NUTRITIONAL REGULATION OF MITOCHONDRIAL BIOGENIC ENERGY-SENSING PATHWAYS IN SKELETAL MUSCLE FOLLOWING ENDURANCE EXERCISE

by

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ABSTRACT

Endurance exercise improves health partly though improvements in skeletal muscle function. Mitochondrial biogenesis is one of the mechanisms that underpin the positive health benefits of endurance exercise. Endurance-exercise and energy sensitive pathways signal to promote transcriptional processes that initiate the adaptive response. Thus the aim of this thesis was to further understand the regulation of post-exercise signalling within skeletal muscle, with specific focus on the activation of energy-sensitive mitochondrial biogenic signalling pathways. It was demonstrated that muscle-specific knockout of p53 does not impair mitochondrial protein content or enzyme activity within mouse skeletal muscle. In human skeletal muscle, fasting and fasted-exercise augment $CREB^{Ser133}$ and $AMPK^{Thr172}$ phosphorylation, while the mRNA expression of *PDK4* but not *PPARGC1A* is also increased in the fasted state. Finally, one week of nicotinamide riboside supplementation did not alter skeletal muscle mitochondrial respiration and whole-body substrate utilisation at rest or during endurance exercise, while SIRT1 and 3 activity and *PPARGC1A* mRNA expression at rest and following endurance-exercise are also unaffected by nicotinamide riboside supplementation. Overall, this thesis contributes novel data to the understanding of metabolism and skeletal muscle signalling following endurance exercise and how nutrition and endurance exercise could be integrated to optimise specific adaptations.

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Declaration

I declare that all of the work contained within this thesis is my own with the following exceptions:

i. Dr Jessica Dent assisted in the writing of sections 1.4.1 and 1.4.2.

ii. Dr Andrew Philp originally designed and created the figures contained within chapter 1, which were then edited by myself.

iii. Muscle samples from p53 mKO and WT mice for chapter 2 were a kind gift from Dr Christopher Adams from the University of Iowa.

iv. Dr Sophie Joanisse advised and assisted during the experimentation and analysis of Figure 2.1C.

v. Dr Jessica Dent undertook the skeletal muscle biopsy procedures for chapter 3. Mr Henry Ogden and Ms Martina Zemp provided assistance during the experimental trials contained within chapter 3.

vi. Dr Gareth Wallis undertook the skeletal muscle biopsy procedures for chapter 4.

vii. Dr Sophie Joanisse, Mr Stephen Ashcroft and myself performed experimentation for Figure 4.2A.

viii. Dr Andrew Philp and Dr Jessica Dent, as supervisors, provided intellectual input throughout.

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LIST OF ABBREVIATIONS

β-HAD	3-hydroxyacyl-CoA dehydrogenase
ACADL	acyl-CoA dehydrogenase long chain
ACADM	acyl-CoA dehydrogenase medium chain
ACADVL	acyl-CoA dehydrogenase very long chain
ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
ADPR	ADP-ribose
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATF2	activating factor 2
ATP	adenosine triphosphate
ATP5o	ATP synthase subunit 5o
BNIP3	BCL2-interacting protein 3
CaM	calmodulin
CAMK	calmodulin-dependent protein kinase
CAMKK	calmodulin-dependent protein kinase kinases
cAMP	cyclic adenosine monophosphate
CD36	fatty acid translocase
cDNA	complementary dioxyribose nucleic acid
$CI+II_E$	maximal electron chain transport capacity

$CI+II_P$	coupled oxidative phosphorylation through complexes one and two
CI_L	leak respiration through complex one
CI_P	coupled respiration through complex one
CO1	cytochrome-c oxidase subunit 1
COX	cytochrome-c oxidase
CRE	cAMP response-element binding protein
CS	citrate synthase
Cyt-c	cytochrome-c
DAPI	4’-6’-diamidino-2-phenylindole
DRP1	dynamin-related protein 1
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N’,N’,-tetraacetic acid
ERR	oestrogen-related receptor
Fis1	fission 1
FOXO	forkhead box protein O
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN5	general control of amino acid synthesis 5
GLUT4	glucose transporter type4
GPR109A	niacin receptor 1
HDAC	histone deacetylase
KO	knockout

LKB1	liver kinase B1
LPL	lipoprotein lipase
MCK	muscle creatine kinase
MEF2	myocyte enhancer factor 2
MeNAM	N^1-methylnicotinamide
MFN2	mitofusin 2
mKO	muscle-specific knockout
MnSOD	superoxide dismutase
mtDNA	mitochondrial deoxyribonucleic acid
NA	nicotinic acid
NAAD	nicotinic acid adenine dinucleotide
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADSYN1	NAD^+ synthase 1
NAM	nicotinamide
NAMN	nicotinic acid mononucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	nicotinic acid phosphoribosyltransferase
NAR	nicotinic acid riboside
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide mononucleotide adneylyltransferase
NNMT	nicotinamide N-methyltransferase
NR	nicotinamide riboside
NRF1	nuclear respiratory factor 1

