7.3.4 4E-BP1 phosphorylation may drive an increase in cytochrome c protein expression

The variability in responses between subjects highlights the importance of analysing changes within individual subjects. It is likely that the majority of the inter-subject variability can be explained by differences in insulin response and glycogen status, as discussed in Chapter 6. An analysis of individuals' responses reveals that there were no relationships between mTOR signalling and downstream events (Table 7-2). In neither the PRO nor the CHO condition did any of the subjects show an increase in phosphorylation of both mTOR and its downstream effector 4E-BP1. However, in the CHO condition, two subjects demonstrated an increase in 4E-BP1 phosphorylation and a later increase in total immunoreactive cytochrome c, while in the PRO condition three subjects demonstrated this relationship.

sub	CHO					PRO				
	phospho-mTOR		phospho-4E-BP1		cyt c	phospho-mTOR		phospho-4E-BP1		cyt c
	3h	6h	3h	6h	6h	3h	6h	3h	6h	6h
2	--	↓	↑	--	↑	--	--	↑	--	--
3	↓	--	↑	↑	--	--	↑	↑	--	↑
4	--	↑	--	--	↑	↓	↑	↑	--	↑
9	↑	↑	↑	--	↑	--	--	↑	--	↑

Table 7-2 Change from baseline in phosphorylation of mTOR (Ser^{2448}) and 4E-BP1 ($Thr^{37/46}$) and total cytochrome c for each subject

There were some correlations between mTOR signalling and downstream events. ↑, increase: increase in ratio of phosphorylated to total protein of greater than 1.2-fold from baseline; ↓, decrease: change in ratio of phosphorylated to total protein of less than 0.7-fold from baseline; -- no change: change in ratio of phosphorylated to total protein of 0.8-1.2-fold from baseline.

7.4 Discussion

There was greater 4E-BP1 phosphorylation at three hours post-exercise in the PRO condition compared to the CHO condition in three of the four subjects. There was also a greater expression of cytochrome c at six hours post-exercise in the PRO condition in three out of the four subjects. However, there were no differences between the CHO and PRO conditions in mTOR phosphorylation at either three or six hours post-exercise.

7.4.1 Protein-plus-carbohydrate feeding after aerobic exercise increases 4E-BP1 phosphorylation and cytochrome c expression more than carbohydrate-only

Since these experiments have been completed, two studies have been published examining the effect of nutrition provided after a bout of aerobic exercise on signalling in the mTOR pathway. In rats, Morrison et al. demonstrated that the effect of protein added to a carbohydrate drink provided after a three-hour bout of swimming, was increased 4E-BP1 phosphorylation . A confounding factor in Morrison et al.'s study was that the drinks were not isocaloric. Such a difference would have influenced their findings, as the protein-plus-carbohydrate drink, which provided the greatest amount of energy, also produced the greatest increase in phosphorylation. The presence of amino acids in the drink may have increased the circulating level of insulin beyond that induced by carbohydrate-only ingestion , which may have had effects on the mTOR signalling pathway. In the second study, undertaken in humans, a protein-plus-carbohydrate drink provided after a roughly hour-long bout of cycling resulted in greater mTOR phosphorylation than the provision of a placebo . Ivy et al.'s finding could simply be an effect of the food provided, because the same increase in mTOR phosphorylation following protein-plus-carbohydrate ingestion would be expected in the non-exercised condition . Regardless, Ivy et al.'s work did not investigate the specific effect of adding protein to a post-aerobic exercise carbohydrate feeding, which was the aim of the work in this thesis.

The 2.1 ± 0.3 fold elevation in 4E-BP1 phosphorylation observed in the PRO condition at three hours post-exercise is in agreement with previous findings following aerobic and resistance exercise, and subsequent protein-plus-carbohydrate feeding . Phosphorylation of 4E-BP1 causes its inactivation, thus allowing eIF4E to associate with eIF4G and translation initiation to proceed (Figure 2-5). Amino acids signal directly to mTOR via an insulin-independent pathway, as well as increasing the circulating level of insulin and sending a signal along the PKB pathway (Figure 2-4). mTOR signals directly to 4E-BP1, phosphorylating and

inactivating it. The greater level of 4E-BP1 phosphorylation in the PRO condition suggests that the additive effect of the carbohydrate, affecting insulin signalling, and the amino acids, affecting mTOR signalling, is greater than the insulin signal provided by carbohydrate alone. This finding is in keeping with the hypothesis but in contrast to Koopman et al.'s observation of no difference in 4E-BP1 phosphorylation at four hours post-resistance exercise between a carbohydrate-only and a protein-plus-carbohydrate condition. It is possible that the response to feeding after a bout of aerobic exercise differs to that after a bout of resistance exercise, which could explain the difference between Koopman et al.'s findings and those reported in the study presented here.

The expression of more immuno-reactive cytochrome c at six hours post-exercise in the PRO condition compared to the CHO condition suggests that the increased 4E-BP1 phosphorylation at three hours post-exercise had an up-regulatory effect on translation of mitochondrial protein, a finding in keeping with the hypothesis. This is the first study to report on the post-exercise expression of cytochrome c in skeletal muscle.

7.4.2 No difference in mTOR phosphorylation between feeding protein-plus-carbohydrate or carbohydrate-only after aerobic exercise

The lack of a difference in mTOR phosphorylation between the PRO and CHO conditions at either time point in the study reported here is in agreement with previous findings in rats and humans. Morrison et al. found the same degree of mTOR phosphorylation in rats fed carbohydrate-only or protein-plus-carbohydrate after a bout of aerobic exercise, which was greater than that in exercised but unfed rats, indicating no difference between the types of nutrition. Unfortunately, Morrison et al. did not use an unexercised control for comparison, so it is not possible to know whether there was an increase in mTOR phosphorylation in the fed conditions or a decrease in the exercise condition. In contrast, Benziane et al. did measure an increase in mTOR phosphorylation after a one-hour bout of cycling. However, Benziane et al.

did not determine the ratio of phosphorylated to total protein, but rather reported the absolute expression of phosphorylated mTOR, a method that may not have reflected a true change in phosphorylation as it does not exclude the possibility of greater expression of mTOR protein.

It is interesting to note that the increase in 4E-BP1 phosphorylation at three hours post-exercise in both the CHO and PRO conditions in this study was not accompanied by a measurable increase in mTOR phosphorylation, as the latter protein lies upstream of the former. It is possible that there was an increase in mTOR phosphorylation, but that it had returned towards baseline levels by the time the first post-exercise muscle sample was taken at three hours. Although it would have been helpful to have taken an earlier sample, the three hour sample was necessary to measure expected changes in mRNA (Chapter 6). Increasing the number of post-exercise biopsies to three would have meant a total of seven biopsies for each subject, which was not a viable option for both ethical and practical reasons. Phosphorylation of both mTOR and 4E-BP1 decreased from three to six hours post-exercise in most of the subjects in both conditions, which lends credence to the notion that phosphorylation of both these proteins may have peaked at some time prior to three hours post-exercise. Ivy et al. measured an 86% increase in mTOR phosphorylation at only 45 minutes after a bout of exercise and provision of nutrition similar to the protocol in this study. As the measured increase at three hours post-exercise in this study was only 50%, it is possible that this measure represented a decline from the 45-minute point in Ivy et al.'s study. Further support for this idea comes from the work of Mascher et al. , who measured a three-fold increase in mTOR phosphorylation in the first two hours of post-exercise recovery, following the same exercise parameters as used in this study, which had returned to baseline levels by three hours post-exercise. Alternatively, it is possible that the sampling point wasn't too late, but simply not at quite the right time, as mTOR is not up-regulated in a simple on/off fashion after exercise but rather exhibits fluctuating levels of phosphorylation over time . Bolster et al. sampled rat skeletal muscle at 5, 10, 15, 30 and 60

minutes after a bout of resistance exercise and found a transient increase in mTOR phosphorylation at 10 minutes post-exercise. It is also possible that the mutation of Ser^{2448} to alanine could have occurred, with no resultant effect on downstream activity. It is for this reason that 4E-BP1 is often accepted as a marker of mTOR activity . As 4E-BP1 can be phosphorylated in an mTOR-independent fashion, it is also possible that the 4E-BP1 phosphorylation measured in this study was not associated with a rise in mTOR phosphorylation at all .

7.4.3 More immuno-reactive mTOR and 4E-BP1 with carbohydrate-only feeding

In the study reported here, there was more immuno-reactive mTOR and 4E-BP1 at six hours post-exercise in the CHO condition than the PRO. As discussed in Chapter 6, protein synthesis is an energy-consuming process therefore it is possible that the greater availability of readily available energy in the CHO condition would have allowed synthesis of more of these proteins.

7.4.4 Multiple signals regulate protein expression

It is possible that the bout of exercise in this study and the nutrition provided were sending conflicting signals, and that one over-rode the other, thus diminishing the observed results. The decreased energy status following the exercise would have increased phosphorylation, and hence activation, of AMPK, which would have increased inhibition on mTOR and its downstream effectors via TSC2 and raptor . The effect of this inhibition may have been stronger than the stimulatory effects of the amino acids and insulin. The only way to clarify this possibility would be to conduct an exercise-only, unfed trial. Considering the extreme feelings of hunger reported by the subjects, even when they were fed, conducting an unfed trial would impose unnecessary discomfort. It has been shown, in rat skeletal muscle, that the insulin-induced stimulation of mTOR by carbohydrate ingestion is enough to over-ride the aerobic exercise-induced inhibition . Similarly, Atherton et al. found that low-frequency

electrical stimulation of rat skeletal muscle, designed to mimic aerobic exercise, down-regulated signalling in the mTOR pathway at both 4E-BP1 and mTOR, but that this decrease was reversed by feeding amino acids. In contrast, it has been shown that in human skeletal muscle, after a bout of resistance exercise, mTOR phosphorylation and AMPK and TSC2 activity can be elevated concomitantly . It is therefore quite possible that the exercise and food were sending conflicting signals in the study reported here.

Alternatively, it may be that the convergence of multiple signals activates the response that leads to muscular adaptation which, in the case of this study, is expected to be mitochondrial biogenesis. It is likely that signalling in the mTOR pathway is not specific to a mode of exercise, but that the specific proteins that get translated are due to the mRNA that is up-regulated being exercise-specific. For example, in Chapter 6 it was shown that a bout of aerobic exercise up-regulated the expression of PGC-1α mRNA, which would lead to increased mRNA expression of genes encoding mitochondrial proteins, and up-regulation in the mTOR pathway would then execute their translation.

7.4.5 Limitations and considerations

The greatest limitation in this part of the second study is that of insufficient power. As none of the trends discussed were statistically significant, any conclusions drawn are tentative. Increasing the number of subjects may have brought the observed trends closer to significance, or it may have produced opposite, significant findings. Using the data obtained, power calculations indicate that a sample size of 19 would have provided for a significant difference in 4E-BP1 phosphorylation between the conditions at three hours post-exercise, while a sample size of ten would have been enough to see a significant difference between the conditions at six hours post-exercise for expression of total immunoreactive cytochrome c .

A confounding factor in this study is that there was no exercise-only trial, as already discussed, so all the measured effects could be due simply to the nutrition provided, with no interactive effect of the exercise. Nutrient provision increases ATP availability, which in turn decreases AMPK signalling. A decrease in AMPK activation subsequently releases inhibition of mTOR by TSC2 and of translation elongation by eEF2 (Figure 2-2). An oral dose of essential amino acids taken in the rested state results in increased phosphorylation of both mTOR and 4E-BP1 at three hours post-ingestion , suggesting that nutrition alone could have produced the results observed in this study. Furthermore, one hour of cycling at 75% VO_{2max} results in no increase in mTOR phosphorylation at three hours post-exercise , suggesting that there may not have been any effect of the exercise on this variable in the study presented here.

As the subjects in this study had not exercised for 24 hours prior to the start of the long testing day, it is unlikely that they began the trial in a glycogen-depleted state. However, the bout of exercise may have been sufficient to deplete their glycogen stores , and the nutrition provided insufficient to replenish them. A test of glycogen content in the muscle samples would have verified this possibility. If this was the case, then muscle glycogen content would have been a confounding factor as this variable affects PKB phosphorylation, a protein upstream of mTOR and 4E-BP1, as well as the rate of MPS via GSK3 . Additionally, muscles that are glycogen-depleted remain more sensitive to insulin until glycogen has been restored , so all insulin-sensitive signalling would have been up-regulated in the muscles of subjects who experienced glycogen depletion.

Time and financial constraints, as well as the quantity of muscle lysate, limited the number of proteins to three that could be studied in this part of the second study. mTOR was chosen as it is the convergence point for so many signals for protein translation (Figure 2-4); 4E-BP1 was chosen as it is a direct inhibitor of translation initiation, and cytochrome c for its property as a mitochondrial protein with a reasonably short half life. The protein expression of cytochrome c

was assumed to be reflective of that of many mitochondrial proteins. There are plenty of other sites for potential regulation between mRNA expression and the formation of new mitochondrial reticulum, such as post-translational modification of proteins other than mTOR and 4E-BP1, and changes in the rates of protein import, folding, and assembly, all of which are affected by exercise . There are many other mitochondrial proteins worthy of study, as well as other sites in the pathway signalling for protein translation. For example, phosphorylation and inactivation of GSK-3β by aerobic exercise relieves inhibition on eIF2B, and so has an up-regulatory effect on translation initiation . Furthermore, mTOR does not account for all leucine-induced stimulation of MPS , and there are other mechanisms and pathways, such as MAPK and eIF2, that influence translation initiation and elongation . Also, 4E-BP1 phosphorylation status is not the only variable controlling eIF4E-eIF4G association; it has been shown that feeding carbohydrate alone leads to increased formation of the active eIF4F complex in the absence of increased mTOR and 4E-BP1 phosphorylation, suggesting an alternate signalling pathway . Measurement of eIF2 activity, eIF4E-eIF4G association, and expression of other mitochondrial proteins such as citrate synthase or succinate dehydrogenase , all could provide additional insights into the regulation of mitochondrial protein translation after a combination of feeding and aerobic exercise.

7.4.6 Conclusion

In conclusion, the provision of a protein-plus-carbohydrate beverage after a high-intensity bout of cycling resulted in greater 4E-BP1 phosphorylation and greater levels of cytochrome c expression than a carbohydrate-only beverage in three out of four subjects. This is the first study to compare the effects of a protein-plus-carbohydrate and a carbohydrate-only beverage provided after a bout of aerobic exercise on signalling for protein translation in human skeletal muscle.

8 Concluding remarks and future directions

8.1 Concluding remarks

The main findings from the studies and experiments in this thesis are that a protein-plus-carbohydrate drink (PRO) provided immediately after a bout of aerobic exercise had a greater effect on WBPS and global signalling for protein translation than a carbohydrate-only one (CHO), while the opposite appeared to be true for signalling specific to mitochondrial biogenesis. From two through to four hours of post-exercise recovery after a bout of moderate-intensity cycling, older individuals consuming the PRO drink exhibited a 19% higher rate of NOLD than those consuming the CHO drink (Chapter 4). NOLD, a measure of WBPS, encompasses the synthesis of all the body's proteins, of which skeletal muscle mitochondria is a component. At three and six hours of post-exercise recovery following a bout of moderately high intensity cycling, young adults consuming the PRO drink exhibited greater phosphorylation of 4E-BP1 and greater protein expression of cytochrome c in skeletal muscle than those consuming the CHO drink (Chapter 7). Phosphorylation of 4E-BP1 is a required step in the initiation of protein translation while cytochrome c is a mitochondrial protein in the electron transport chain. Other researchers have shown a correlation between 4E-BP1 phosphorylation and mixed muscle FSR after a bout of resistance exercise, so it is possible that the 4E-BP1 phosphorylation measured in Chapter 7 could be reflective of mitochondrial FSR . As the rate of mitochondrial FSR was not determined successfully in Chapter 5, such a connection could not be evaluated from the work in this thesis. At three and six hours of post-exercise recovery following a bout of moderately high intensity cycling, subjects consuming the CHO drink tended to have higher mRNA expression of PGC-1α, PPARδ, and COXI than those consuming the PRO drink (Chapter 6). Up-regulation of PGC-1α and PPARδ has been implicated in increased mitochondrial biogenesis, while COXI is a mitochondrial enzyme involved in ATP synthesis.

The adaptations described in this thesis occur at the cellular, muscular, and whole body level. After a single bout of exercise, cellular signalling produces a response in muscle which is small but which accumulates incrementally with those occurring after subsequent bouts. Over a period of training, the responses accumulate to drive a process of skeletal muscle remodelling . As reviewed in 2.1.1, the type of exercise stimulus drives the type of adaptation. The magnitude and nature of the molecular signalling response is related to the intensity and duration of the exercise bout. The energetic demands of the one-hour bouts of moderate or moderately-high intensity cycling used in this work would lead to enhanced mitochondrial biogenesis, in order to better meet the demands of the next exercise bout . Although a measure of mitochondrial FSR was not achieved successfully in the work reported in this thesis, several other measures suggest that the rate was up-regulated in response to the exercise and nutrition stimuli provided.

Over a period of training, the increase in muscle mitochondrial protein content would provide greater muscular endurance capacity. For the older individual, such a change could provide a much better quality of life and a level of fitness commensurate with that of a younger person. A training programme designed to increase mitochondrial content to a new, higher steady state amount would last at least six weeks . Each exercise bout would be of a moderate to moderately high intensity, and last between 20 and 60 minutes with three to five sessions being performed each week . Once achieved, maintenance of this higher level of content could occur with only thrice-weekly bouts of similar exercise.

Post-exercise nutrient availability seems to maximise the response of protein synthesis to exercise. Cuthbertson et al. found that the effect of different types of resistance exercise on signalling for protein synthesis was the same when post-exercise nutrition was provided. Cuthbertson et al.'s findings are in contrast to those of Eliasson et al. , who found a difference

between subjects performing the different modalities in the post-absorptive state, indicating that a maximal response is obtained when post-exercise nutrition is available. The question of which of the two drink conditions tested in this thesis provided the greatest increase in mitochondrial protein has not been answered conclusively. From the results collected in this work, it appears that the rate of synthesis of many of the body's proteins was up-regulated more in the PRO condition, but that intracellular signalling for new skeletal muscle mitochondria was stronger in the CHO condition.

8.2 Future directions

There is considerable scope for future research, both in repeating some portions of the work presented in this thesis, and also in extending it. The work described here provides promise and direction for further studies with broad applications in human health, successful ageing, and sports performance.

8.2.1 Scope for future research

The next step in the pathway of work described in this thesis is a repeat of the experiment reported in Chapter 5 with sound procedures in place for operation of the IRMS and GC-C-IRMS. Repeating this work would provide specific information regarding the effect of the PRO and CHO conditions on the rate of skeletal muscle mitochondrial protein synthesis which is an essential piece of the puzzle. Ideally, large enough muscle biopsy samples could be obtained so that the analyses described in Chapter 7 could be repeated as well. Information regarding protein expression and phosphorylation status of a larger number of subjects than that possible in Chapter 7 would provide sufficient power to test for significance. It may also be helpful to include a third, unfed condition in these experiments, to isolate the effects of the nutrition from those of the exercise.

Training studies, comprised of a period of several weeks, in which subjects exercised three to five times each week and then received the PRO or CHO drink immediately after each exercise session, are another area for future work. Measurements of skeletal muscle mitochondrial gene expression and protein content, as well as whole body VO_{2max} and endurance capacity, could be made both before and after the training period. Such studies would reveal whether the acute changes described in this thesis are indicative of longer term adaptations. The exercise sessions could consist of either the sustained bouts described here, or investigate the effects of other intensities and durations. The effect of high-intensity interval training on mitochondrial adaptation is a currently emerging area of research (e.g.).

It would also be interesting to explore the combination of signalling in the PGC-1α and mTOR pathways following a bout of exercise comprised of both aerobic and resistance exercise in a circuit style. Whether the increase in AMPK signalling caused by the high energetic demand of such exercise could over-ride the hypertrophic response to the resistance component is a fascinating question. A measurement of AMPK activity as well as phosphorylation would be informative, as it has been shown in rat skeletal muscle that this variable varies inversely with the rate of MPS . Bouts of 'mixed' exercise such as this could be part of either an acute or a training study with each exercise session followed by the type of nutrition determined in the preceding work to provide the greatest response.

The question of whether it is essential to provide nutrition immediately following a bout of exercise to achieve the maximum response is one that has been answered equivocally in the literature. It would be informative, therefore, to conduct a study in which nutrition was provided either immediately, or a few hours after the cessation of a bout of aerobic exercise in a training programme. The effect on the rate of mitochondrial protein synthesis and signalling

in the PGC-1α and mTOR pathways could all be examined after an acute intervention, or gene and protein expression examined in a training study.

Ideally the studies described above would be conducted in populations of older or elderly individuals. As reviewed in Chapter 2, the anabolic effects of amino acids and insulin on the rate of MPS and the activation of translation initiation in elderly may be impaired . Therefore, it seems pertinent to determine the above-mentioned effects of post-aerobic exercise nutrition on the population of interest, i.e. adults aged over 55.

To further extend the information obtained in this thesis, muscles other than the *m. vastus lateralis* could be sampled, as the effect of age on muscle and mitochondrial function may vary between muscle groups . To this point, mRNA and protein synthesis have been considered, and an examination of degradation also would contribute to a more complete picture. Furthermore, signalling at more points along the pathways described in this thesis would be informative. For example, in the mTOR pathway phosphorylation of PKB, TSC2, $p70^{S6K1}$, eEF2, and GSK-3 could be measured during the post-exercise period. In the PGC-1α pathway, phosphorylation of p38MAPK and ATF2, gene expression of more mitochondrial proteins such as the subunits of COX and NADH, and protein expression of those genes studied in Chapter 6, are all areas which would provide useful information. A biopsy taken within the first hour of post-exercise recovery, and one taken at 18 hours following cessation of exercise, would both add information to the picture obtained at three and six hours post-exercise. Rat skeletal muscle harvested at the later time point has shown a measurable increase in PGC-1α protein expression .

8.2.2 Application of information

An improved understanding of how to promote mitochondrial biogenesis has a number of applications. The information in this thesis and subsequent experiments it may prompt, could

be used in the development of training and nutrition methodologies to enhance sporting performance. The greater the quantity of mitochondria in skeletal muscle, the greater that muscle's endurance capacity. Enhanced endurance capacity would lead to greater performance in such endurance sports as cycling, running, and triathlon. Mitochondrial biogenesis has implications beyond the sporting arena however, as it affects human health in a number of ways.

The information gained from this work regarding changes in protein synthesis and signalling caused by post-exercise nutrition provides insights into creating interventions to slow, halt, or even reverse some aspects of sarcopenia. An aerobic exercise training programme combined with protein-plus-carbohydrate post-exercise nutrition could slow or reverse the accumulation of muscle dysfunction with increasing age, and provide older individuals with a practical method of attenuating the age-related decline in mitochondrial protein synthesis. Theoretically, the right combination of exercise and nutrition will increase mitochondrial biogenesis and function, which will lead to increased ATP production and decrease ROS production. In this way the vicious cycle of ageing and inactivity can be broken, and healthy ageing, with a maintenance of functional capacity, can be promoted.

At the molecular level, a better understanding of regulation in the PGC-1α and mTOR signalling pathways will help in the development of new therapies to treat muscular and mitochondrial dysfunction and disease. Currently, very little is known about the role that exercise-induced mitochondrial biogenesis could play in attenuating age-related mitochondrial dysfunction and its attendant myopathies. Measurements of MPS and intracellular signalling in older and elderly individuals may provide insight into the molecular basis for the age-related defects in muscle protein metabolism that contribute to sarcopenia, and may suggest relevant targets in the development of therapeutic interventions. Specific drug targets could be

developed to inhibit the age-related decline in skeletal muscle quality and quantity. For example, a CaMK agonist could be used to increase oxidative capacity, while targets in the mTOR pathway may increase skeletal muscle protein content. Such a notion is not just wishful thinking, as demonstrated by Narkar et al. , who have shown that agonists of AMPK and PPARδ taken orally enhance endurance capacity in mice. Similarly, Lagouge et al. have shown that resveratrol-treated mice have greater expression of PGC-1α mRNA and protein, and higher endurance and aerobic capacities. Therapies such as these may provide for the possibility of returning normal muscle function to people who cannot exercise at intensities sufficient to induce adaptive mitochondrial biogenesis.

Understanding the mechanisms by which skeletal MPS is modulated during periods of energy stress, such as occur during a bout of aerobic exercise, may provide information for the development of nutritional strategies to use in metabolic disease therapy. The progression of other diseases associated with ageing, such as osteoporosis and non-insulin dependent diabetes, could also be slowed or even halted with the use of a regular programme of exercise and nutrition as described in this thesis.

9 Appendices

Appendix A Stable isotope methodology

Stable isotope methodology can be used to investigate such metabolic processes as protein metabolism by introducing a labelled amino acid into the body and then following the fates of that label. Enrichment is defined as the ratio of labelled to unlabelled amino acid within a body's pool and can be measured using mass spectrometry. Blood is often sampled as a representative of the body water pool, which includes intracellular and interstitial fluid as well as plasma. A number of assumptions and simplified models are inherent in the use of metabolic tracers which permit accurate results within certain defined parameters of experimental design . Tracers can be introduced either orally or intravenously, with there being a number of choices of dose and infusion time, depending on experimental conditions and the amino acid being used .

When using stable isotopes in metabolic research, the following assumptions must hold true if findings are to be considered accurate. The first assumption is that the tracer is metabolised in the same way as the trace, and does not change the latter's metabolism. Because it is the electron configuration of an atom that affects its chemical properties, rather than the number of neutrons, isotopes are roughly analogous in chemical and functional properties, and therefore should not have any significant effects on metabolic pathways . This assumption is supported by experimental data . Further assumptions are that the size of the tracee pool remains constant throughout the study, and that the tracer is incorporated into body protein at a linear rate . Factors affecting the size of the pool include ingestion of food, and changes in the rates of WBPS and breakdown. Finally, it is assumed that the tracer is not recycled during the time course of the experiment .

A1 Whole body protein turnover

Whole body protein turnover is determined using the single pool model. Tracer studies must be performed in the steady state, a situation in which the rate of disappearance (R_d) of tracee from the pool is equal to the rate of appearance (R_a), such that the size of the pool is constant. Enrichment is not affected by R_d because it is assumed that there is no selectivity of uptake between the tracer and tracee.

Figure A-1 illustrates the change in enrichment of a steady-state pool during continuous infusion of a tracer. When a tracer is infused, it diffuses throughout the entire pool, is diluted by all the unlabelled tracee, and the enrichment of the pool is initially low (A-1A). Both tracer and tracee leave the pool in a ratio roughly equal to the pool's enrichment. After some time the pool's enrichment increases gradually, as more and more tracer is infused and diffused throughout the pool (A-1B). Finally, a plateau in enrichment is reached, at which point the R_a and R_d of the tracer are equal (A-1C). A situation in which the enrichment of a pool is constant is referred to as isotopic equilibrium . It is important to understand that physiological changes, such as those that occur during exercise , continue to occur during isotopic equilibrium; only the enrichment value is unchanged.

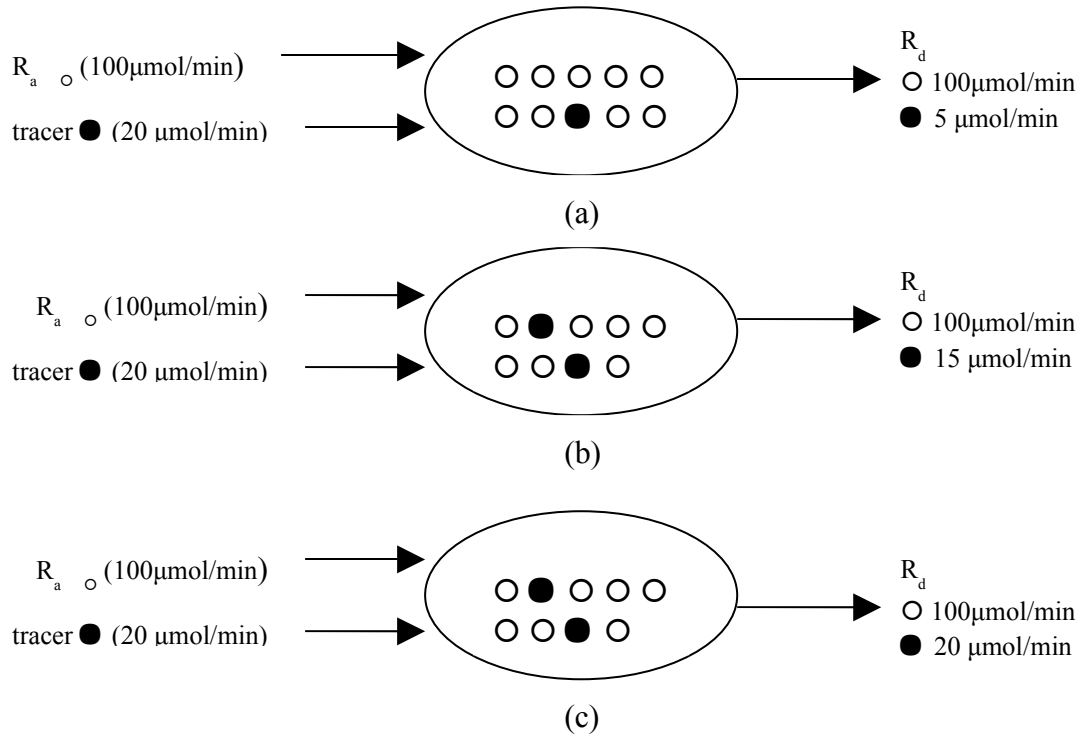


Figure A-1 Change in enrichment of a steady state pool during continuous infusion of a tracer

When a tracer (●) is infused into an unlabelled (○) pool, enrichment is initially low, and gradually increases until a steady state enrichment is reached at which $R_a = R_d$. R_a, rate of appearance; R_d, rate of disappearance. Adapted from .

Once isotopic equilibrium is reached, R_d can be assumed to be equal to a calculated value of R_a using the following equation;

$$R_a = F / E_p$$

where F is the infusion rate of the tracer and E_p is the enrichment of the pool being studied. Such a calculation is accurate if the tracee appears directly into, and is irreversibly lost from, the pool being studied. Sources of disappearance from the body's free pool are oxidation and protein synthesis. In isotopic equilibrium, calculations of whole body protein synthesis can be made according to the standard equation;

$$R_a = R_d = \text{oxidation} + \text{WBPS}$$

which can be rearranged to give;

$$\text{WBPS} = R_a - \text{oxidation}$$

In the case of a leucine tracer;

$$\text{oxidation} = \frac{(E_B * \text{total } CO_2 \text{ production}/0.81)}{E_P}$$

where E_B is the enrichment of expired breath.

A2 Fractional synthesis rate

The whole body protein turnover method provides information about all the body's proteins considered together as one pool. Often, specific information regarding the turnover of a particular fraction, such as muscle, is required, and the FSR method is used.

Because human skeletal muscle turns over relatively slowly, it is not practical to wait for the precursor and product pools to reach equilibrium enrichment. FSR in skeletal muscle is always determined from two time points between which the enrichment of the product pool is increasing linearly (Figure A-2). In order for linear tracer incorporation to occur, the size of the product pool, the FSR, and the enrichment of the precursor pool must all be constant . Tracer infusion must occur for long enough before the first biopsy that an isotopic equilibrium in the precursor pool is reached.

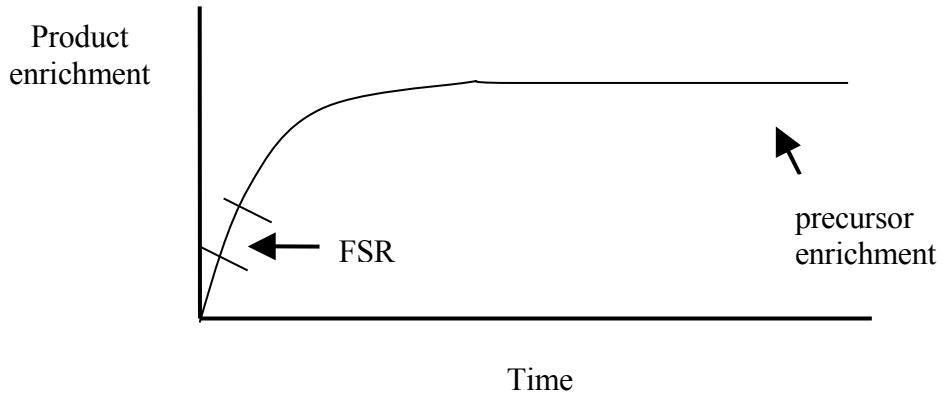


Figure A-2 Calculation point of FSR during the continuous infusion method

FSR is calculated during a period of linear increase in product enrichment. Enrichment is measured as the ratio of tracer to tracee. Enrichment of the product pool is equal to that of the precursor at the plateau of product enrichment. Adapted from .

Because FSR is calculated over the initial phase of product enrichment when absolute values are extremely low, it is highly unlikely that a labelled amino acid would be released during this phase, therefore making the assumption of no tracer recycling valid .

FSR is calculated according to the standard equation;

$$FSR = \frac{E_b(t_2) - E_b(t_1)}{E_p * t}$$

where E_b is the enrichment of the bound tissue protein at the time of the biopsy, E_p is the enrichment of the precursor pool, and t is the time between the biopsies.

A number of studies have raised the question of which precursor pool to use as a surrogate measure of tRNA enrichment in calculations of FSR. Amino acid enrichment may vary between pools, as the rate of equilibration between intracellular fluid and plasma is dependent upon transmembrane transport and can vary between amino acids . Baumann et al. infused [1-^{13}C]leucine and [*ring*-$^{2}H_5$]phenylalanine into swine and sampled a number of pools. They found a considerable difference in enrichments between possible precursor pools and tRNA, with arterial enrichment of both labels 30% higher, plasma α-KIC enrichment closer but still higher, and intracellular enrichments very close. An artificially high value for precursor enrichment will result in an erroneously low calculated value for FSR. In the case of the leucine tracer it is likely that the amount by which the tissue fluid enrichment exceeded that of the tRNA represents that leucine which will be oxidised rather than incorporated into muscle protein . In a study using cultured skeletal muscle cells and an infusion of [1-^{14}C]leucine, Schneible et al. determined that 60% of intracellular leucine derived from protein degradation is used for protein synthesis at 0.5 mM extracellular leucine. Varying the concentration of the medium did affect the source of leucine for its various fates, which appears to underscore the need to sample the correct precursor pool when determining FSR in animals.

In humans, the choice of precursor pool may not be as important as it is in animals. Ljungqvist et al. infused [1-^{13}C]leucine in the fasted and a variety of fed states. The authors found the same enrichments in the tissue fluid leucine and leucyl-tRNA pools, while plasma leucine and α-KIC, and tissue fluid α-KIC enrichments, were all higher. In agreement with both Ljungqvist et al. and Baumann et al., Martini et al. found that using tissue free enrichment best approximated true rates of protein synthesis. In contrast to the above results, Watt et al. found that labelling of human skeletal muscle tRNA following primed, continuous infusion of [1-^{13}C]leucine was lower than venous plasma leucine and α-KIC, but higher than that of tissue free leucine. They suggested that a specific pool exists from which tRNA is charged, which is fed equally by both extra- and intracellular sources. Their use of either plasma α-KIC or tissue fluid leucine as the precursor pool resulted in over- or underestimation of MPS rate by equal amounts, which suggests that sampling either pool is appropriate.