NRK	nicotinamide riboside kinase
OPA1	dynamin-like 120 kDA protein
p160MBP	p160 myb binding protein
p38 MAPK	p38 mitogen-activated protein kinase
PAR	poly (ADP-ribose)
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cell
PDH	pyruvate dehydrogenase
PDK4	pyruvate dehydrogenase kinase 4
PGC1α	peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PHF20	PHD finger protein 20
PINK1	PTEN-induced putative kinase 1
PKA	protein kinase A
POLG1	ploymerase γ 1
PPAR	peroxisome proliferator-activated receptor
RB	retinoblastoma protein
RER	respiratory exchange ratio
RPE	ratings of perceived exertion
RT-PCR	reverse transcription polymerase chain reaction
SCO2	cytochrome-c assembly protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRT	sirtuin

TBST	tris-buffered saline with tween
THBS1	thrombospondin 1
TIGAR	tp53-inducible glycolysis and apoptosis regulator
TLR	toll-like receptors
TNFα	tumour necrosis factor α
TR	thyroid hormone receptor
ULK1	unc-51 like autophagy activating kinase 1
VEGF	vascular endothelial growth factor
$\dot{V}O_{2max}$	maximal oxygen uptake
VWR	voluntary wheel running
WT	wild-type

LIST OF GENE SYMBOLS

Atp5a1	ATP synthase F1 subunit α
Atp6v0a2	ATPase H^+ transporting V0 subunit A2
Cox10	cytochrome-c oxidase assembly factor COX10
Cox18	cytochrome-c oxidase assembly factor COX18
Cox4i1	cytochrome-c oxidase subunit 4I1
Cox4i2	cytochrome-c oxidase subunit 4I2
Cpt1b	carnitine palmitoyltransferase 1B
Cs	citrate synthase
Cyc1	cytochrome-c1
Esr1	estrogen receptor 1
Fis1	fission, mitochondrial 1
Hk2	hexokinase 2
Ldh1	lactate dehydrogenase 1
Ldh2	lactate dehydrogenase 2
Mdh1	malate dehydrogenase 1
Mdh2	malate dehydrogenase 2
Mfn1	mitofusin 1
Mfn2	mitofusin 2
Myc	MYC proto-oncogene, bHLH transcription factor
Myod1	myogenic differentiation 1
NADSYN1	nicotinamide adenine dinucleotide synthetase 1
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	*n*icotinic acid phosphorybosyltransferase

NMNAT1	nicotinamide mononucleotide adenylyltransferase 1
NMNAT2	nicotinamide mononucleotide adenylyltransferase 2
NMNAT3	nicotinamide mononucleotide adenylyltransferase 3
NMRK1	nicotinamide riboside kinase 1
NMRK2	nicotinamide riboside kinase 2
NNMT	nicotinamide N-methyltransferase
Ncoa1	nuclear receptor coactivator 1
Ncoa2	nuclear receptor coactivator 2
Nr1d2	nuclear receptor subfamily 1 group D member 2
Ndufa1	NADH:ubiquinone oxidoreductase subunit A1
Ndufc1	NADH:ubiquinone oxidoreductase subunit C1
Opa1	OPA1 mitochondrial dynamin like GTPase
Ppara	peroxisome proliferator activated receptor α
Ppard	peroxisome proliferator activated receptor δ
PPARGC1A	peroxisome proliferator activated receptor gamma coactivator 1α
Ppargc1b	peroxisome proliferator activated receptor gamma coactivator 1β
PDK4	*pyruvate dehydrogenase kinase 4*
Sdhb	succinate dehydrogenase complex iron sulfur subunit B
Slc2a4	glucose transporter type 4
Tfam	transcription factor A, mitochondrial
Tfb1m	transcription factor B1, mitochondrial
Tfb2m	transcription factor B2, mitochondrial

Timm8a1	translocase of inner mitochondrial membrane 8A1
Timm9	translocase of inner mitochondrial membrane 9
Tomm20	translocase of outer mitochondrial membrane 20
Tomm22	translocase of outer mitochondrial membrane 22
Uqcrc1	ubiquinol-cytochrome-c reductase core protein 1
Uqcrc2	ubiquinol-cytochrome-c reductase core protein 2
Vegfa	vascular endothelial growth factor A
Vegfb	vascular endothelial growth factor B
Vegfc	vascular endothelial growth factor C

1 GENERAL INTRODUCTION: ENERGY-SENSING AND MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE

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1.1 Abstract

Endurance exercise is well established to improve health, with enhanced skeletal muscle mitochondrial biogenesis one of the mechanisms that underpins this adaptation. This process is regulated by complex signalling pathways that sense changes in metabolites, energy availability and contraction to initiate the transcriptional mitochondrial biogenic process. When repeated regularly, chronic activation of mitochondrial biogenesis results in a greater capacity for aerobic ATP synthesis, particularly from lipid sources, enhanced fatigue resistance and accelerated recovery in skeletal muscle. Importantly, the activation of these signalling pathways can be manipulated by exercise intensity, intermittency, duration and the nutritional environment that exercise is performed in. Thus developing strategies to enhance the activation of these signalling pathways holds therapeutic potential by augmenting mitochondrial biogenesis and oxidative capacity of skeletal muscle.

1.2 Endurance exercise for health

Physical activity and endurance exercise training has long been known to improve health and wellbeing as well as improve prolonged athletic performance. Seminal work by Morris *et al* (223) described lower mortality rates in active bus conductors versus sedentary drivers. Subsequently, an enormous body of literature has demonstrated how physical activity, exercise or higher levels of cardiovascular fitness lower rates of or mortality from cardiovascular disease (28), type II diabetes (170), obesity (148), sarcopenia (89) and certain cancers (235), among other conditions.

It is now beyond contention that maintaining adequate levels of physical activity throughout the lifespan is an important mediator of maintaining health. Many of the gross mechanisms have been elucidated (e.g. greater stroke volume, reduced blood pressure, reduced blood glucose concentration, greater muscle mass, increased skeletal muscle mitochondrial volume, etc.) and are described in section 1.2.1. However, many of the molecular mechanisms underpinning these adaptations are either not fully understood or methods of targeting these molecular pathways have not been completely established. It will therefore be the aim of this thesis to further understand the molecular mechanisms conferring skeletal muscle adaptations following endurance exercise, with a particular focus on targeting energy-sensing pathways to promote mitochondrial biogenesis. Mitochondria are the cellular site of aerobic ATP production, providing the majority of energy for cellular processes and as such are often referred to as the ‘powerhouses of the cell’. Within this thesis ‘mitochondrial

biogenesis' is ultimately defined as an increase in capacity for oxidative phosphorylation and ATP production. Within this definition, 'mitochondrial biogenesis' encompasses both an increase in mitochondrial mass or volume *per se* and increases in respiratory efficiency of existing mitochondria.

1.2.1 Adaptations to endurance exercise training

Regular physical activity in the form of endurance training can substantially improve endurance capacity in a range of populations (38, 56, 88, 99, 224). This is achieved both by an increased maximal oxygen uptake ($\dot{V}O_{2max}$) and an ability to work at a given submaximal intensity with a smaller homeostatic disturbance (21). These outcomes are the result of the interaction between a series of physiological adaptations that ultimately result in an increased capacity for delivery and utilisation of oxygen and lipids for the synthesis of adenosine triphosphate (ATP) by oxidative phosphorylation in skeletal muscle. Furthermore, many of these adaptations have positive benefits not just in terms of endurance performance but also in general health.

1.2.1.1 Cardiovascular adaptations to endurance exercise training

Endurance exercise training results in an increase in $\dot{V}O_{2max}$ principally by increases in cardiac output, via elevated stroke volume and reduced vascular systemic resistance (30, 153, 165), and elevated oxygen carrying capacity via increased red blood cell volume (30, 289). In addition, training-induced capillarisation of the musculature (144, 225) results in shorter diffusion

distances (286), which alongside possible increases in myoglobin concentration within working muscles (125) increases the oxygen extraction capacity of the musculature. Together, increased cardiac output and greater oxygen extraction by exercising muscles increases $\dot{V}O_{2max}$ (307).

1.2.1.2 Skeletal muscle adaptations to endurance exercise training

While the cardiovascular system may limit maximal aerobic capacity, oxygen uptake at a given submaximal intensity is the same in the trained and untrained state (125). Therefore, exercise capacity at submaximal workloads is more closely related to adaptations in skeletal muscle (21), which demonstrates considerable plasticity when exposed to different functional demands. Following endurance training, the shift in whole-body substrate oxidation towards greater lipid oxidation (167) and reduced glycolysis (113) allows for a greater exercise intensity to be supported predominantly by aerobic energy production. This results in reduced lactate accumulation in blood and muscle (157, 285) and sparing of muscle glycogen stores (113), which play a pivotal role in the increased exercise capacity and performance following endurance training.

Endurance exercise training results in a shift towards a more oxidative, fatigue-resistant, phenotype of the trained muscle. An increased proportion of slow-twitch type I, fast oxidative type IIa and hybrid fibres is apparent, with a reduction in rapidly fatiguing fast glycolytic type IIx and IIb fibres (9, 60, 301). This is caused by hypertrophy of type I and type IIa fibres (60) and a

transformation of fibres to a slower phenotype, by an altered expression of myosin heavy chain isomers (262). The shift towards a slower muscular phenotype is of physiological importance to endurance performance given the close relationship between muscle fibre composition and both the oxygen cost of locomotion (66) and lactate threshold (146). Moreover, a shift in fibre-type distribution may affect glucose uptake and therefore insulin sensitivity because slower-oxidative fibres have a higher content of glucose transporter type 4 (GLUT4) and mitochondrial metabolic proteins (69, 197), resulting in more effective removal of glucose from the blood and oxidation in mitochondria.