In addition to methodological considerations regarding choosing the correct precursor pool and ensuring the assumptions of the FSR method are met, there are some practical considerations with regards to the infusate in experiments that calculate FSR. An essential amino acid should be chosen that does not have a large gradient between extra- and intracellular enrichment because some extracellular fluid will inevitably be included in the biopsy sample (Figure A-2). An amino acid with a low endogenous turnover rate is desirable in order to maintain a reasonable approximation of isotopic equilibrium. With these two considerations, and the findings of Baumann et al. in mind, leucine is a good choice of tracer for FSR studies. Leucine has a low concentration gradient from the outside to the inside of cells and, in studies where whole body protein turnover is also of interest, is a logical choice. It is also sensible to choose an amino acid that is abundant in the target protein so that detectable amounts of tracer are incorporated . Leucine is abundant in muscle but not in the body's free pool , further enhancing its suitability for muscle study. A final consideration is the rate of infusion, which

should be about 7% of the plasma R_a for the infused amino acid. 7% plasma enrichment results in 4-5% intracellular enrichment, which is the optimal range for measurement via GC-MS .

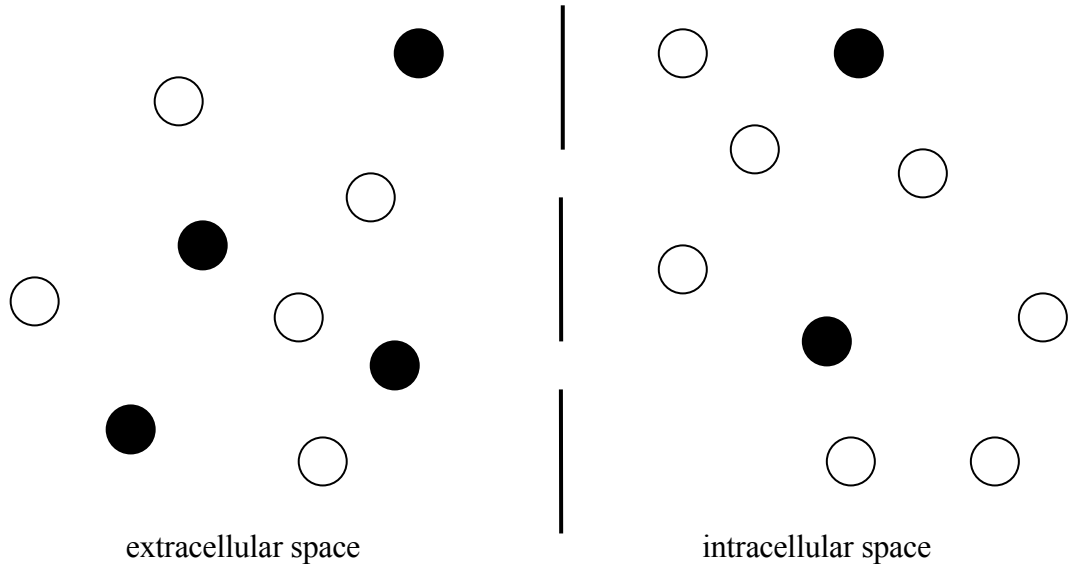


Figure A-3 Representation of enrichment gradient between the muscle extracellular and intracellular space
If the ratio of labelled (●) to unlabelled (○) molecules is very different between the two spaces then inadvertent inclusion of extracellular fluid in the intracellular sample during muscle biopsy processing, will introduce error into the value for intracellular enrichment.

A3 Other methods: arterio-venous balance and 3-methylhistidine

Another method for the determination of muscle protein turnover is the A-V balance method. Theoretically, muscle protein turnover accounts for the majority of protein turnover within a limb because 60-70% of limb volume is muscle, and the other tissues such as bone, skin, and adipose contribute either not at all, or an insignificant amount to protein metabolism . In the A-V balance method, catheters are inserted into the main artery leading into, and the main vein draining from, the limb of interest, commonly the femoral artery and vein in the leg. Determining the R_d of a tracer from the artery, and the R_a into the vein, thus gives an indication of muscle protein metabolism in the limb, using similar equations to those used for determination of whole body protein kinetics (Figure A-4). In the two-pool model R_d is an indicator of MPS, while R_a is an indicator of MPB. In the three-pool model a third

compartment, muscle, is sampled via biopsy, and rates of intracellular appearance and disappearance used to determine MPB and MPS respectively .

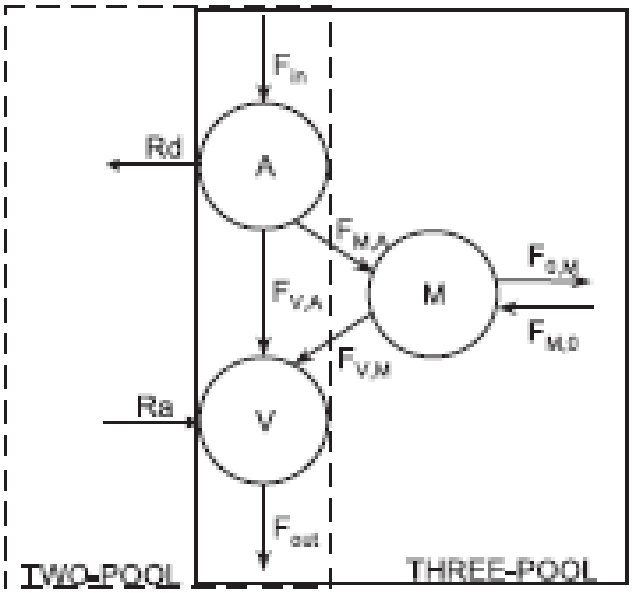


Figure A-4 Two- and three-pool models using the arterio-venous balance method
Free pools in the artery (A), vein (V), and muscle (M) are connected by arrows indicating unidirectional flow (F) between each compartment. In the three-pool model O denotes the intracellular fluid. From .

There is considerable room for error in values of MPS and MPB determined using the A-V balance technique. The first is that the R_d does not take into account amino acids in the intracellular fluid being used for MPS, and so underestimates this value. Underestimation of MPS leads to underestimation of MPB, which is calculated by subtracting net balance of the tracee across the limb from the value for MPS. In physiologically non-steady-state conditions, such as those stimulated by feeding or exercise, one of either MPS or MPB will be underestimated more, relative to the other, resulting in conclusions of questionable validity . On the other hand, the two-pool model assumes that the difference in amino acid concentration between the artery and the vein represents amino acids being used for MPS when, in fact, the concentration of the intracellular pool may just be increasing. Such a situation would cause an overestimation of the rate of MPS. Furthermore, amino acids may leave the intracellular pool and return to plasma without having any effect on synthesis.

In contrast to the FSR method described in A2, which provides a direct measurement of the rate of protein synthesis, the A-V balance method relies on proxy values . The FSR method determines actual incorporation while the net uptake from plasma and net balance of the A-V method infer synthesis, which is only true if there is subsequent uptake from the intracellular pool.

A second problem with the A-V balance method is that it relies on values of amino acid concentration and enrichment in the blood, which are very small relative to a large amount of blood flow. When blood flow increases during exercise, percentage error is compounded, further compounding inaccuracies . Furthermore, calculations are made based on measurements taken simultaneously from the artery and vein, while the delay in flow between these two compartments could be as much as 15 minutes. Only in the steady state would it be appropriate to assume that conditions are constant over this time frame.

Another technique for measuring whole body protein breakdown involves the use of 3-MH, a natural tracer. 3-MH is found in actin and myosin, the two main contractile proteins in human skeletal muscle, and when released as a result of MPB cannot be reused for MPS and so is excreted . Measurement of urinary 3-MH concentration is therefore an indicator of MPB. The problem with this method is that dietary, skin and gut proteins also contribute to urinary 3-MH excretion, with actin being present in all cell types . The gut has rates of protein turnover up to 20 times that of skeletal muscle, particularly in the elderly, thereby contributing to a greater percentage of excretion than its presence in the protein-bound pool. Urinary 3-MH therefore likely overestimates MPB .

Appendix B Common protocols

B1 Bruce Protocol

1. Apply electrodes and connect leads for 12-lead ECG (Figure B-1). Obtain resting printout and blood pressure with subject in supine and standing positions.
2. Allow subject to familiarise himself with stepping off moving treadmill.
3. Explain test and questions that will be asked.
4. Start treadmill on first stage; each stage lasts for three minutes (Table B-1).
5. In the last minute of each stage record heart rate, rating of perceived exertion (RPE), and ask subject if they are experiencing any chest pain, shortness of breath or dizziness. If subject reports chest pain ask them to rate it on a scale from one to ten with one being mild and ten being severe. If subject reports shortness of breath ask them to rate it on a scale from one to four with one being mild and four being severe.
6. In the last 30 seconds of each stage record blood pressure and ask subject if they think they can go on to the next stage.
7. In the last 15 seconds of each stage print ECG and warn subject that the next stage is about to start.
8. Test finishes when subject reaches 85% of age-predicted heart rate maximum (HR_{max}) or there are indications for terminating exercise testing (Figure B-2).
9. Remove subject from treadmill and have them lie down in a supine position.
10. At one, three, and five minutes of recovery record heart rate and blood pressure, print ECG, and ask subject if they are experiencing any chest pain, shortness of breath or dizziness.

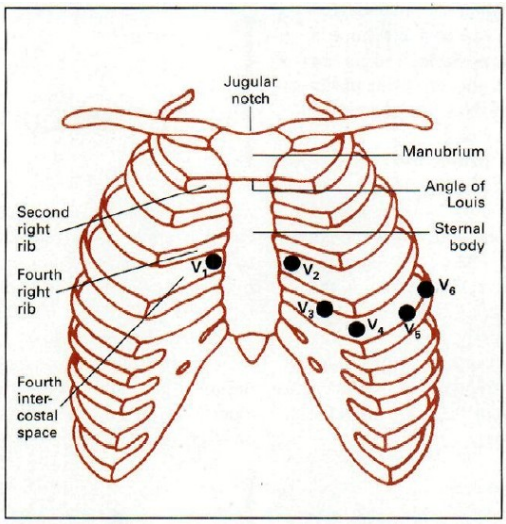


Figure B-1 Electrode placement for 12-lead ECG

Chest electrodes are placed at V1 through V6. Left and right arm electrodes are placed below the midpoint of the clavicle on the left and right sides respectively. Left and right leg electrodes are placed two finger-widths below the bottom rib on the left and right sides respectively. From .

Stage	Speed (km/h)	Gradient (%)
1	2.7	10
2	4.0	12
3	5.4	14
4	6.7	16

Table B-1 Stages of Bruce Protocol

Absolute indications

- Drop in systolic blood pressure of >10 mg Hg from baseline blood pressure despite an increase in work load, when accompanied by other evidence of ischemia
- Moderate to severe angina
- Increasing nervous system symptoms (eg, ataxia, dizziness, or near-syncope)
- Signs of poor perfusion (cyanosis or pallor)
- Technical difficulties in monitoring ECG or systolic blood pressure
- Subject's desire to stop
- Sustained ventricular tachycardia
- ST elevation (≥1.0 mm) in leads without diagnostic Q-waves (other than V_1 or aVR)

Relative indications

- Drop in systolic blood pressure of ≥10 mm Hg from baseline blood pressure despite an increase in workload, in the absence of other evidence of ischemia
- ST or QRS changes such as excessive ST depression (>2 mm of horizontal or downsloping ST-segment depression) or marked axis shift
- Arrhythmias other than sustained ventricular tachycardia, including multifocal PVCs, triplets of PVCs, supraventricular tachycardia, heart block, or bradyarrhythmias
- Fatigue, shortness of breath, wheezing, leg cramps, or claudication
- Development of bundle branch block or IVCD that cannot be distinguished from ventricular tachycardia
- Increasing chest pain
- Hypertensive response*

*In the absence of definitive evidence, the committee suggests systolic blood pressure of >250 mm Hg and/or a diastolic blood pressure of >115 mm Hg. ECG indicates electrocardiogram; PVCs, premature ventricular contractions; ICD, implantable cardioverter-defibrillator discharge; and IVCD, intraventricular conduction delay. Modified from Fletcher et al.[7]

Figure B-2 Indications for terminating exercise testing

From .

B2 Blood draw

1. Lie subject supine on collection table.
2. Warm area surrounding vein by wrapping in electric blanket.
3. Place spill sheet under arm. Apply tourniquet proximal to vein.
4. Wipe vein and surrounding area clean with alcohol swab.
5. Assemble needle and vacutainer holder. Insert needle into vein at a roughly 30 ° angle.
6. Insert vacutainer into holder. Remove tourniquet and allow vacutainer to fill.
7. Gently remove vacutainer while holding needle steady.
8. Insert and fill additional vacutainers as required.
9. Once final vacutainer has been removed, remove needle and replace immediately with gauze. Apply pressure then tape gauze in place.
10. Gently invert vacutainers five or six times then store as appropriate for relevant test.

B3 VO_{2max} test

1. Turn on and warm up metabolic cart. Calibrate analysers for both gas (room and standard gas) and volume fractions.
2. Assemble clean mouthpiece and headgear. Double check to ensure Hans Rudolph one-way valves open the correct way.
3. Attach heart rate monitor strap to participant.
4. Allow participant to warm up for as long as desired.
5. When participant is ready, allow them a drink of water, then put on the headgear. Double check that gas analysis and heart rate equipment is recording accurately.
6. Begin test with a 3-2-1 countdown.
7. Increase intensity as required by experimental protocol.
8. Record heart rate and RPE every minute.
9. Test stops at volitional fatigue.
10. Upon termination remove workload and allow participant to cool down for as long as desired.
11. A successful maximum is indicated by any of respiratory exchange ratio (RER) > 1.1, heart rate within 10 beats of age-predicted maximum, RPE > 18, a plateau in VO_2 with increasing workload.
12. Clean and sterilise mouthpiece, valves and headgear. Clean tubes and bike.

B4 Skinfolds

Landmarks

Acromiale

Point at superior and external border of acromion when subject is standing erect with relaxed arms.

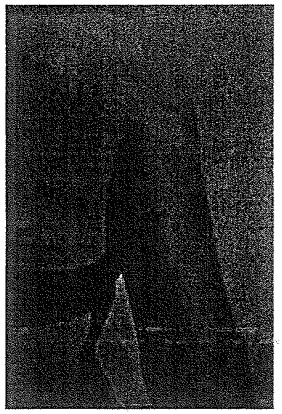
1. A marking pencil as a straight edge is applied to the lateral aspect of the acromion process at an angle of 45° to depress tissue and identify the superior border
2. The most lateral point on the superior border is identified by the left thumb
3. The pencil pressure is removed and the landmark is confirmed on the uncompressed surface
4. The landmark is marked
5. The landmark is re-identified as a check, using steps 1 to 3

Landmark identification

Radiale (r)

The point at the upper and lateral border of the head of the radius.

1. Using the left thumb or index finger, the anthropometrist palpates downward in the lower portion of the lateral dimple of the elbow
2. Slight pronation/supination of the forearm is reflected by a rotary movement of the head of the radius
3. Marking and checking the landmark are done as above

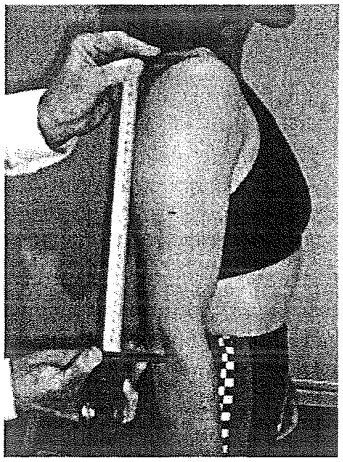


Landmark identification

Mid-acromiale-radiale

Arm girth, triceps and biceps skin fold sites.

1. A line is marked horizontal to the long axis of the humerus at the mid-acromiale-radiale distance, as determined by an anthropometric tape
2. The horizontal line is extended to the posterior surface of the arm, where a vertical line at the most posterior surface is made to intersect with the horizontal line, to mark the site where the triceps skin fold is raised.
3. The biceps site is marked anteriorly

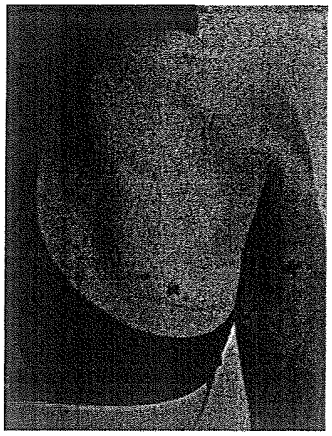


Landmark identification

Subscapulare (ssc)

The under most tip of the inferior angle of the scapula.

1. Palpate the inferior angle of the scapula with the left thumb
2. If there is difficulty locating the inferior angle, the subject can assist by reaching behind the back with the right arm
3. The site must not be marked, however, until the arm is returned to the side in the functional position
4. The mark is made 2 cm distal to a 45° angle from the tip

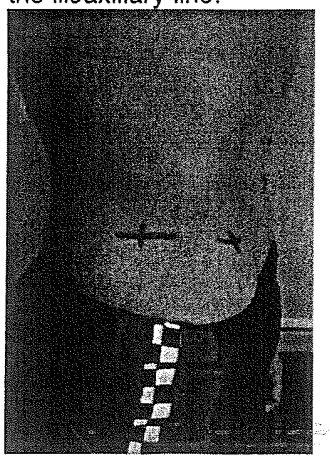


Landmark identification

Iliocristale

The point on the most lateral aspect of the iliac tubercle on the ilioaxillary line.

1. With the subject's arms placed horizontally in a lateral position, locate the most lateral superior edge of the ilium using the right hand
2. Use the left hand to stabilize the body on the opposite ilium
3. The landmark is at the edge of the ilium which is intersected by the imaginary vertical line from the mid-axilla

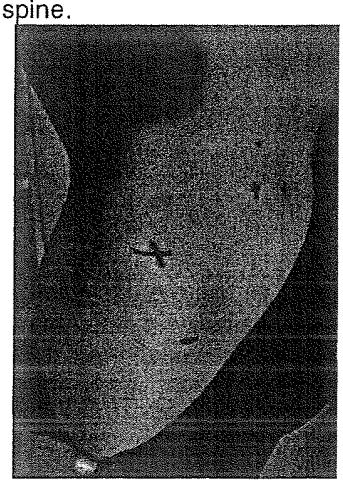


Landmark identification

Iliospinale (ispi)

The inferior aspect of the tip of the anterior superior iliac spine.