1.2.1.3 Skeletal muscle mitochondrial adaptations to endurance exercise training

Aerobic exercise promotes a large increase in mitochondrial mass, mitochondrial enzyme activity and oxidation efficiency (135, 138, 221, 236, 308). Holloszy first demonstrated an increased mitochondrial enzyme activity in rats following progressive endurance training (135), a finding that has subsequently been replicated in numerous human studies (60, 99, 106, 138, 191, 308). The activity of enzymes in the electron transport chain can increase up to two-fold in response to training (60, 135). Concentrations of cytochrome-c (Cyt-c) also increase by approximately two-fold, suggesting the increased enzyme activity is due to an increase in mitochondrial enzyme protein content (135). Crucially, in this study oxidative phosphorylation was tightly coupled, suggesting that the increase in electron transport capacity was associated with a proportional increase in the capacity for ATP production by oxidative

phosphorylation (135). Enzymes involved in the citric acid cycle (137), fatty acid oxidation (221) and ketone oxidation (353) also increase. However, mitochondrial enzymes do not respond in a uniform manner to endurance training. In response to the same exercise stimulus in rats, enzymes involved in the oxidation of fatty acids increase by approximately two-fold (221), whereas enzymes of the citric acid cycle only increase by up to 50% (137). Glycolytic enzymes remained unchanged, or even decrease in activity when expressed per milligram of mitochondrial protein content (136, 236). Therefore, regular endurance exercise results in an adaptive response to increase the capacity for ATP resynthesis by oxidative phosphorylation, especially from the oxidation of fatty acids, and in doing so reduces the reliance upon glycolysis.

In an electron microscopy study, Gollnick and King (107) demonstrated an increased size, number and density of mitochondria following endurance exercise training in rats. Hoppeler *et al* (138) replicated this finding in a human cross-sectional study comparing the skeletal muscle of well-trained orienteers versus untrained controls, with further longitudinal studies confirming this training effect in humans (140, 323). Thus endurance exercise increases the absolute volume of the mitochondrial pool.

In addition to increases in absolute mitochondrial volume, mitochondrial morphology and connectivity can also be modified by exercise (164). Mitochondria can exist as both a highly organised reticulum and as fragmented organelles, with mitochondria remodelled by fusion and fission events

dependent upon the metabolic state of the cell (186, 263, 370). Fragmented mitochondria have lower maximal rates of carbohydrate and fat oxidation (14, 250), thus an alteration in the mitochondrial morphology, as well as overall content, may play a crucial role in the adaption to endurance exercise. There is growing evidence in support of endurance exercise as a stimulus for the proliferation of the mitochondrial reticulum (46, 77, 164, 242, 249). Endurance exercise training proliferates the mitochondrial reticulum in rat skeletal muscle (164) and increases the mRNA and protein expression of mitochondrial fusion proteins (164, 242). Acutely, Picard *et al* (249) have demonstrated that endurance exercise increases the number of electron dense contact sites between adjacent mitochondria in skeletal muscle of mice, hypothesised to increase electrical coupling between mitochondria (102). Furthermore, evidence of interconnecting mitochondria linked by matrix-filled membrane-bound bridges in the skeletal muscle of the exercising mice was apparent (249). However, while it is speculated that these processes may serve as pre-fusion events in mitochondria, no differences in mitochondrial fusion proteins or mitochondrial morphology were apparent between sedentary and exercising mice.

Conversely to mitochondrial fusion, acute fission is also likely to occur following endurance exercise, which is required for removal of damaged and dysfunctional sections of mitochondria by mitophagy (332). Endurance exercise acutely activates the mitochondrial fission proteins fission 1 (Fis1) and dynamin-related protein 1 (DRP1) in rodents and humans (77, 149, 150, 237). Critically, formation of highly oxidised isolated mitochondria can be visualised following

endurance exercise in mice (172). These mitochondria co-localise with the lysosome suggesting evidence of post-exercise mitophagy (172). However, there is currently no evidence of exercise-induced mitophagy in human skeletal muscle. Indeed, the expression of the mitophagy markers BCL2-interacting protein 3 (BNIP3), PTEN-induced putative kinase 1 (PINK1), or parkin do not change after ultra-endurance treadmill running (149) or cycling at 70% $\dot{V}O_{2peak}$ for 30 minutes (303) or 2 hours (292) in humans.

In addition to morphological changes in the overall mitochondrial reticulum, endurance exercise training may also alter the internal morphology of mitochondria. The density of mitochondrial cristae could influence mitochondrial efficiency (i.e. greater respiratory rates per mitochondrial volume or content) by determining the surface area for electron transfer (61). In a cross-sectional study, Nielsen *et al* (226) demonstrated increased cristae density in skeletal muscle mitochondria from endurance-trained athletes compared to inactive controls. However, it must be noted that earlier studies have reported no difference in cristae density between training statuses (138). Furthermore, Nielsen *et al* (226) found no change in cristae density of previously sedentary individuals following 10-weeks of endurance exercise training, while 28-days of skeletal muscle electrical stimulation in cats does not alter cristae density (295). Thus elevated cristae density with endurance exercise training remains controversial and, given the short-term resistance to changes in cristae density, is unlikely to explain evidence of increased mitochondrial respiratory capacity without changes in mitochondrial volume following exercise training (32, 244).

Another potential explanation for increased efficiency of mitochondrial respiration following endurance training (32, 244) could come from formation of supercomplexes within the electron transport chain. Complexes of the electron transport chain can assemble together to form supercomplexes, thereby minimising diffusion distances, increasing respiratory efficiency and reducing reactive oxygen species production (97). Following 16-weeks of endurance exercise training in humans, the overall content of supercomplexes increased in skeletal muscle, with a particularly striking redistribution of complex III and complex IV into SC $I+III_2+IV_n$ supercomplexes (114). Indeed, exercise efficiency at baseline and the change with training is related to the fraction of complex IV in supercomplexes, while the proportion of complex III in supercomplexes was related to fat oxidation during exercise (114). Furthermore, the absolute amount of supercomplexes as well as the proportion of complex III and complex IV in supercomplexes is related to mitochondrial respiratory capacity in skeletal muscle (114). Thus, formation of supercomplexes within skeletal muscle following endurance exercise training is likely related to increased respiratory efficiency during exercise.

Ultimately, endurance exercise training results in an increased skeletal muscle mitochondrial function. However, it is unclear whether this is driven by increased mitochondrial content or efficiency. In most instances it is likely to be a combination of the two, with the predominance dependent on exercise intensity, duration and volume (110, 111, 212, 267).

1.3 Role of skeletal muscle mitochondria in health and disease

Deficiencies in skeletal muscle mitochondria result in glycolytic phenotypes displaying reduced oxidative phosphorylation, increased lactate production and reduced lipid oxidation. Animals displaying impaired skeletal muscle mitochondrial function can exhibit reduced endurance capacity (281), muscle atrophy (268) and accelerated ageing (79). Furthermore, a reduction in mitochondrial content and capacity for fatty acid oxidation occurs concomitantly with the development of insulin resistance and is associated with the degree of insulin resistance in humans (160, 300). Thus reduced mitochondrial mass, function and oxidative capacity appears related to metabolic inflexibility and the development of chronic metabolic diseases.

During ageing there appears to be a natural decline in mitochondrial content and capacity (298, 335), a finding that is mirrored in studies of muscular disuse (31). However, this is a partially reversible process mediated by nutrition and physical (in)activity (335). Thus, conversely, increases in mitochondrial function can result in greater oxidative capacity and increased rates of lipid oxidation (135, 221). Importantly, exercise-induced increases in lipid oxidation can reduce intramuscular lipid accumulation and improve insulin sensitivity (35). Furthermore, training-induced increases in lipid oxidation during exercise and, especially, non-exercise activity thermogenesis have the potential to increase energy expenditure at the same relative intensity (325), thus itself becoming protective of obesity and the associated health implications. Therefore, understanding the fundamental process of mitochondrial biogenesis, of which

endurance exercise is a potent stimulator, holds great promise as a therapeutic tool for preventing and treating chronic diseases. Section 1.4 will discuss the current knowledge of the mechanisms that lead from acute and chronic exercise stimuli to mitochondrial biogenesis in skeletal muscle. Thus, hopefully, exercise regimes can be developed to specifically target adaptations in mitochondrial mass, morphology and, crucially, function.

1.4 Energy-sensing in skeletal muscle

During exercise, specialised energy, nutrient and contractile 'sensors' detect the metabolic disturbance caused by the increased demand for energy and subsequent utilisation of stored fuels. These exercise responsive sensor-proteins initiate intracellular signalling networks that ultimately converge on transcriptional co-activators and transcription factors (240) that upregulate the expression of mitochondrial genes. If repeated chronically, these molecular signals promote an increased mitochondrial biogenesis.

1.4.1 Alterations in adenine nucleotide availability drive remodelling through AMP-activated protein kinase (AMPK).

ATP turnover is directly proportional to the work rate of skeletal muscle during sustained submaximal exercise (12). However, despite large increases in demand for energy during exercise, ATP concentration is tightly regulated within skeletal muscle (17, 40, 127, 128). For example, muscle ATP concentration only decreases by ~10-15% during prolonged (~100 min) or high-

intensity (to failure) leg extensor exercise in humans (127, 128). Instead, it is metabolites of ATP (e.g. adenosine diphosphate (ADP) and adenosine monophosphate (AMP)) that undergo changes in cellular concentration, albeit within narrow ranges. To simplify somewhat, the reversible ATP reaction (2ADP ↔ ATP + AMP) is maintained close to equilibrium under aerobic conditions (359). Therefore, a rise in the ADP:ATP ratio during exercise causes a shift towards ATP and AMP production in a manner related to the intensity of the muscle contraction (17, 52, 133). In addition to important feedback mechanisms in the control of glycolysis and respiration (68, 116), changes in free AMP and ADP can be sensed by AMP-activated protein kinase (AMPK). AMPK can then initiate intracellular signalling pathways that culminate in the activation of transcription factors and their co-activators involved in the regulation of mitochondrial biogenesis (Figure 1.1).