1. The designated landmark is the under surface of the tip of the anterior superior spine and not the most frontally curved aspect
2. The subject takes the body weight on his/her left foot, lifts the right heel and rotates the femur outward
3. The anthropometrist grasps the hip with his/her left hand, and locates the landmark with the thumb
4. Since the sartorius muscle arises from the spinale, slight movement of the thigh enables identification of the muscle, which can then identify the landmark
5. Once the landmark is identified, the subject stands erect, with feet together, while the spinale is marked and checked



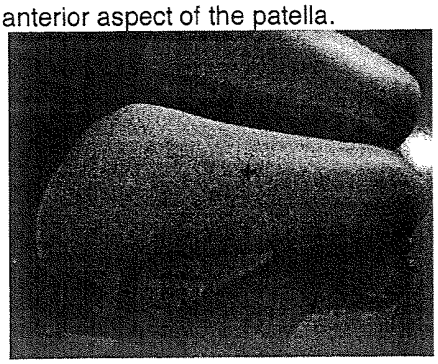
Landmark identification

Skin folds

1. Mid thigh

The mid point between the inguinal fold and the anterior aspect of the patella.

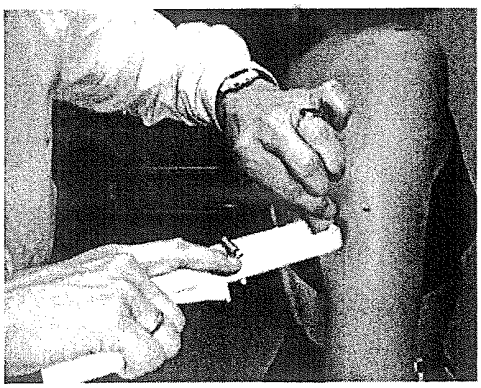
1. Locate the inguinal fold and the most anterior aspect of the patella
2. Use an anthropometric tape to identify the mid-point between the two landmarks
3. Mark this point
4. Verticle fold



Landmark identification

3. Triceps

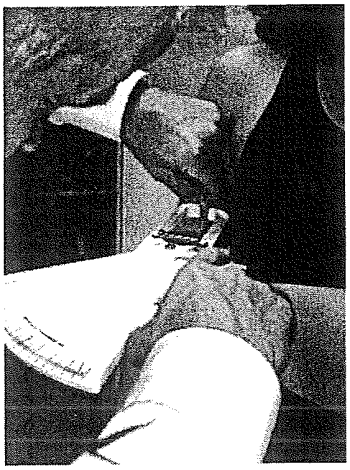
1. The calliper is applied 1 cm distally from the left thumb and index finger raising a vertical fold at the marked mid-acromiale-radiale line on the posterior surface of the right arm



Skin fold reading

4. Biceps

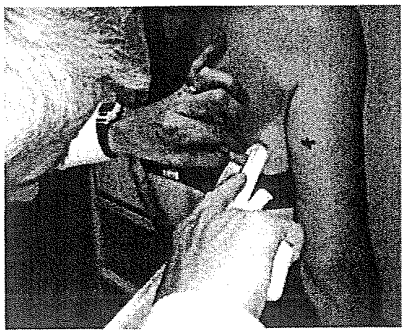
1. The calliper is applied 1cm distally from the left thumb and index finger raising a vertical fold at the marked mid-acromiale-radiale line on the anterior surface of the right arm



Skin fold reading

5. Subscapular

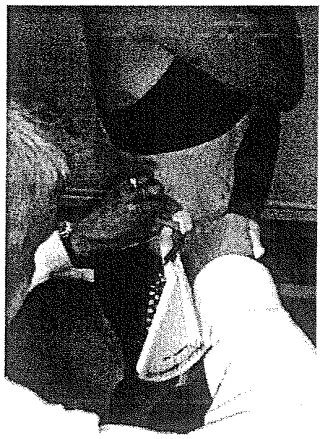
1. The fold is raised from the inferior angle of the scapula in a direction running obliquely downwards in a lateral direction at an angle of about 45° from the horizontal along the natural fold (Langer line)
2. The calliper is applied 1 cm distally from the left thumb and index finger



Skin fold reading

6. Iliac crest

1. The calliper is applied 1cm anteriorly from the left thumb and index finger raising a fold immediately superior to the iliac crest at the mid-axillary line (i.e. above the crest on the mid-line of the body)
2. The fold runs anteriorly downwards and usually is progressively smaller as one moves in this direction away from the designated site

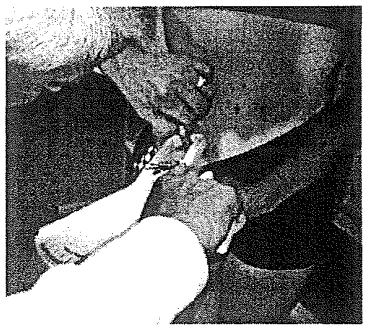


Skin fold reading

7. Supraspinale

Formerly Heath-Carter Suprailiac

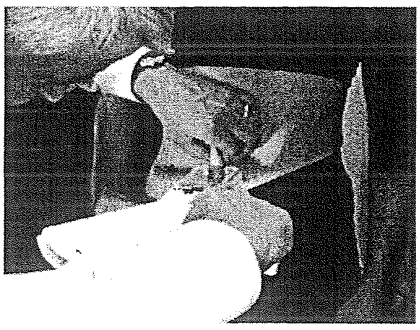
1. The calliper is applied 1cm anterior to the left thumb and index finger
2. The fold is raised at the intersection of the border of the ilium and a line from the spinale to the anterior axillary border
3. To mark ilium, project a horizontal line from the iliac crest mark. The fold follows the natural fold lines running medially downwards at about a 45° angle from horizontal



Skin fold reading

8. Abdominal

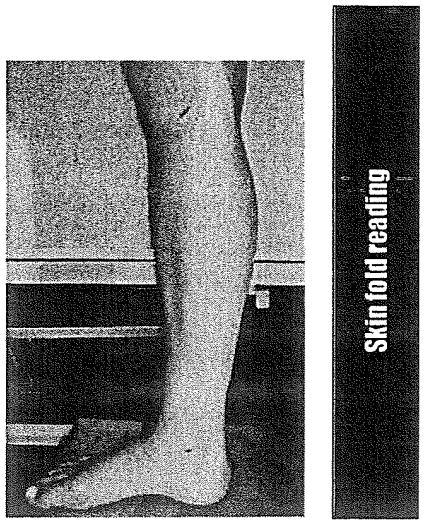
1. A vertical fold is raised on the right side 5 cm lateral to, and at the level of, the omphalion (midpoint of the navel)
2. The calliper is applied 1 cm inferiorly to the left thumb and index finger



Skin fold reading

9. Medial calf

1. The calliper is applied 1cm distally to the left thumb and index finger, raising a vertical fold on the relaxed medial right calf at the estimated level of the greatest circumference
2. This is easiest to obtain when the subject's leg is flexed to an angle of 90° at the knee by placing the foot on a box



B5 Catheter insertion

1. Assemble appropriate extension/burette line.
2. Prepare subject as per blood draw steps 1-4.
3. Ensure rubber tubing is moving freely on needle guide. Insert needle into vein at a shallow angle.
4. Watch for barrel of catheter to fill with blood to ensure good placement. May need to probe around and/or withdraw needle slightly.
5. Remove tourniquet.
6. Press down on vein proximal to needle insertion point to minimise blood flow. Retract needle guide.
7. Immediately attach extension.
8. Secure plastic tube with tape and then catheter dressing. Secure extension to subject's arm with tape to prevent tugging on catheter.
9. To remove catheter gently remove all tape, then remove catheter and replace immediately with gauze. Apply pressure then tape gauze in place.

B6 Percutaneous needle biopsy

1. Mark biopsy site with fingernail.
2. Shave site if necessary.
3. Clean site with iodine.
4. Cover site with sterile, fenestrated drape.
5. Spray skin with ethyl chloride to provide topical numbness.
6. Inject lidocaine with a fine gauge needle for skin area, then with a heavier gauge to reach muscle fascia.
7. Cover with sterile gauze and wait 5 minutes for anaesthetic to take hold.
8. Make a 1 cm incision at a constant depth of 5 cm with sterile scalpel blade.
9. Insert biopsy needle and make 3 passes under constant suction.
10. Remove biopsy needle and apply pressure with sterile gauze for 10 minutes then with ice for a further 5 minutes.
11. Clean site with alcohol swab.

12. Seal incision with Steri-Strip and plaster.
13. Apply pressure wrap using gauze and elastic bandage.

B7 Determination of protein concentration

1. In a 96-well microplate, prepare the first three rows as a series dilution of BSA or albumin standard in Milli-Q water.
2. Using Milli-Q water, prepare a series dilution of each sample to be tested in microtubes. Pipette triplicate samples into the remaining rows of the microplate.
3. If samples contain detergent, make reagent A'. To do so, add 20 µL reagent S for each 1 mL reagent A.
4. In each microplate well, add 25 µL reagent A' and 200 µL reagent B.
5. Mix by rocking plate for 15 minutes at room temperature.
6. Read protein concentration of each well in microplate reader.
7. Repeat assay if variation within triplicates is greater than 0.01, or correlation coefficient of standard curve is less than 0.95.

B8 Pouring gels

1. Mix 30% acrylamide, 4x resolving buffer (1.5 M Tris HCl, pH 8.8) and Milli-Q water in flask according to recipe (Table B-2). Set on vacuum for at least 10' to de-gas the solution.
2. Wipe five alumina plates and five 8 x 10 cm glass plates with acetone or ethanol.
3. Put foam gasket and Spectra/Por closures in cast (Mighty Small multiple gel caster SE 200 series, Hoefer Pharmacia Biotechnology, San Francisco CA, USA). Fill cast with two plastic sheets, then alumina plate, two spacers (8 x 0.75 mm T spacers, Hoefer SE 2119T-2-.75, Amersham Bio, San Francisco CA, USA), and a glass plate. Repeat plates and spacers five times.
4. Make 10% ammonium persulphate (APS) solution in Milli-Q water.
5. Add 10% SDS, 10% APS, and tetramethylethylenediamine (TEMED) to flask according to recipe (Table B-2). Put pipette very near the surface of the solution in the flask so as not to add too many bubbles to the de-gassed solution.
6. Pour the solution gently into a beaker and then immediately into the cast. Fill the cast to 1 cm below the bottom of the comb. Tilt the cast to each side to ensure all spaces have filled equally.
7. Add 100 µL water-saturated butanol to each space.
8. Draw leftover solution into transfer pipette. When this has set, your gels are set (roughly 90 minutes).
9. Make stacking gel according to recipe (stacking buffer: 0.5 M Tris HCl, pH 6.8). De-gas and prepare as resolving layer. Don't add TEMED until the last possible second.
10. Pour butanol out of gel cast (hold glass in place when tipping cast upside down). Dab with paper towel. Rinse gels with Milli-Q water and dab again with paper towel.
11. Pour in stacking gel. Put combs in. Again wait to set or put in fridge to set wrapped in cling film.

	resolving layer			
	7%	12%	15%	stacking layer
water	5.05	10.05	7.05	12.2
4x resolving buffer	7.5	7.5	7.5	5
30% acrylamide	7	12	15	2.66
10% SDS	300	300	300	200
10% APS	150	150	150	100
TEMED	9.9	9.9	9.9	20

Table B-2 Gel pouring recipe

Quantities of acrylamide and water vary according to the desired percentage of the gel. APS, ammonium persulphate; TEMED, tetramethylethylenediamene.

B9 Western blot

1. Turn on heat block to 95 ºC.
2. Put 4x SDS reducing load buffer (0.25 M Tris HCl pH6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.004% bromophenol blue), molecular weight ladder, and samples for the day on ice.
3. Plan loads. 20 µL per lane: 5 µL load buffer, sample to provide required µg protein, water to 20 µL. Run blanks in outermost lanes if less than 10 samples to be loaded.
4. Assemble the gel onto the apparatus. Tighten the clamps. Mark the glass at the bottom of each comb tooth, then remove the comb. Fill the reservoir at the back to the top and the reservoir at the bottom to 1-2 cm up from the bottom of the gel with 1 x SDS running buffer (25 mM Tris HCl pH 8.3, 192 mM glycine, 0.1% SDS). Rinse all wells with 50 µL running buffer.
5. Heat sample tubes for 3' (**not** standard tube).
6. Load 20 µL from each sample tube into the gel lanes. Rinse the syringe six times in Milli-Q water between each sample.
7. Plug the apparatus into the power supply (BioRad Laboratories, Hercules CA, USA). Run the gel at 10 mA, 150 V until dye front is migrated into the resolving gel. Press stop. Increase the current to 20 mA, 250 V and run until the dye front is at the bottom of the gel. For mTOR run at 10 mA, 100V to begin, then increase to 20 mA, 200V. For a double gel run at 20 mA to begin, then increase to 40 mA.
8. Remove gel by pulling out the spacers and lifting off the glass plate. Cut the wells and stacking gel and top right hand corner of resolving gel off. Soak the gel in electroblot buffer (25mM Tris HCl pH8.3, 192mM glycine, 20% methanol) for ten minutes.
9. Soak membrane in Milli-Q water and electroblot buffer for 3-4 minutes each.
10. Load cassette with two fibre pads, two pieces of filter paper, a little buffer, the gel with the cut corner on the left, the membrane with the cut corner on the left, two more pieces of filter paper, and two more fibre pads. Roll the bubbles out between each layer.
11. Clip the cassette together and place in transfer cell with an ice block and a stirring bar. Fill with electroblot buffer. Cover the tank and place on magnetic stirrer. Plug into PowerPac (BioRad Laboratories, Hercules CA, USA), set to 100 V and press run. Check the current does not exceed 300 mA (if it does then change the buffer or make new buffer).
12. Place the membrane in a lunchbox with enough Ponceau S (0.2% Ponceau S, 1% acetic acid) to cover the membrane on orbital shaker for five minutes. Discard Ponceau S.

Rinse the membrane with Milli-Q water until the bands can be clearly seen, but not for so long that the bands are washed out. Smooth off any remaining lumps of gel.
13. Put the stained membrane between two pieces of mylar and scan.
14. Mark where you want to cut the membrane with pencil.
15. Block by incubating in 10 mL 5% milk in (LS)TTBS (20 mM Tris HCl pH7.4, 0.5 M (0.15 M) NaCl, 0.05% Tween 20) for one hour at room temperature with shaking.
16. Add primary antibody to milk and TTBS solution. Incubate overnight at 4 ºC with shaking.
17. Rinse membrane twice with Milli-Q water, then wash four times for eight minutes each.
18. Add 2 µL secondary antibody to 10 mL milk and TTBS solution. Incubate for two hours at room temperature with shaking.
19. Rinse membrane twice with Milli-Q water, then wash four times for eight minutes each.
20. Prepare electrochemiluminescence (ECL) reagent (500 µL of each of two reagents into an eppendorf tube). Place drained membrane on transparency. Pipette ECL reagent onto membrane. Incubate for five minutes without shaking, but keep an eye on the membrane and make sure the reagent stays covering the whole membrane.
21. Drain excess liquid from membrane. Cover with another piece of transparency. Blot with toilet paper. Expose in LAS3000 until blots start to show red spots. Save every interval.

B10 Western blot analysis

1. Open file using MultiGauge software.
2. Go to the Measure window.
3. Select the rectangle from the toolbar on the left.
4. Draw a box around a band on the Western blot. Check that this box will suit all the bands on the blot being analysed by moving it using the arrow from the toolbar. Resize if necessary.
5. Press Ctrl + D to duplicate the box enough times for all the bands on the blot.
6. Move the duplicated boxes so that each band is boxed.
7. Move one more box to an empty space on the Western blot which will be set as background.
8. Go to the Analysis window.
9. It is possible to divide the lanes into groups.
10. In the Quant Result table tick the B box for the background lane.

B11 DEXA scan

1. Measure subject's height and weight.
2. Ask subject to remove any metal on their body, e.g. zippers, underwire bras, jewellery, etc.
3. Female subjects to take pregnancy test. Do not put a pregnant woman in the DEXA scanner.
4. Align subject with head at the top line on the bed, centred on table, with hands and feet inside the outside lines on the bed. Provide a strap for ankles if necessary. Have subject then sit up and slowly roll back down again to have spine as straight as possible.
5. Start scanner.

B12 Reverse transcription

Always use barrier tips!

DNase treatment:

1. Calculate how much re-suspended RNA will be needed in each tube to provide 2.5 µg RNA (µL re-suspended RNA = 2.5 ÷ RNA conc'n in µg/µL).
2. Fill each tube with DEPC-treated water so that the final volume with the re-suspended RNA added will be 9.8 µL.
3. Prepare 1 : 1.2 DNase : DNase buffer mix i.e. for every 1 µL DNase I mix 1.2 µL buffer, add 2.2 µL to each tube. Pipette the buffer mix first as it is ok to reuse this tip in the DNase and mix.
4. Add required volume of re-suspended RNA. Flick and spin.
5. Incubate in PCR machine (GeneAmp PCR System 9700, Perkin Elmer, Norwalk CT, USA) 15 minutes at 25 °C.
6. Add three µL 25 mM EDTA (to give 5 mM EDTA in solution).
7. Incubate 10 minutes at 65 °C.

The above gives 15 µL DNase-treated RNA at 166 ng/µL.
This product can be stored at -85 °C or on ice to continue RT^+.