AMPK is ubiquitously expressed and exists as heterotrimeric complexes, consisting of a catalytic α and regulatory β and γ sub-units (124). Within skeletal muscle, three AMPK heterotrimers are abundant (α1/β2/γ1, α2/β2/γ1 and α2/β2/γ3) (27). Furthermore, endurance exercise appears to preferentially activate the AMPKα2 isoform within human skeletal muscle (27, 95), particularly the α2/β2/γ3 complex (27, 329). Increases in the kinase activity of AMPK complexes occur upon allosteric activation by AMP at two Bateman domains contained within the γ-subunit (317). This allosteric activation results in a conformational change to the AMPK complex, aiding accessibility of upstream kinases to threonine 172 (230) and opposing dephosphorylation by protein phosphatases (288). Together these markedly increase AMPK enzymatic

activity (230). In addition to AMP, ADP binding may also alter cyclical AMPK phosphorylation patterns (231). The amplification of AMPK activity is therefore potently sensitive to both sustained increases in AMP and parallel fluctuations in the ADP:ATP ratio (315).

In mammalian skeletal muscle, liver kinase B1 (LKB1) is generally considered the principal AMPKα2 kinase. Muscle-specific knockout (mKO) of LKB1 reduces AMPK and Acetyl-CoA carboxylase (ACC) (a classical downstream substrate of AMPK) phosphorylation at rest, while activation of AMPK α2 is diminished in response to *in situ* hind limb muscle contraction (280, 327). However, LKB1 activity is unaltered by many AMPK activating stimuli, including exercise (279, 311), suggesting it may be constitutively active and is unlikely to be an energy or nutrient sensor *per se*. Instead, it is likely that AMP and ADP-mediated conformational changes to AMPK allow additional LKB1-mediated phosphorylation, as well as opposing dephosphorylation at threonine 172 (231, 288).

AMPK can also be activated through a Ca^{2+}-dependent phosphorylation via the calcium-calmodulin dependent kinase kinases (CAMKK) α/β (1, 126, 143, 151). Indeed, AMPK phosphorylation and activity is reduced in response to contraction in the presence of CAMKK inhibitors (1, 151). However, although CaMKKs may play a role in exercise-induced AMPK activation, it is unlikely to confer AMPK's energy-sensing properties, as CAMKKs are likely sensitive to contractility rather than energy stress.

In addition to nucleotide fluctuations, muscle glycogen content can also regulate skeletal muscle AMPK activity (246). Elevating skeletal muscle glycogen content, via super-compensation, inhibits both contraction and pharmacological-induced activation of AMPK (76, 355), even when AMP/ATP ratios are similar to a comparable low glycogen state (355). AMPK can bind glycogen via a glycogen-binding domain in its β-subunits (141, 204), regulated via an autophosphorylation site on threonine 148 in the glycogen binding domain that opposes glycogen binding (233, 234). The inhibition of binding and therefore release of AMPK from the glycogen particle may subsequently render AMPK more accessible for phosphorylation and activation. This idea is supported by reduced AMPK-glycogen binding and augmentation of $AMPK^{Thr172}$ phosphorylation, AMPKα2 nuclear abundance and AMPK kinase activity when exercise is commenced with depleted glycogen (20, 171, 247, 316, 356, 365).

AMPK activity is also sensitive to exercise intensity (52, 83, 95, 256). Cycling exercise at moderate (59 ± 1% $\dot{V}O_{2peak}$), and high (79 ± 1% $\dot{V}O_{2peak}$) but not low (40 ± 2% $\dot{V}O_{2peak}$) intensity is associated with increases in the AMP:ATP ratio (52), while glycogen utilization is greater in high (80% $\dot{V}O_{2peak}$) versus low (40% $\dot{V}O_{2peak}$) intensity cycling exercise (24). Concomitantly, AMPKα2 activity is increased sequentially from low to high exercise intensities (52, 83).

A role for AMPK in skeletal muscle endurance capacity is supported by the observation that exercise capacity and adaptation to exercise training is dramatically reduced in AMPK β1/β2 mKO mice (229) and AMPK-α1/α2 mKO

mice (87). However, it should also be noted that impairments in mitochondrial adaptions in AMPK-α1/α2 mKO mice appeared to be more closely related to impaired exercise performance and, thus, reduced work achieved as opposed to reduced AMPK signalling (87). In support, when AMPK-α1/α2 mKO mice were matched with wild-type (WT) mice for training volume, the majority of chronic gene and protein responses were similar between genotypes (87). Recently, an inducible muscle-specific AMPK-α1/α2 KO mouse was developed that, due to the acute ablation of AMPK, do not display the mitochondrial dysfunction seen in all of the previously studied AMPK deficient models (130). This model therefore provides the best opportunity to date, to investigate the direct contribution of AMPK to exercise-induced signalling transduction and mitochondrial biogenesis.