Reverse transcription**:**

1. 7.5 µL DEPC-treated water in each 0.6 mL RT- tube.
2. Prepare 1 : 1 : 1 : 1 mix of dNTP, oligo dT, random hexamers, DEPC-treated water, 0.5 µL of each per ½ reaction.
3. Prepare cDNA synthesis mix (volumes are µL per ½ reaction):

10X RT buffer	1.0
$MgCl_2$ 25 mM	2.0
DTT 0.1 M	1.0
RNase OUT	0.5
SuperScript III polymerase	0.5

$\underline{RT^+}$

4. Add 2 µL 1 : 1 : 1 : 1 mix and 3 µL DNase-treated RNA to each tube. Flick and spin.
5. Incubate five minutes at 65 °C.
6. Set on ice for one minute or more.
7. Add five µL cDNA synthesis mix. Flick and spin.
8. Incubate ten minutes at 25 °C, 50 minutes at 50 °C, five minutes at 85 °C.
9. Chill on ice, flick and spin.
10. Add 0.5 µL RNase H.
11. Incubate 20 minutes at 37 °C.
12. Gives 10.5 µL 47.6 ng/µL cDNA.
13. Transfer 3 µL to a 0.6 mL tube, add 95.8 µL DEPC H_2O to give 1.45 ng/µL. This gives 98 µL diluted cDNA which is enough to run 27 wells in qPCR (9 genes).
14. Aliquot remaining 2 x 3.5 µL of 47.6 ng/µL into 0.6 m L tubes and store all at -25 °C.

RT-

4. Add 3 µL DNase-treated RNA to each tube.
5. Gives 10.5 µL 47.6 ng/µL RNA.
6. Add 33 µL DEPC-H_2O to give 11.5 ng/µL. This gives 43.5 µL diluted RT- which is enough to run 12 wells in qPCR (6 genes).
7. Store at -25 °C.

B13 Real time quantitative polymerase chain reaction

1. Thaw all reagents, cDNA, RT-, primer/probe mix on ice. Flick and spin once thawed.
2. Place open boxes of barrier tips, open RNase-free microtubes, PCR plate, and electronic pipettes in UV hood. Turn hood on for 30 minutes. Close tubes and tip boxes then leave hood running for 15-30 minutes to clear ozone.
3. Plan plate layout.
4. Calculate number of wells that will be used in the run.
5. Calculate the total amounts of PCR reaction mix and each sub-master mix needed.

IN THE HOOD:

6. All reagents should only ever be opened in the hood, including DEPC-treated H_2O. Prepare reaction mix. Flick and spin.
7. Prepare sub-master mixes. Flick and spin.
8. Mark the plate to help with orientation.
9. Add 8.7 µL of sub-master mixes to appropriate well using one tip.
10. Add 3.3 µL of cDNA or RT- or DEPC-treated H_2O (NTCs) to appropriate wells. Use one 10 µL tip per triplicate, on dispense mode.
11. Cover used wells with optical adhesive cover.

IN THE PCR KITCHEN:

12. Tap plate for 15-30 seconds to ensure contents mix.
13. Spin plate for two minutes at 1 000 g.
14. Open SDS software (version 2.3).
15. Set run type to standard curve.
16. Set up detectors for each gene.
17. Set passive reference to Invitrogen ROX.
18. Select 9600 emulation in thermal cycler and ensure the reaction volume is set to 12 µL.

*Appendix **C** **Materials***

C1 General materials

Chemicals

- ammonium persulfate (Bio-Rad Laboratories, Hercules CA, USA)
- aprotinin, β-mercaptoethanol, dithiothreitol, [^{12}C]leucine, leupeptin, OPD, phenylmethylsulfonyl fluoride, sodium orthovanadate, (Sigma-Alrich, St Louis MO, USA)
- BSTFA-TCMS (Sylon BFT) (Supelco, Bellafonte PA, USA)
- TEMED (PlusOne) (Amersham Biosciences, Little Chalfont, UK)

Isotope infusion

- latex-free infusion set with Smartsite needle-free valve port (Alaris Medical Systems Inc., San Diego CA, USA)
- Millex syringe driven filter unit 0.2 μm (Millipore Corporation, Bedford, MA, USA)

Intravenous catheter

- Blunt Plastic Cannula (Becton, Dickinson and company, Franklin Lakes NJ, USA)
- Connecta Plus three-way valve (Becton, Dickinson Infusion Therapy AB, Helsingborg, Sweden)
- Extension Set (Baxter Healthcare Corporation, Deerfield IL, USA)
- Insyte Autoguard shielded I.V. catheter (Becton, Dickinson Infusion Therapy Systems Inc., Sandy UT, USA)
- Interlink System Injection site (Baxter Healthcare Corporation, Deerfield IL, USA)
- IV3000 moisture responsive catheter dressing (Smith and Nephew Medical Limited, Hull, UK)

Muscle biopsy

- Med-X sterile G.P. drape (Fabri-Cell International, Auckland, NZ)
- Providone Iodine Prep Pads (Dynarex Corporation, Brewster NY, USA)
- Opsite Post-Op dressing (Smith and Nephew Medical Limited, Hull, UK)

Polymerase chain reaction

- ESCO ultraviolet hood (Airpro Scientific Ltd, Auckland, NZ)
- Maxymum Recovery certified DNase/RNase and pyrogen safe barrier tips (Axygen supplied by Global Science & Technology Ltd, Auckland, NZ)
- MicroAmp Optical 384-well reaction plate (Applied Biosystems, Foster City CA, USA)
- Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad CA, USA)
- Research Pro electronic pipettes (eppendorf)
- ROX Reference Dye (Invitrogen, Carlsbad CA, USA)

Reverse transcription

- dNTP set, PCR Grade (Roche, Penzberg, Germany)
- SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad CA, USA)

RNA extraction

- DEPC-treated water (Applied Biosystems, Foster City CA, USA)
- RNase *Zap* Solution (Ambion supplied by Applied Biosystems, Foster City CA, USA)

Western blotting

- Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, UK)
- Hybond – LFP PVDF membrane (Amersham Biosciences, UK)
- Lumi-Light Western Blotting Substrate (Roche, Penzberg, Germany) Lot no. 12 015 200 001
- mini trans-blot gel holder cassette (Bio-Rad Laboratories, Hercules CA, USA)
- Precision Plus Protein Standards: All Blue (Bio-Rad Laboratories, Hercules CA, USA)
- SuperSignal west femto maximum sensitivity substrate (Pierce Biotechnology, Rockford IL, USA) Lot nos J112413 and IJ 116735

Venupuncture

- BD Vacutainer Systems Precision Glide 21G1.5" needle (Becton, Dickinson, Plymouth, UK)
- vacutainer holder (Vacutainer, Becton, Dickinson, Plymouth, UK)

C2 Primers and probes

The *Taqman* primer/probe mixes in Table C-1 were purchased from Applied Biosystems (Foster City CA, USA).

Gene	catalogue number	lot number(s)
PGC-1α	Hs01016726_m1	684014
TFam	Hs01082775_m1	684013
PPARα	Hs00231882_m1	492371
PPARδ	Hs00606407_m1	451200
COXI	Hs02596864_g1	493160
COXIV	Hs00266371_m1	448060
β2m	Hs99999907_m1	494425, 497248
GAPDH	Hs99999905_m1	492902, 524927

Table C-1 Order information for primer/probe mixes used in polymerase chain reaction analysis

PGC-1α, peroxisome proliferation-activated receptor gamma co-activator - 1 alpha; TFam, mitochondrial transcription factor A; PPAR, peroxisome proliferation-activated receptor; COX, cytochrome c oxidase; β2m, beta-2-microglobulin.

C3 Antibodies

The primary antibodies in Table C-2 were purchased from Cell Signalling (Cell Signalling Technology Inc., Boston MA, USA) or Abcam (supplied by Sapphire Bioscience, Redfern NSW, Australia).

protein	catalogue number	lot number
phospho-mTOR Ser^{2448}	CS2971	10/2007
mTOR	CS2972	01/2008
phospho-4E-BP1 $Thr^{37/46}$	CS9459	02/2008
4E-BP1	CS9452	01/2008
cytochrome c	ab18738	170477
GAPDH	120-82450	287601

Table C-2 Order information for primary antibodies used in Western blotting analysis in Chapter 7
mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor binding protein.

Appendix D Subject information

D1 Recruiting advertisements

The following flyers were used to recruit subjects to the first and second studies respectively.

DEPARTMENT OF SPORT AND EXERCISE SCIENCE

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Building 734, Tamaki Campus
Morrin Road, Glen Innes
Auckland, New Zealand
Telephone 64 9 373 7599 ext 86887
Facsimile 64 9 373 7043

The University of Auckland
Private Bag 92019
Auckland, New Zealand

Participants needed for a study on Exercise and Ageing

Participants Required For Research Study

Seeking: Healthy, non-smoking males and females, over 55 years of age, for a study about how to maintain muscle quality with ageing.

You would be required to give samples of blood and muscle on 2 occasions, each lasting approximately 8 hours, at the Dept. of Sport and Exercise Science, University of Auckland Tamaki Campus.

Completion of the study will lead to information on how to prevent decreases in muscle mass and function in older individuals and will aid in the formation of a senior exercise group at the university.

If you think you might be interested, please contact us for more information.

Cheryl Murphy

Tel. 373 7599 extn. 88559, email: cmur042@ec.auckland.ac.nz

Dr. Benjamin Miller
Tel. 373 7599 extn. 86607, e-mail: b.miller@auckland.ac.nz

INTERESTED IN AGING SUCCESSFULLY?

Participants needed for a research study on exercise and nutrition

Seeking: healthy, active, non-pregnant, non-smoking males and females, 18-35 years of age, for a study about how to improve muscle quality.

You will receive a VO_{2max} test and a DEXA scan and provide samples of muscle as part of this study.

Study participation will involve two short visits and two full days of testing. The study will take place at the Dept. of Health and Exercise Science and Hartshorn Health Services, Colorado State University.

Completion of the study will lead to information on how to prevent decreases in muscle function with age.

If you think you might be interested, please contact us for more information.

Cheryl Murphy

Tel. 970-689-9904, email: cherylmurph@gmail.com

Benjamin Miller, PhD

Tel. 970-491-3291, e-mail: bfmiller@cahs.colostate.edu

D2 Participant information sheet

The following form was provided to subjects in the first study, prior to their providing consent. In the second study, the consent form (Appendix D3) and participant information sheet were a single document.

DEPARTMENT OF SPORT AND EXERCISE SCIENCE

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Building 734, Tamaki Campus
Morrin Road, Glen Innes
Auckland, New Zealand
Telephone 64 9 373 7599 ext 86887
Facsimile 64 9 373 7043

The University of Auckland
Private Bag 92019
Auckland, New Zealand

PARTICIPANT INFORMATION SHEET

Project title: **Interaction of nutrition and exercise on muscle function**

Researcher name: Benjamin F Miller, PhD

To: Potential research participant:

I am a researcher and lecturer at the University of Auckland's Department of Sport and Exercise Science. You are invited to participate in a research study exploring how to use diet and exercise to maintain healthy muscle. Your participation is entirely voluntary (your choice); you do not have to take part if you do not wish to. If you agree to take part you are free to withdraw from the study at any time, without having to give a reason.

Why are we doing this study?

Over the course of 24 hours, body-mass is determined by whether a person is in positive or negative energy balance (sum of calories in from food and calories out from energy expenditure). Therefore when individuals are in prolonged periods of underfeeding, your body starts to break itself down. Underfeeding can be involuntary as in diseased individuals (e.g. HIV), elderly or those at high altitude, or voluntary as with those involved in a weight loss program. Regardless of mechanism, underfeeding results in muscle wasting which is undesirable in all causes. Since muscle mass is critical for the maintenance of metabolic rate (in overweight individuals), standard of living (diseased and elderly participants), and performance (healthy active participants) it would be desirable to maintain muscle mass or function when other body components, such as fat, are breaking down.

It is known that muscle responds differently to different exercise types. Further, whereas it is known that resistance training and feeding has an additive effect making muscle bigger, it is yet unexplored whether endurance exercise and feeding will have an additive effect on the ability to perform prolonged activity. Therefore, this proposal seeks to explore whether endurance capacity of the muscle responds to exercise and feeding in the same manner as growth after resistance training. Further, the proposal will explore the signals within the muscle responsible for the changes.

Who may participate in this study:

To be part of this study, you must be over 55 years old, physically healthy and preferably active. You must not have or have had any problems with bleeding, or be on medication that prolongs bleeding time, or be allergic to local anaesthetics. Because smoking is known to affect the factors being investigated, you can not be a smoker. Males and females are invited to participate. Dr. Benjamin Miller will select 20 subjects that meet the criteria to participate.

What is involved?

We will ask you to answer some questions about your past and current participation in exercise, and will measure your body weight, height, and body composition (% body fat). Your gender and date of birth will also be recorded. Before undergoing the sampling procedures, you will be asked a few questions relating to your present state of health, current medication and past medical history. This is to exclude the presence of any condition or medication that might prolong your bleeding time or make the blood or muscle sampling unsafe for you.

If the initial screening is successful, we will determine your maximal oxygen consumption (VO_2 max). This involves riding on a bicycle while breathing into a mouth piece that samples the breathed air. The exercise will start easy and get progressively more difficult until you indicate that you can not go further. We will also measure your heart rate throughout the test.

About a week later you will come in overnight fasted for the first of two testing sessions. The testing session will consist of 2 hours resting while lying down, a 1 hour bout, and 4 hours of recovery. Both testing sessions will be conducted exactly the same.

Upon arrival you will lie in a bed and we will insert an intravenous cannula in each arm. One will be used for an infusion of a stable isotope (discussed below) and one will be for blood sampling. After the initial discomfort of inserting the needle, you will not feel the catheter. You will lie for 2 hours while we periodically take blood samples (5 ml at a time) and feed you a drink consisting of energy and protein or energy only. At the completion of the resting period, you will receive the first of two muscle biopsies. The muscle sample will be taken under local anaesthesia from the thigh of your leg. This will be done under sterile conditions and will be carried out by an experienced physician. It will involve making a small skin incision ($\approx$ 1 cm long) through which the biopsy needle is placed to a depth of about 5 cm, and a small piece of muscle (40-100 mg, about the size of a pea) is removed. Following this, the incision will be secured by a Steri-strip, then firmly bandaged with an elasticised cylinder.

After the biopsy, you will start a one hour bout of exercise on a stationary bike at an intensity determined from your previous bike test. Throughout the test we will continue to draw blood samples and take periodic breath and heart rate measurements to ensure that you are exercising at the correct intensity. After completion of the exercise period you will lie back down and resume the feeding and blood sampling protocols as preformed before the exercise bout. At the end of 4 hours, a second muscle sample will be taken in the opposite leg as before. The procedures for the biopsy are the same except that now we will secure the incisions in a bandage that should be worn for about 3 days.

The biopsy procedure will take no more than 5-10 minutes, and will not prevent you from performing any of your normal daily activities afterwards. After 2 days the incision will be inspected by health clinic staff and by 3 or 4 days the Steri-strip and bandage can be removed. The resultant scar will gradually fade and be hardly noticed.

The entire experimental procedure (rest, exercise, recovery) will be preformed twice a minimum of one week apart. The only difference will be the drink consumed throughout the trial. Therefore, upon completion you will have had 2 muscle biopsies in each leg for a total of 4.

You are likely to experience temporary physical discomfort during the blood sampling and muscle biopsy procedures. Discomfort during the blood sampling is minor and brief (<30s). During the biopsy, you will sense the administration of the local anaesthetic (like a bee sting), and will feel some pressure in the muscle during the biopsy needle insertion and sampling. Afterwards, you may feel some muscle tightness and soreness (like a bruise) around the biopsy site that may persist for 1-4 days. The muscle repairs itself fully within two weeks.

The associated risks of the muscle biopsy technique are minimal. There is a small risk of muscle bleeding and bruising around the area of the biopsy. There is also a small risk, as with any incision, of local infection. In the unlikely event that you have any unanticipated physical injury during or following the procedures, you will be directed to a physician for medical advice and/or treatment. If any abnormal or unusual results are found during the analyses of your samples, we will notify you of this and refer you to appropriate medical personnel.

What is a stable isotope? It is important that you realize that a stable isotope is **NOT** radioactive. Stable isotopes are naturally occurring and at any given time are present in your body at variable amounts. The amount of stable isotope in your body at any given time is largely determined by what you eat. For instance corn (maize) has a large amount of the stable isotope of carbon. We will be infusing a stable isotope attached to an amino acid. Again, both the stable isotope and the amino acid are naturally occurring in your body and we are simply increasing the amount. You will feel nothing nor is it possible for you to detect that it is there. The amount we infuse will be out of your system with 2 weeks.

Compensation:

It will not cost you any money to participate in the study and travel expenses to and from the facilities will be reimbursed. As stated below, it is your right to withdraw at any time from the study.

The research will be conducted in the Dept of Sport and Exercise Science, at the University of Auckland Tamaki campus in Glen Innes.

Your rights:

- Your participation is entirely voluntary.
- If you need an interpreter, one will be provided.
- You may have a friend, family or whanau support to help you understand the risks and/or benefits of this study and any other explanation you may require
- You may have your own data with some comparative values from normal and athletic populations sent to you upon completion of the study. You may also request the resultant publication although there is usually some delay until this is completed.
- Your identity will be kept strictly confidential, and no identification of you or your data will be made in any subsequent publication of the research findings.
- You are free to withdraw from the project at any time, without giving any reason, and without any disadvantage to yourself of any kind.
- You may withdraw information that you have provided at any time prior to completion of data collection.

- If you are a student at the University of Auckland your participation or refusal of participation in this study will ***not*** affect your grades in any way. Nor will it affect any academic relationship with the department or members of staff.

Your information and data will be used for this project only. However, if you agree, after this study any remaining sample of your blood and/or muscle may be used for further studies on molecular aspects of muscle growth. The analysis would be standard biochemical assays not currently done by our laboratory. If the extra analysis is deemed appropriate, these samples may be sent to collaborators in Scotland or Denmark. If you do not agree, any remaining samples will be destroyed at the end of this study.

Data identifiable to you will be stored in a locked office for up to ten years, after which it will be destroyed. You will not be identified on any electronically stored data, data that will be retained indefinitely and used for publication.

Your consent to participate will be indicated by your signing and dating the consent form. Signing the consent form indicates that you have freely given your consent to participate, and that there has been no coercion or inducement to participate.

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

Thank you very much for your time and help in making this study possible. If you have any queries, wish to know more, or have medical concerns, please phone Dr. Miller at:

373 7599 extn 86607, or write to:

Dr. Benjamin Miller
Department of Sport and Exercise Science
The University of Auckland
Private Bag 92019
Auckland Tel. 373-7599 extn. 84681
b.miller@auckland.ac.nz

The Head of Department is:
Professor Alan Lee
Department of Sport and Exercise Science
The University of Auckland
Private Bag 92019
Auckland Tel. 373-7599 extn 86844

This study has ethical approval from the Northern X Ethics Committee.