Mechanistically, AMPK can increase skeletal muscle mitochondrial biogenesis (199) through both direct and indirect processes. For example, chronic activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) enhances transcription of peroxisome proliferator-activated receptor (PPAR) gamma, PPARγ coactivator 1-alpha (PGC1α) and nuclear respiratory factor 1 (NRF1), with subsequent increases in mitochondrial biogenesis in rodent skeletal muscle (25, 178, 354). It appears as though this effect is reliant upon functional PGC1α, potentially through the direct phosphorylation of threonine 177 and serine 538 on PGC1α (147). However, although increases in AMPKα2 activity are associated with an exercise intensity dependent regulation of PGC1α mRNA abundance in human skeletal muscle (83), AMPK-mediated

phosphorylation of PGC1α has not been confirmed *in vivo* and so the relevance of this process for exercising skeletal muscle remains unknown (the role of PGC1α in mitochondrial biogenesis is further discussed in section 1.4.4.1).

Whilst the direct phosphorylation of PGC1α by AMPK remains to be determined in skeletal muscle *in vivo*, there is clear evidence that AMPK regulates PGC1α transcription in exercising skeletal muscle through a histone deacetylase 5 (HDAC5)/ myocyte enhancer factor 2 (MEF2) pathway (207). Exercise-induced nuclear translocation of AMPK (208, 247, 316) may lead to the phosphorylation of HDAC5 at serines 259 and 498, which inactivates HDAC5 and leads to HDAC5 nuclear export (257). This removes HDAC5-mediated inhibition of MEF2, allowing MEF2 to transcriptionally activate numerous genes involved in oxidative metabolism including PGC1α and GLUT4 gene transcription (8, 209).

AMPK has also been identified as an upstream kinase of the transcription factor cyclic adenosine monophosphate (cAMP) response-element binding protein (CREB) (326). Typically phosphorylated by protein kinase A (PKA), a downstream substrate of cAMP (119), Thomson *et al* (326) demonstrated AMPK phosphorylation of CREB serine 133 in both rat liver and skeletal muscle. Additionally, AICAR induces phosphorylation of $CREB^{Ser133}$ in incubated epitrochlearis muscles (326). Furthermore, knock out (KO) of AMPKα2 results in decreased basal transcription of HKII and PGC1α, both genes containing cAMP response-element (CRE) promoters (155) (the role of CREB in mitochondrial biogenesis is discussed further in section 1.4.4.3).

AMPK activation also contributes to the activation of autophagy and mitophagy through phosphorylation of unc-51 like autophagy activating kinase 1 (ULK1) at multiple sites (serines 317, 467, 555, 637 and 777 and threonine 575) (84). Utilising electroporation of the mitophagy reporter construct MitoTimer in mouse skeletal muscle, Laker *et al* (172) have recently reported that exercise-induced ULK1 phosphorylation, lysosomal biogenesis and mitophagy are abolished in mice expressing a dominant-negative form of the AMPKα2-subunit (and thus lacking catalytic activity), while skeletal muscle ULK1 was also required for effective mitophagy (172). Together these data highlight the importance of the AMPK-ULK1 interaction for mitochondrial quality control following endurance exercise.