D3 Consent forms

The following forms were used to obtain subjects' informed consent for the first and second studies respectively.

DEPARTMENT OF SPORT AND EXERCISE SCIENCE

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Building 734, Tamaki Campus
Morrin Road, Glen Innes
Auckland, New Zealand
Telephone 64 9 373 7599 ext 86887
Facsimile 64 9 373 7043

The University of Auckland
Private Bag 92019
Auckland, New Zealand

CONSENT FORM

THIS CONSENT FORM WILL BE HELD FOR A PERIOD OF SIX YEARS

Research Project: **Interaction of nutrition and exercise on muscle function**
Researcher: Dr Benjamin Miller

English	I wish to have an interpreter.	Yes	No
Maori	E hiahia ana ahau ki tetahi kaiwhakamaori/kaiwhaka pakeha korero.	Ae	Kao
Samoan	Ou te mana'o ia i ai se fa'amatala upu.	Ioe	Leai
Tongan	Oku ou fiema'u ha fakatonulea.	Io	Ikai
Cook Island	Ka inangaro au i tetai tangata uri reo.	Ae	Kare
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu.	E	Nakai
	Other languages to be added following consultation with relevant communities.		

I have read and understand the information in the Participant Information Sheet. I have had the opportunity to ask any questions about the project and have had them answered. My participation in this research is voluntary and I have had time to consider whether to take part. I have been given and have understood the explanation of this research project and my role as a participant.

In particular, I have been informed and understand that:

1. If I request, I can obtain results regarding the outcome of the project upon completion of the study.
2. My personal information and my identity will remain confidential.
3. Data from my participation will be kept secure and used for research purposes only.
4. I will not be identified on any electronically stored data, data that will be retained indefinitely and used for publication

5. I am free to withdraw from participation at any time, without giving a reason, irrespective of whether or not payment is involved, and to withdraw any data traceable to me up to the completion of the data collection.
6. If I am a student at the University of Auckland, participation or non-participation will not affect grades in any way.
7. I accept that any of my blood or muscle remaining after this study may be used for future studies on muscle in humans. **YES**☐ **NO**☐ (Please check one).
8. I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.

I know who to contact if I have any side effects or if I have any questions about the study. I have read and I understand the information sheet dated 11/08/05 for volunteers taking part in the study designed to explore the interaction of nutrition and exercise on muscle function. I have had the opportunity to discuss this study and I am satisfied with the answers I have been given.

I _____ (full name) hereby consent to take part in this study.

Date: _____

Signature: _____

Principle Researcher: Dr. Benjamin F Miller (9) 373 7599 x 86607

Project explained by: _____

Project role: _____

Signature: _____

Date: _____

This study has ethical approval from the Northern X Ethics Committee.

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY:
Influence of post-aerobic exercise nutrient composition on the mitochondrial biogenesis pathway

PRINCIPAL INVESTIGATOR: Benjamin F. Miller PhD, bfmiller@cahs.colostate.edu, 491-3291

CO-PRINCIPAL INVESTIGATOR: Cheryl Murphy, cherylmurph@gmail.com, 689-9904

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH?
If you are 18-35 years old, healthy, not pregnant, and physically active, then we are interested in you taking part in this study. Active means that you currently participate in more than two dedicated aerobic exercise sessions per week. Males and females are invited to participate. Twelve subjects that meet the criteria to participate will be selected.

WHO IS DOING THE STUDY?
The primary investigator for this study is Benjamin Miller PhD, a faculty member in the department of Health and Exercise Science at CSU. Day-to-day experimental procedures will be performed by the co-principal investigator, Cheryl Murphy, who is a PhD student at the University of Auckland in New Zealand, mentored by Dr. Miller, who has traveled to Colorado to conduct this study. Some procedures will be performed by Matt Hickey PhD who is also a faculty member in the department of Health and Exercise Science at CSU.

WHAT IS THE PURPOSE OF THIS STUDY?
It is known that muscle responds differently to different types of exercise. Further, whereas it is known that lifting weights and eating has an additive effect on making muscle bigger, it is yet unknown whether endurance exercise and eating will have an additive effect on endurance capacity. The more mitochondria a person has, the greater their endurance capacity. Therefore, this project seeks to explore whether muscle mitochondria respond to exercise and eating in a similar manner to muscle growth after weight-lifting. Specific parts of the pathway of signals from exercise to new mitochondria will be studied.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
The study will take place in the Human Performance Clinical and Research Laboratory and Hartshorn Health Centre at Colorado State University. The whole study will last for two months; each individual's involvement will last about three weeks.

WHAT WILL I BE ASKED TO DO?
We will ask you to answer some questions about your past and current participation in exercise, and will measure your body weight and height. Your gender and date of birth will also be recorded. You will be asked a few questions relating to your present state of health, current medication and past medical history. This is to exclude the presence of any condition or medication that might prolong your bleeding time or make the muscle sampling or exercise unsafe for you. This part of the visit will last about 20 minutes.

If anything adverse is found in any of the medical screening, you will be advised.

If the initial screening is successful, your body composition will be determined by DEXA scan. This is like an x-ray that works out how much of your body is muscle. You will also perform an exercise test in which we will determine your maximal oxygen consumption (VO_{2max}). This involves riding on a stationary bicycle while breathing into a mouthpiece that samples the breathed air. The exercise will start easy and get progressively more difficult until you indicate that you can not go further. We will also measure your heart rate throughout the test. These tests will take about one hour.

In another visit a small (100mg, about the size of a pea) sample of muscle will be taken from your thigh. This will be done under a local anesthetic. A small (6-8mm) cut will be made in your skin and another cut of the same size in your muscle. The muscle sample will be taken under sterile conditions and will be carried out by an experienced investigator (Matt Hickey PhD) with medical supervision. Following this, pressure and ice will be applied, the incision will be secured by steri-strips and band-aids, then firmly

bandaged with gauze. The biopsy procedure will not prevent you from performing any of your normal daily activities afterwards. After 12 hours you can remove the gauze, by three or four days the steri-strips, and after one week the incision will be inspected. The resultant scar will gradually fade and be hardly noticeable. This visit will last about half an hour.

About a week later you will come in overnight fasted for the first of two nine-hour testing sessions. Both testing sessions will be conducted exactly the same. You will be given breakfast and then asked to rest for 90 minutes. You will then start a one-hour bout of exercise on a stationary bike at an intensity determined from your VO_{2max} test. After completion of the exercise you will lie back down and receive a drink consisting of energy only or energy and protein. An hour later a muscle sample will be taken from your leg. At the end of six hours, a second muscle sample will be taken from the opposite leg as before. You will not be allowed to eat or drink anything other than water and the drink we provide you for 90 minutes before, and six hours after, the exercise bout. The entire testing procedure (rest, exercise, recovery) will be performed again at least one week later. The only difference will be the drink consumed. Therefore, upon completion of the study you will have had a total of five muscle biopsies.

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
You must not have any heart disease, diabetes or other major illness. You must not be pregnant. You also must not have or have had any problems with bleeding, or be on medication that prolongs bleeding time. Because smoking is known to affect the factors being investigated, you can not be a smoker. Because the biopsies require the use of lidocaine as a local anesthetic, you can not be allergic to lidocaine. The drinks will consist of a combination of any or all of the following: maltodextrin, whey protein, vegetable gum. If you are allergic to any of these things, you should not participate in this study.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?
These procedures are all low risk in a healthy population.

- During exercise testing (VO_{2max}) there is a small risk (less than 1 in 10 000) of illness or death due to a cardiac event. There are also risks of fatigue and muscle strains. You may experience temporary breathlessness or dizziness towards the end of the VO_{2max} test. These feelings are transient and pass once the test is finished.
- During vigorous exercise there is also a risk of cardiac complications but in individuals with good cardiac health this risk is extremely low (1 in 1 000 000). There are also risks of fatigue, boredom, and muscle soreness.
- There is a small risk of infection at the site of the incision for the biopsy, and a small risk of this incision reopening or bleeding after you leave the lab. The risk of allergic reaction to lidocaine (the anaesthetic used for the biopsy incision) is extremely low. You will have a small scar at the incision site. The rate and degree of healing varies considerably, but it is expected that scars will be difficult to see within 6-12 months after the procedure.
- It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

ARE THERE ANY BENEFITS FROM TAKING PART IN THIS STUDY?
You will receive free information regarding your health status, a free test of maximal aerobic capacity and a free DEXA scan. The results of this study may provide practical information regarding the use of exercise and nutrition to postpone chronic disease, illness and death. The results may also help other people in the future wishing or needing to increase muscle function. The results of this study may benefit all adults seeking to improve their quality of life through diet and exercise.

DO I HAVE TO TAKE PART IN THE STUDY?
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE?
It will not cost you any money to participate in the study. Any treatment or medical costs that arise as a result of your participation in this study are your responsibility.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.
In the researchers' records you will be identified according to either your initials or a number. For example, if John Smith was the first subject recruited his data will be labelled as either 1 or js. We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court.

CAN MY TAKING PART IN THE STUDY END EARLY?
If you decide that you do not want to complete any of the procedures, for example the VO_{2max} test or the muscle biopsies, then you will be removed from the study. If you decide you do not want to adhere to the study protocol, for example arrive at the laboratory overnight fasted, or rest for the time between the biopsies, then you will be removed from the study.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?
You will receive a free maximal oxygen consumption test, and DEXA scan which would normally cost a substantial amount. Upon completion of the study you will also receive $25 for each biopsy, up to a maximum of $125, as follows: $25 after the first biopsy, $50 after the third biopsy, $50 after the fifth biopsy.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH?
Because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries incurred as a result of participation in this study under a Colorado law known as the Colorado Governmental Immunity Act (Colorado Revised Statues, Section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury.

In light of these laws, you are encouraged to evaluate your own health and disability insurance to determine whether you are covered for any physical injuries or emotional distresses you might sustain by participating in this research, since it may be necessary for you to rely on your individual coverage for any such injuries. Some health care coverages will not cover research-related expenses. If you sustain injuries, which you believe were caused by Colorado State University or its employees, we advise you to consult an attorney.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the investigator, Cheryl Murphy at 970-689-9904. If you have any questions about your rights as a volunteer in this research, contact Janell Meldrem, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

WHAT ELSE DO I NEED TO KNOW?
Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 4 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

D4 Questionnaires

The following questions were asked in the first study during initial contact to determine whether subjects were eligible to proceed to screening.

1. Are you over 55?
2. Are you healthy with no known heart disease, diabetes, high blood pressure or other major illness?
3. Do you have any known bleeding disorders, a history of bleeding, or on medications known to effect bleeding e.g. anticoagulants?
4. Are you on any other medications?
5. Are you allergic to anaesthetics?
6. Are you currently free of illness and infection?
7. Do you consider yourself active or inactive?

The following questions were asked in the second study during initial contact to determine whether subjects were eligible to proceed to screening.

1. Are you between the ages of 20 and 35?
2. Do you consider yourself active or inactive?
3. How much exercise do you do in a week? How many times? How long? What sort?
4. Are you healthy with no known heart disease, diabetes, or other major illness?
5. Do you have any known bleeding disorder, a history of bleeding, or on medications known to affect bleeding (e.g. anticoagulants)?
6. Are you on any other medications?
7. Are you allergic to lidocaine?
8. Are you currently free of illness and infection?

The following questionnaire was provided to all subjects in both studies who progressed beyond the initial screening questions.

MEDICAL AND EXERCISE HISTORY

NAME_____ GENDER_____ DATE_____

BIRTHDATE_____ AGE_____ HEIGHT_____ WEIGHT_____

ADDRESS_____

TELEPHONE_____ EMAIL_____

1. How often do you exercise? _____ times/week

2. Describe the intensity of your exercise (circle one)
   1 = none
   2 = light (e.g. casual walking, golf)
   3 = moderate (e.g. brisk walking, jogging, cycling, swimming)
   4 = heavy (e.g. running, high intensity sport activity)

3. What types of exercise do you engage in and how much do you do each session? (circle all that apply)
   1 = none
   2 = walking _____ km or minutes
   3 = jogging/running _____ km or minutes
   4 = swimming _____ meters or minutes
   5 = cycling _____ km or minutes
   6 = team sports (rugby, cricket, soccer, etc.) _____minutes _____ intensity
   7 = racquet sports _____minutes
   8 = weight training _____minutes _____# reps _____# sets
   9 = other _____

4. How much time per week do you spend exercising? _____ hours/week

5. Do you measure your heart rate during exercise? _____
   If yes:
   a. How high does it get during your typical workout? _____ beats/min
   b. What heart rate is maintained throughout most of your workout? _____beats/min

6. How long have you had a regular exercise program?_____

7. What condition or shape do you consider yourself to be in now (in terms of physical fitness)?
   1 = poor
   2 = fair
   3 = good
   4 = excellent

8. Do you or have you ever smoked? _____
   If yes: How long ago?_____ For how many years?_____ How many packs/day?_____

9. Has a close blood relative had or died from heart disease or related disorders (Heart Attack, Stroke, High Blood Pressure, Diabetes etc.)?
1=Mother
2=Father
3=Brother - Sister
4=Aunt - Uncle
5=Grandmother - Grandfather
6=None
If yes- Give ages at which they died or had the event and the problem they had.
_____
_____
_____

10. Have you ever had your cholesterol measured?
1=yes
2=no

If yes- write the date and value (or if it was normal or abnormal)
_____

11. Indicate which of the following apply to you (circle all that apply).
1 = high blood pressure
2 = high blood fats or cholesterol
3 = known heart disease or abnormalities
4 = stressful lifestyle at home or at work
5 = diabetes mellitus
6 = gout (high uric acid)
7 = kidney problems
8 = thyroid problems
9 = lung problems
10 = cancer

12. Any medical complaints now (illness, injury, limitations)?
1 = yes If yes, describe completely_____
2 = no _____
_____

13. Any bleeding disorders?
1 = yes If yes, describe completely_____
2 = no _____
_____

14. Are you allergic to lidocaine?
1 = yes
2 = no

15. Any major illness in the past?
1 = yes If yes, describe completely_____
2 = no _____
_____
_____

16. Any surgery or hospitalization in the past?
1 = yes If yes, describe completely_____
2 = no _____
_____
_____

17. Are you currently taking any medications (prescription or over-the-counter: including birth control)?
1 = yes If yes, list drugs and dosages _____
2 = no _____

18. Are you allergic to any medications?
1 = yes If yes, list medications_____
2 = no _____
_____
_____

19. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = heart murmurs
2 = any chest pain at rest
3 = any chest pain upon exertion
4 = pain in left arm, jaw, neck
5 = any palpitations
6 = fainting or dizziness
7 = daily coughing
8 = difficulty breathing at rest or during exercise
9 = any known respiratory diseases

Please describe fully any items you circled_____
_____
_____
_____

21. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = any bone or joint injuries
2 = any muscular injuries
3 = muscle or joint pain following exercise
4 = limited flexibility
5 = any musculoskeletal problems which might limit your ability to exercise

Please describe fully any items you circled_____
_____
_____
_____

The questions on the following PAR-Q physical activity readiness questionnaire were asked and answered orally prior to the beginning of the Bruce Protocol in the first study.

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of any other reason why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

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DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

The following food and exercise diary was completed three times by all subjects participating in the second study.

Food and Exercise Diary

- *Keep this record for the 2 days before your muscle biopsy*
- *No one will look at this record other than you and the researcher*
- *Do not change from your regular eating and exercise habits*
- *Record enough detail that you will be able to eat exactly the same thing and do the same exercise at the roughly same times in the 2 days before your next testing day*

Day One		
Time	*Food/Exercise*	*Quantity*
Day Two		
Time	*Food/Exercise*	*Quantity*

- *Please remember not to eat anything in the morning before you come in for testing*

*Appendix **E Sample calculations***

E1 Whole body protein synthesis

The following is a sample calculation for subject 1 at five hours following the start of infusion in the CHO condition:

$$R_a = \frac{(F / BW) * 0.99}{E_{plasma}} = \frac{(9.4625 / 85.3) * 0.99}{0.079} = 1.39 \text{ μmol/kg.min}$$

$$V_{CO2\,(rel)} = \frac{V_{CO2\,(abs)} * 1\,000\,000}{22.4 * BW} = \frac{0.191 * 1\,000\,000}{22.4 * 85.3} = 99.96 \text{ μmol/kg.min}$$

$$\text{oxidation} = \frac{E_{breath} * (V_{CO2\,(rel)}/0.81)}{E_{plasma}} = \frac{0.00011 * (99.96 / 0.81)}{0.079} = 0.17 \text{ μmol/kg.min}$$

WBPS = R_a - oxidation = 1.39 - 0.17 = 1.22 μmol/kg.min

E2 Fractional synthesis rate

The following is a sample calculation for subject 9 in the PRO condition using plasma α-KIC as the precursor pool:

$$FSR = \frac{(E_{t2} / 100) - (E_{t1} / 100) * 100\%}{E_{pret12} * (t_2 - t_1)}$$

$$= \frac{(-0.023381929 / 100) - (-0.029938519 / 100) * 100}{0.05 * (7 - 3)}$$

$$= \frac{0.00655659}{0.2}$$

$$= 0.033 \text{ %/h}$$

E3 Relative mRNA expression

The following is a sample calculation for subject 5 in the CHO condition at six hours post-exercise for PGC-1α mRNA expression:

$$\text{mean of housekeeping genes} = \frac{\text{mean raw } C_{t\beta 2m} + \text{mean raw } C_{tGAPDH}}{2}$$

$$= \frac{23.8104 + 20.53659}{2} = 22.17$$

mean normalised C_t = mean (raw C_t - mean of housekeeping genes)

$$= \frac{(23.64472 - 22.17) + (23.65575 - 22.17) + (23.93552 - 22.17)}{3}$$

$$= 1.58$$

expression = $2^{-\text{mean normalised Ct}} = 2^{-1.58} = 0.42$

$$\text{relative expression} = \frac{\text{expression}}{\text{baseline expression}} = \frac{0.42}{0.047} = 8.9$$

E4 Protein expression

The following is a sample calculation for subject 3 in the PRO condition at three hours post-exercise for total 4EBP1:

$$\text{normalised expression for a given lane} = \frac{\text{expression of total 4E-BP1}}{\text{expression of GAPDH}}$$

$$= \frac{21886571}{63629596} = 0.34 \text{ (for example)}$$

Phosphorylation is calculated in the same way by dividing the expression of phosphorylated protein to that of total protein:

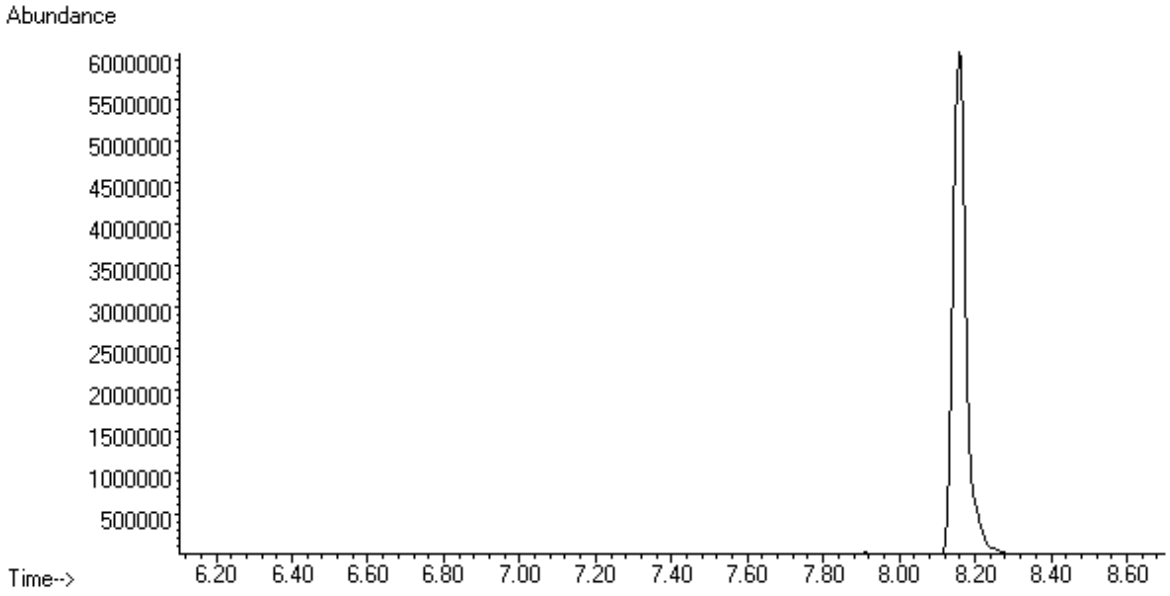
$$\text{phosphorylation} = \frac{\text{expression of phospho-4E-BP1}}{\text{expression of total 4E-BP1}} = \frac{1.38}{0.34} = 4.06$$

$$\text{fold-change from baseline} = \frac{\text{mean phosphorylation at 3h}}{\text{mean phosphorylation at baseline}} = 8.32 / 5.87 = 1.42$$

Appendix F Sample raw data

F1 Mass spectrometry traces

A



B

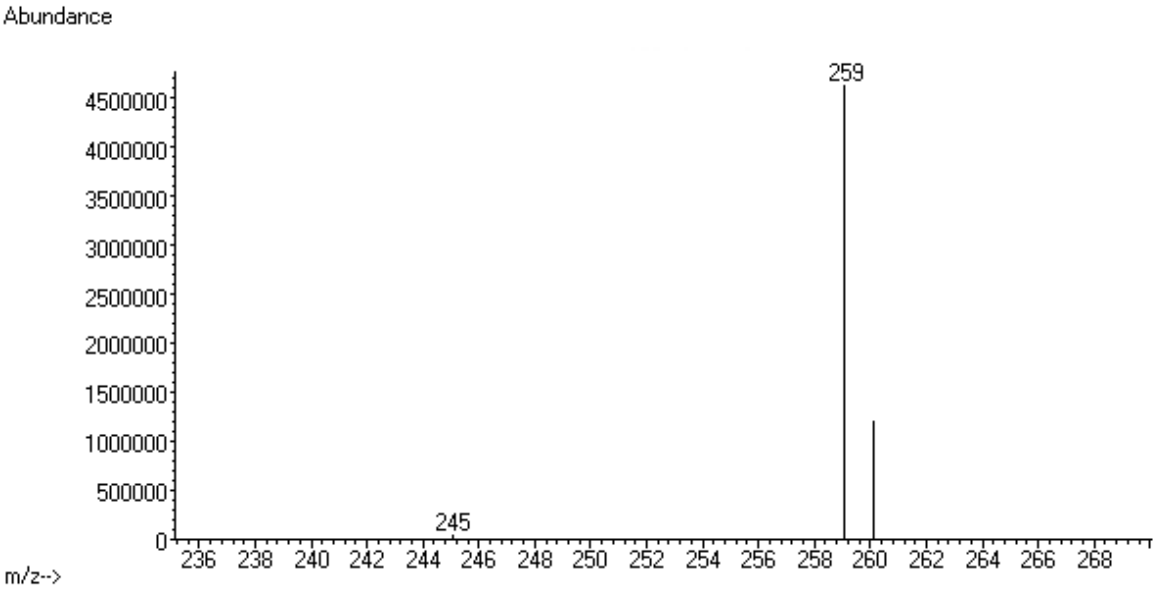


Figure F-1 Representative GC-MS trace for plasma sample subjected to selected ion monitoring for α-KIC

Aliquots of plasma were subjected to derivatisation with BSTFA-TCMS prior to loading onto GC column. A) absorbance spectrum from substances eluting between 6.2 and 8.6 minutes. Elution time of KIC is 8.16 min. B) mass spectrum showing results of selected ion monitoring at 245, 259 and 260 m/z. BSTFA-TCMS, N,o-Bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane.

F2 RNA absorbance spectra

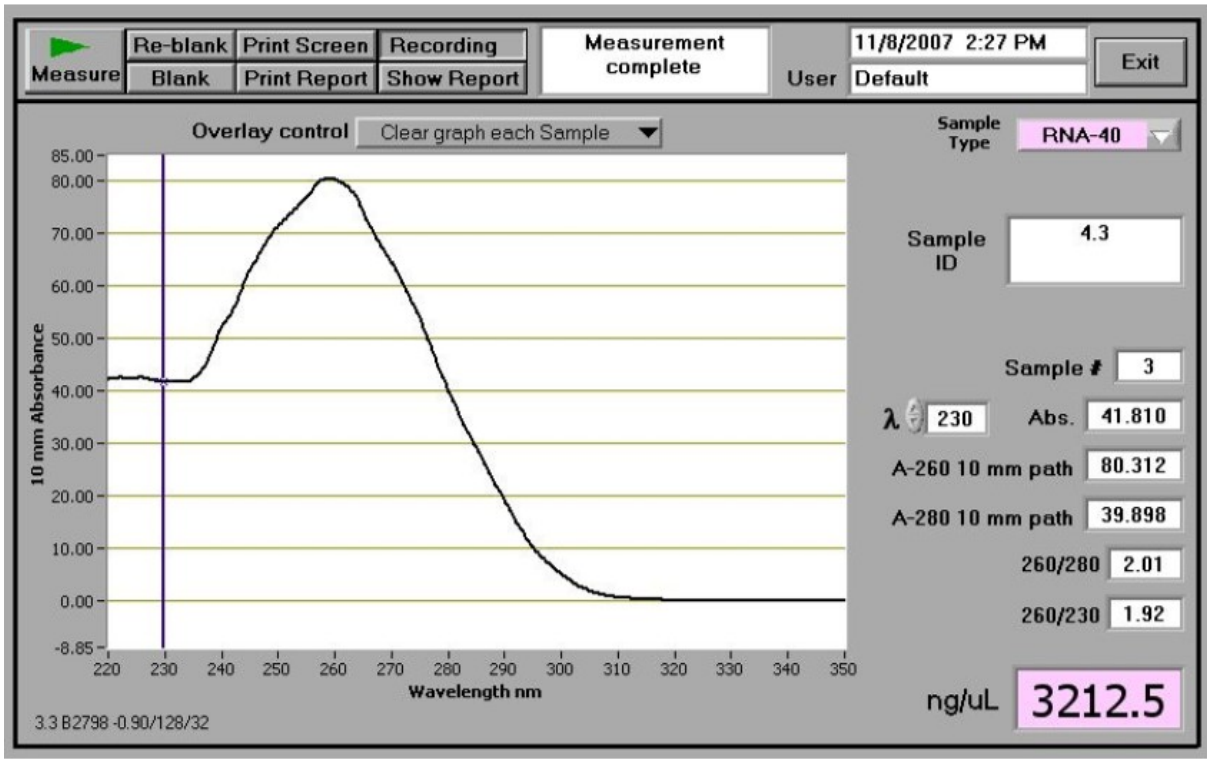


Figure F-2 Absorbance spectrum from a sample yielding substantial RNA of a high quality
A 260/280 ratio of greater than a1.9 was considered acceptable. Mean RNA yield was 940 ng/µL.

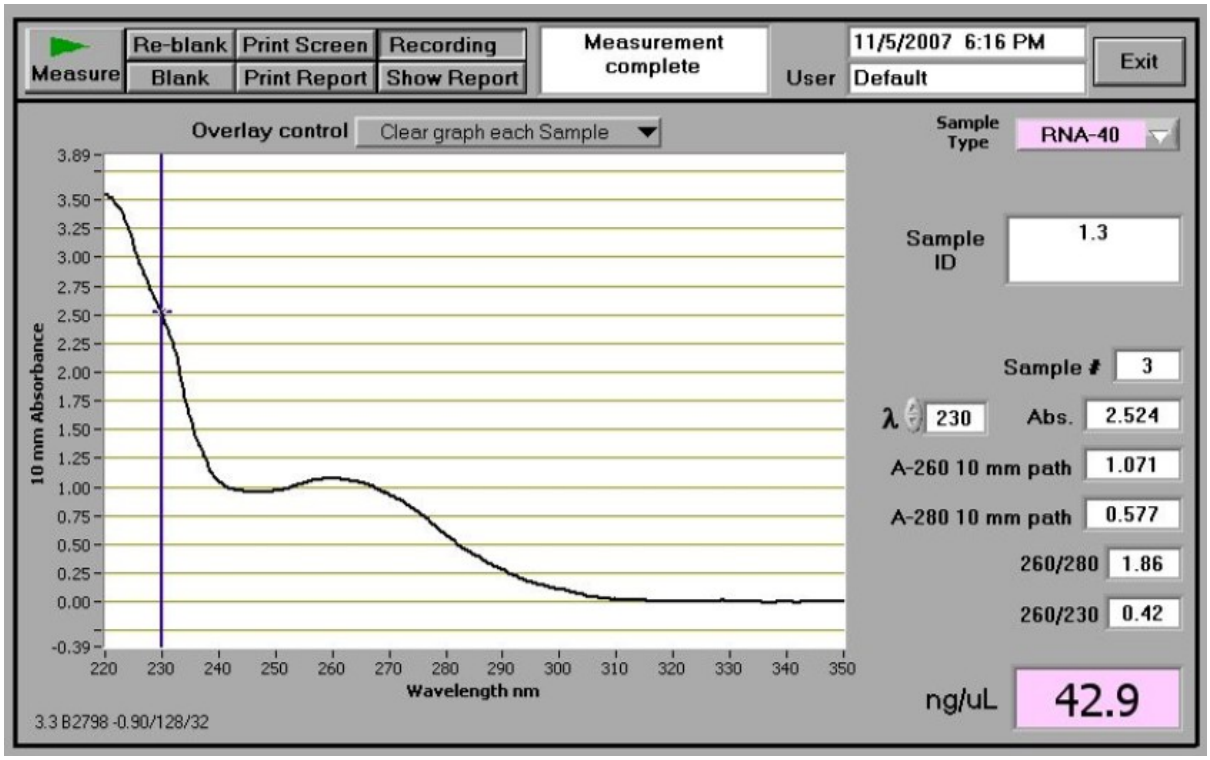


Figure F-3 Absorbance spectrum from a sample yielding very little RNA and of poor quality
A 260/280 ratio of greater than a1.9 was considered acceptable. Mean RNA yield was 940 ng/µL.

F3 PCR amplification curves

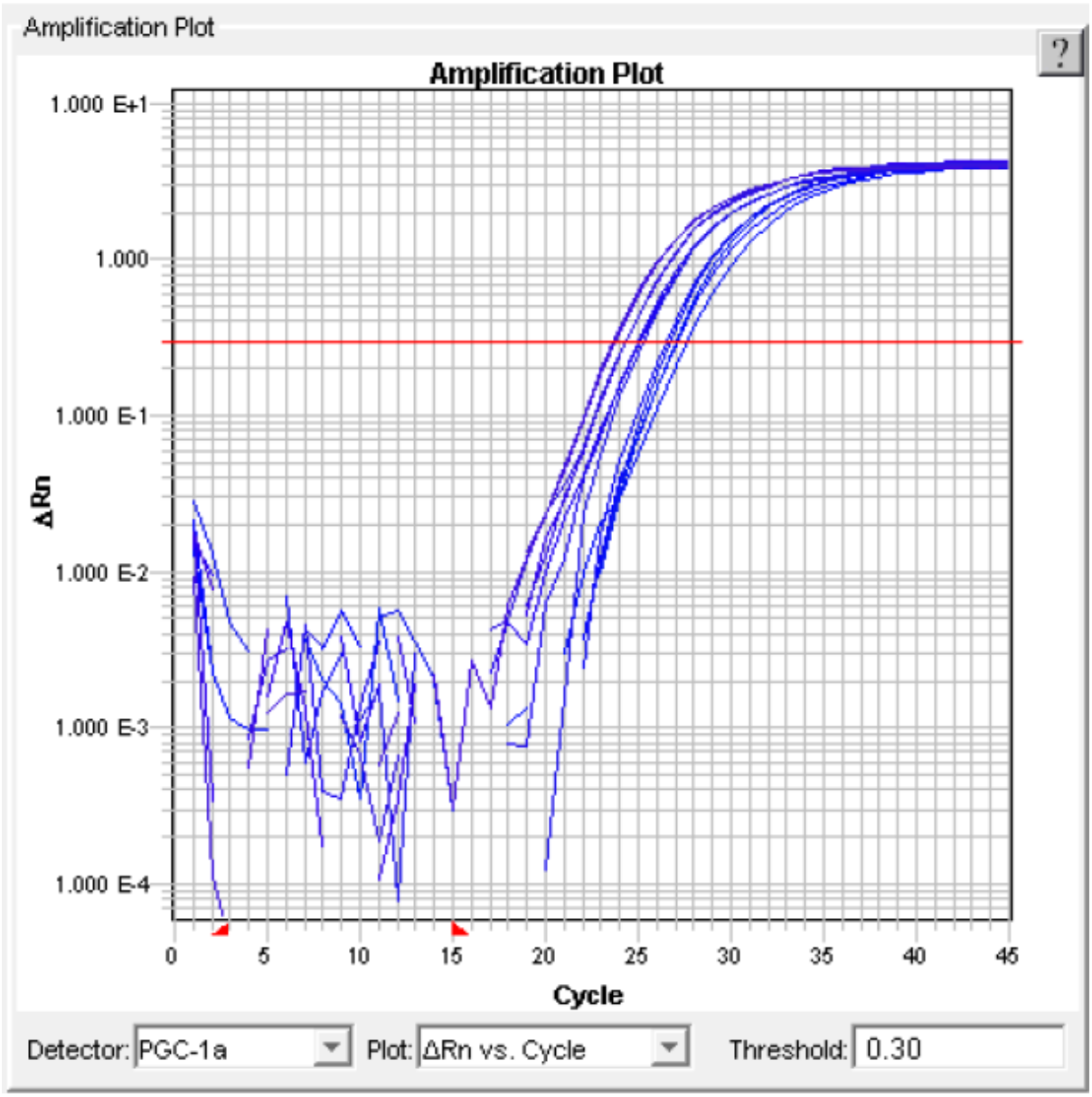


Figure F-4 Amplification curve for PGC-1α for all samples and replicates for a single subject
Threshold is set to 0.3 and background cycles to 3-15.

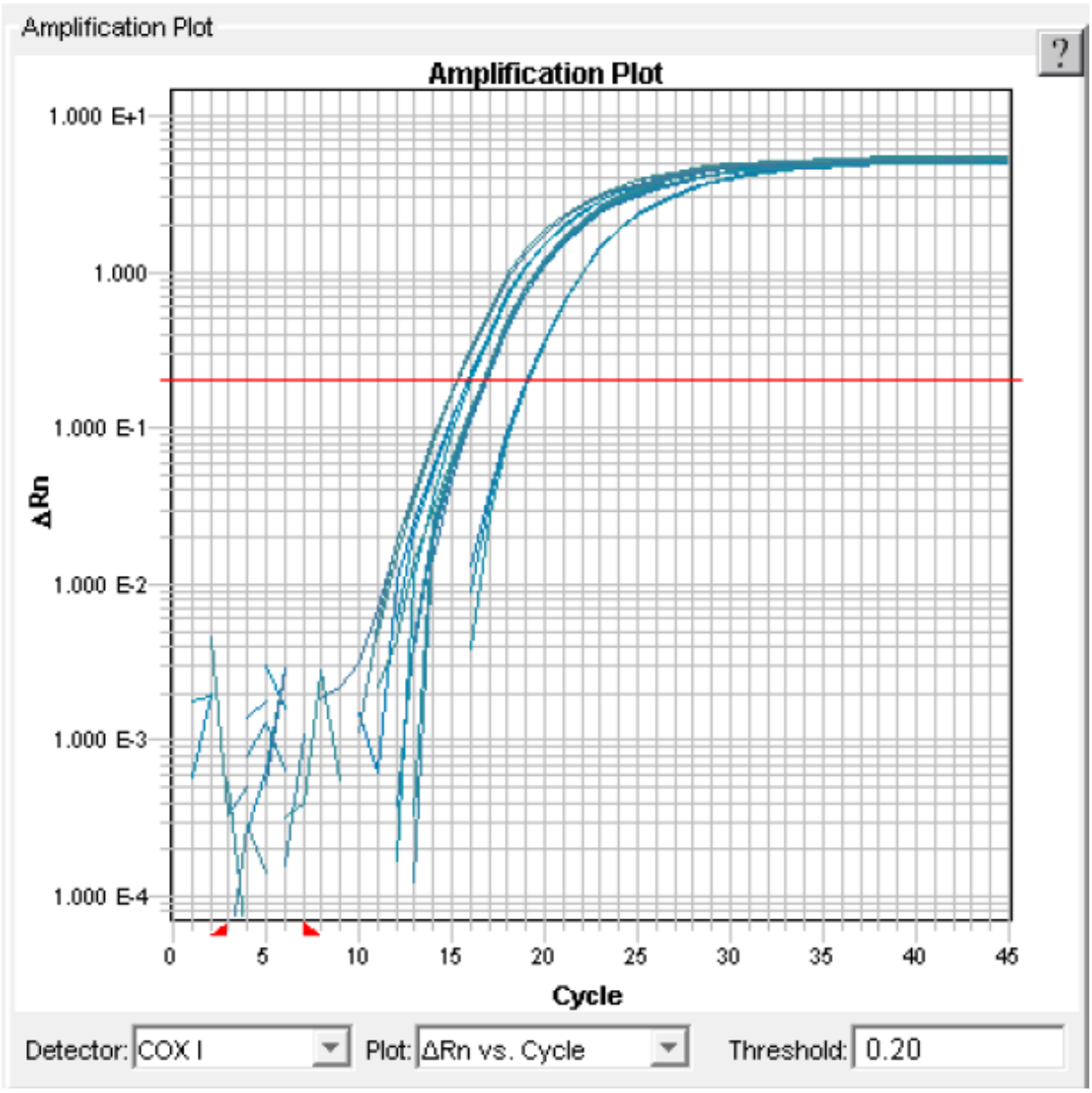


Figure F-5 Amplification curve for COXI for all samples and replicates for a single subject
Threshold is set to 0.2 and background cycles to 3-7.

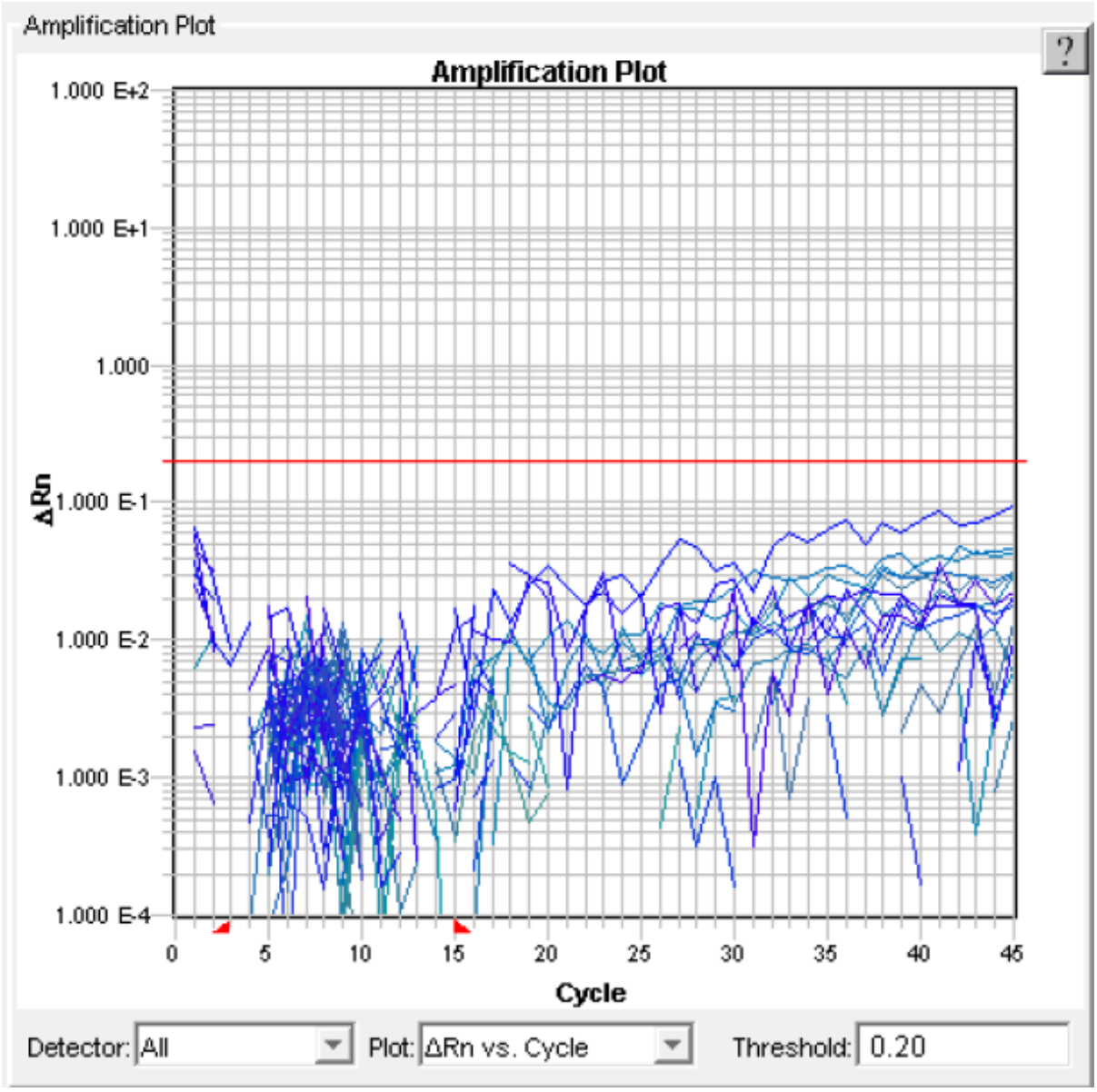


Figure F-6 Amplification curve for all reverse transcription negative control samples and replicates for a single subject

None of the samples amplified which indicates that there was no residual genomic DNA in the DNase treated samples prior to reverse transcription. Threshold is set to 0.2 and background cycles to 3-15.

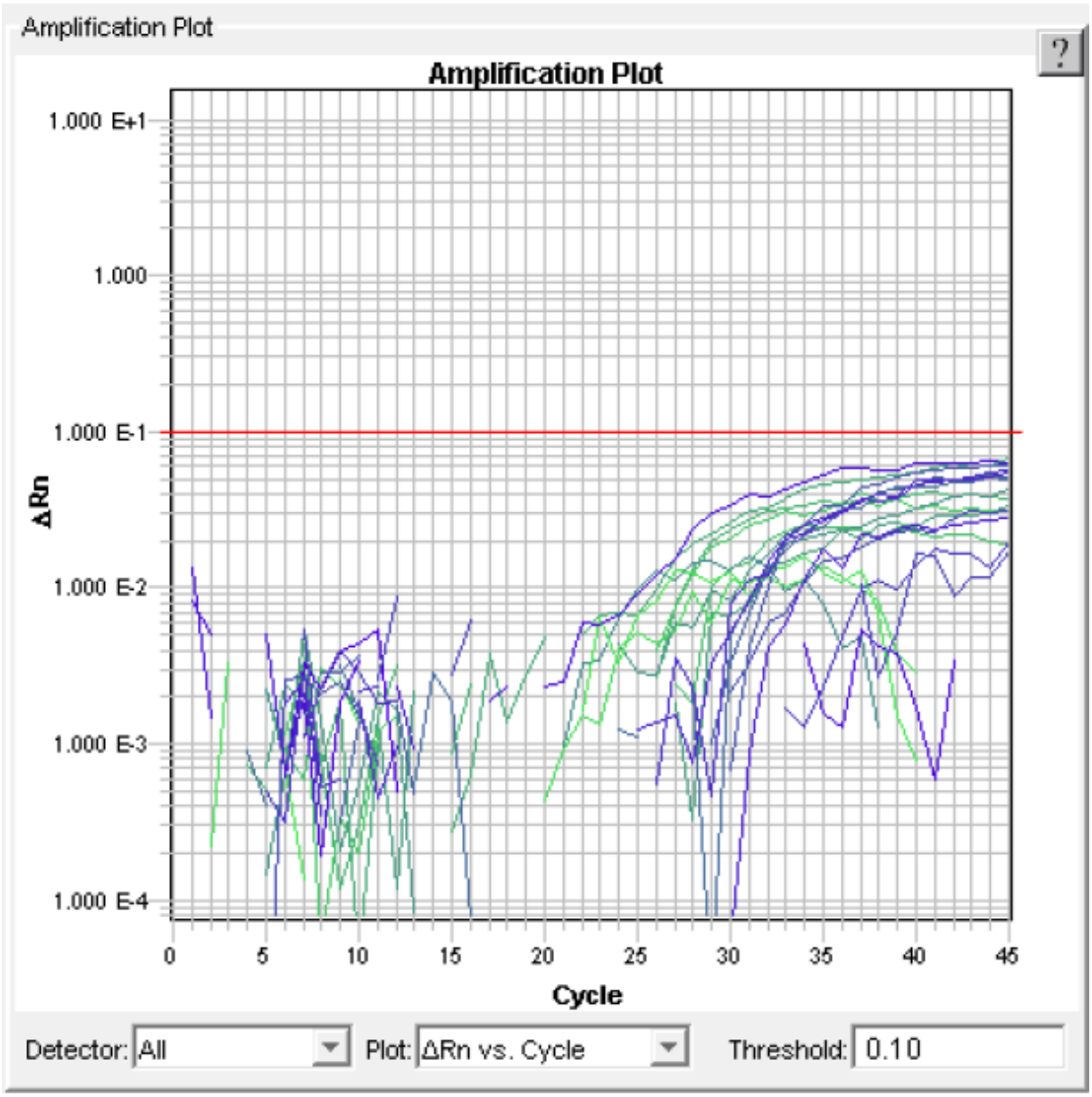


Figure F-7 Amplification curve for all non-template control samples in a single plate

None of the samples amplified which indicates that there was no contaminating genomic DNA in the PCR mix. Threshold is set to 0.1.

Appendix G Supplementary data

G1 RNA extraction

RNA yield and quality for each muscle sample are shown in tables G-1 and G-2 respectively.

	Rest	CHO		PRO	
Subject		sample 1	sample 2	sample 1	sample 2
1	31.168	63.956	26.552	11.902	.858
2	33.520	15.303	2.163	1.669	2.168
3	8.002	9.035	24.188	6.814	13.095
4	1.125	14.642	32.125	1.381	12.473
5	6.608	78.952	11.512	6.054	14.480
6	8.902	14.548	18.778	27.089	28.815
8	3.957	11.258	1.858	1.304	8.280
9	21.140	9.118	26.549	9.407	11.384
10	9.516	24.440	0.975	8.142	3.750
11	8.880	14.495	1.925	4.335	27.073
12	6.760	2.091	2.079	12.161	24.008

Table G-1 RNA yield (µg) for each muscle sample obtained in the second study

RNA was extracted from muscle samples using TRIzol according to the manufacturer's instructions with some modifications (see 6.2.3.1 for details). Yield was determined on a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington DE, USA). CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition.

	Rest	CHO		PRO	
subject		sample 1	sample 2	sample 1	sample 2
1	2.04	2.0	2.01	2.03	1.86
2	1.98	2.05	1.98	1.92	1.97
3	1.95	1.92	1.98	2.01	2.05
4	1.95	1.99	2.01	1.95	2.01
5	1.93	1.91	2.00	2.02	2.04
6	1.95	1.97	2.01	2.01	2.02
8	1.85	2.04	1.80	1.92	1.96
9	2.05	2.02	2.04	1.98	1.98
10	1.95	2.04	1.80	1.98	1.97
11	2.01	2.05	1.96	2.01	2.03
12	1.96	1.89	1.92	2.02	2.04

Table G-2 Absorbance ratio (260:280 nm) of extracted RNA for each muscle sample obtained in the second study

RNA was extracted from muscle samples using TRIzol according to the manufacturer's instructions with some modifications (see 6.2.3.1 for details). Absorbance was determined on a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington DE, USA). CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition.

G2 Protein extraction

The protein yield from each muscle sample is shown in Table G-3. In general, the greater the wet weight of the sample before homogenisation, the greater the concentration of protein in the lysate.

		CHO		PRO	
subject	baseline	3h	6h	3h	6h
2	6.59	4.22	4.43	4.95	7.94
3	5.33	6.68	4.31	11.35	6.94
4	7.73	8.05	6.55	4.21	7.02
9	16.96	13.10	15.87	7.35	8.06

Table G-3 Protein concentration (µg/µL) of muscle lysate from each sample

Protein concentration was determined using a detergent compatible assay (Bio-Rad Laboratories, Hercules CA, USA, see 7.2.1.1 for details). CHO, carbohydrate-only condition; PRO, protein-plus-carbohydrate condition.

10 References

11 Glossary of terms and definitions

aerobic exercise: the repeated application of a small amount of force over a long duration in a cyclic and dynamic fashion such as occurs in cycling, running, and swimming.

ageing: a decline in structure and function over time.

bolus: a large dose given at one time, designed to prime a system; used in this thesis in reference to isotope infusion.

deltaR$_n$: the fluorescence signal generated by a polymerase chain reaction; obtained by subtracting R$_n$$^-$ from R$_n$ for that sample.

decline in function: a decrease in the capacity to perform a desired function. This thesis is concerned with the capacity of skeletal muscle to perform its required tasks, and the efficiency with which it does so, per gram or litre or cross-sectional area.

disability: a loss of physical ability that prevents normal function

disease: an interruption or disturbance in the function of an organ or organelle; a condition characterised by an identifiable set of signs or symptoms. This thesis is concerned with skeletal muscle and its mitochondria.

distal: further away from the torso.

dysfunction: an impairment in function.

endogenous control: an active reference used in polymerase chain reaction to which the sample signal is normalised in order to account for differences in the amount of RNA added to a reaction, aka housekeeping gene.

endurance capacity: the length of time that exercise of a given intensity can be sustained.

epidemiological: a study concerned with the incidence and prevalence of disease in large populations.

euglycemic: having the same concentration of glucose.

fractional synthesis rate: the rate of synthesis of a fraction of the body's protein; used in this thesis to describe the rates of synthesis of mixed skeletal muscle and skeletal muscle mitochondrial protein

functional capacity: the ability to carry out desired tasks. This thesis is concerned with the ability to perform activities of daily living.

gluconeogenesis: the production of glucose by the liver using such precursors as amino acids, lactate, and glycerol.

hyperaminoacidemic: having a higher concentration of amino acids than at baseline, i.e. prior to intervention.

hyperinsulinemic: having a higher concentration of insulin than at baseline, i.e. prior to intervention.

kinase: a transferase that catalyses the phosphorylation of a substrate by ATP.

kinetics: study of rates and rates of change.

macronutrient: one of carbohydrate, protein, fat, or water; any nutrient required by the human body in doses of greater than one gram per day.

mitochondrial biogenesis: synthesis of mitochondrial protein; results in expansion of the reticulum.

mitochondrial content: the quantity of mitochondrial protein present in a muscle.

mitochondrial protein synthesis: the production of mitochondrial protein; usually expressed as a rate.

murine: of a mouse.

mitochondrial volume: the size of the mitochondrial reticulum; usually expressed as density; determined with an electron microscope.

muscle oxidative capacity: the capacity of a particular muscle to extract oxygen from the blood per unit volume of muscle; expressed in units of mg/mL/min.

muscle oxidative enzyme activity: the activity of mitochondrial enzymes involved in oxygen extraction by working muscle.

muscle protein breakdown: the degradation of muscle protein; includes all sub-fractions such as sarcolemmal, myofibrillar, and mitochondrial; usually expressed as a rate.

muscle protein synthesis: the production of muscle protein; includes all sub-fractions such as sarcolemmal, myofibrillar, and mitochondrial; usually expressed as a rate.

muscle protein turnover: the combination of muscle protein breakdown and synthesis.

muscle quality: the ability of muscle to carry out its designated functions, and the efficiency with which it does so, per gram or litre or cross-sectional area. Some examples of muscle functions are enzymes catalysing reactions, the oxidation of fuel, and the production of force.

myopathy: a disease of muscle.

no template control: a sample in the polymerase chain reaction plate that does not contain any complementary DNA; used to verify the quality of the amplification process; provides a value for R_n^-.

normalised reporter: the emission intensity of the reporter dye in polymerase chain reaction divided by that of the passive reference dye.

passive reference dye: used in polymerase chain reaction to provide an internal reference; the reporter dye signal is normalised to this reference during data analysis in order to correct for changes in concentration or volume.

pathology: any deviation from a healthy or efficient condition; the functional manifestations of a disease.

physical function: the ability to perform the activities of daily living.

proximal: closer to the torso.

R_n: measured fluorescence of a sample in a polymerase chain reaction.

R_n^-: baseline fluorescence in a polymerase chain reaction.

resistance exercise: multiple repetitions of lifting a load which requires muscles to move at a high force output over a brief period of time.

ribosome: RNA-protein complex that functions in protein assembly by binding to mRNA.

sarcopenia: decline in muscle fibre size and/or number with age accompanied by structural and biochemical changes; loss of skeletal muscle mass.

stoichiometry: the quantitative relationship between reactants and products in a chemical reaction.

threshold: ΔR_n value used to determine C_T in a polymerase chain reaction; set within the exponential phase of an amplification curve.

threshold cycle (C_T): amplification cycle number at which fluorescence passes the fixed threshold in a polymerase chain reaction.

whole body oxidative capacity: maximal rate of oxygen utilisation by skeletal muscle; aka whole body aerobic capacity; aka VO_{2max}.

whole body protein turnover: the combination of breakdown and synthesis of all proteins in the body.