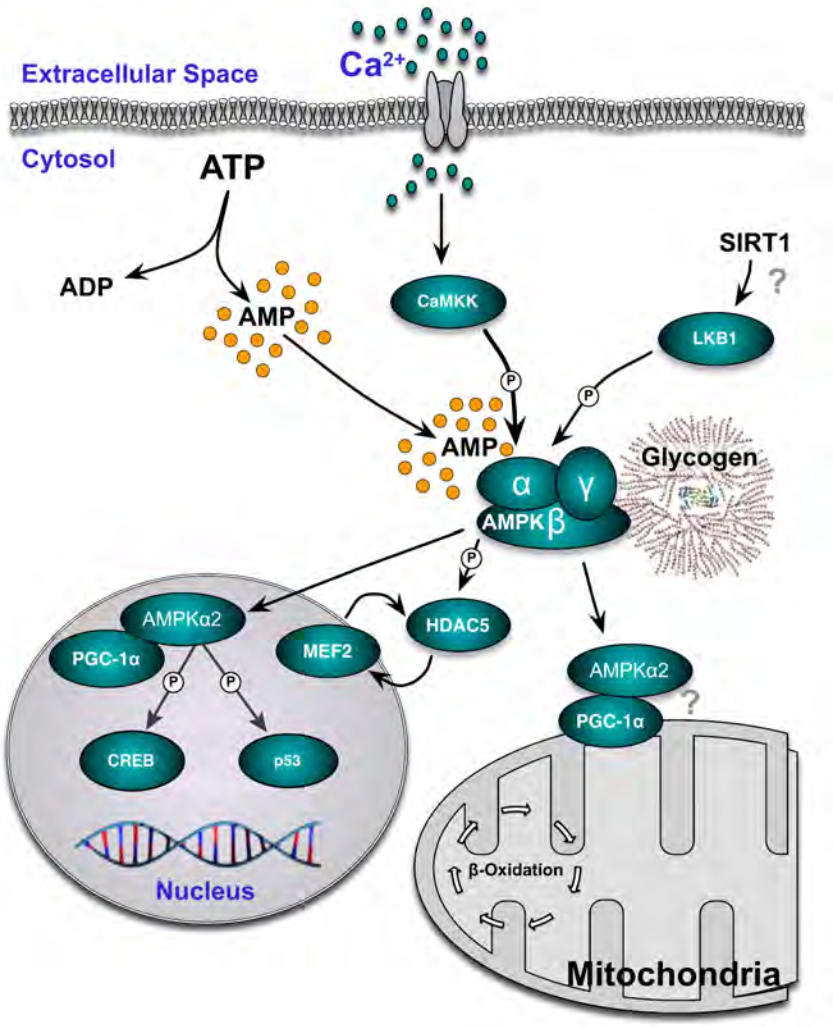

Figure 1.1 AMPK signalling pathway

AMPK senses cellular energy flux via allosteric activation by AMP and by inactivating binding to glycogen. AMPK^{Thr172} phosphorylation by upstream kinases increases AMPK catalytic activity. AMPK induces mitochondrial biogenesis principally via direct and indirect activation of PGC1α. Direct phosphorylation of PGC1α increases PGC1α transcriptional co-activation. Furthermore, phosphorylation of HDACs release repression of MEF2 and, alongside direct phosphorylation of CREB by AMPK, enhances expression of PGC1α and nuclear-encoded mitochondrial proteins.

1.4.2 Alterations in NAD+/NADH availability promote mitochondrial biogenesis through NAD+-dependent deacetylases

Nicotinamide adenine dinucleotide (NAD^+) and its reduced product NADH (NADH) are well characterised for their roles in energy metabolism. More recently, a role for NAD^+ as a signalling moiety within skeletal muscle has emerged (139). During glycolysis and oxidative phosphorylation, substantial inter-conversion of NADH and NAD^+ are required (309). These reducing equivalents participate in reduction-oxidation reactions, regulating metabolism both in the cytosol and mitochondria and are consumed as co-substrates for NAD^+-dependent reactions involving sirtuins (SIRTs), poly (ADP-ribose (ADPR)) polymerases (PARPs) and cyclic ADPR synthases, producing nicotinamide (NAM) and ADPR (23, 192, 306).

Continual synthesis or salvage of NAD^+ is required to preserve cellular NAD^+ concentrations. *De novo* synthesis occurs through a multistep process from tryptophan or via pathways from forms of vitamin B3; NAM, nicotinic acid (NA) or nicotinamide riboside (NR), collectively termed niacin (29). Each NAD^+ precursor has it's own cellular pathway for NAD^+ synthesis (Figure 1.2). NA enters the Preiss-Handler pathway, relying on nicotinic acid phosphoribosyltransferase (NAPRT)-mediated conversion to nicotinic acid mononucleotide (NAMN) and nicotinic acid adenine dinucleotide (NAAD) by nicotinamide mononucleotide adenylyltransferases (NMNATs) prior to synthesis of NAD^+ by NAD^+ synthase 1 (NADSYN1). Via the salvage pathway, NAM and

NR are converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide riboside kinases (NRKs), respectively, prior to synthesis of NAD^+ by NMNATs (29). In addition, intracellular salvage of NAD^+ can occur from NAM via the same pathway. NAM salvage can be prevented by nicotinamide N-methyltransferase (NNMT)-mediated methylation to N^1-methylnicotinamide (MeNAM) (29). Thus the efficiency of salvage of NAM versus methylation to MeNAM should regulate cellular NAD+ concentrations and, potentially, global metabolism (65, 168).

Different tissues display different expression and reliance upon NAD+ synthesis/salvage pathways (91, 222). Within skeletal muscle, metabolites of the Preiss-Handler pathway and NADSYN1 activity are mostly undetectable, with NAD+ synthesis/salvage mediated predominantly via the salvage pathway (91, 222). NAD+ precursors relying upon the Preiss-Handler pathway (e.g. nicotinic acid riboside (NAR)) or de novo synthesis pathway (e.g. tryptophan) fail to increase myocyte NAD+ (91, 192), while robust increases are apparent with precursors mediated by the salvage pathway (e.g. NR, NAM and NMN) (91, 192). NAMPT is the rate-limiting enzyme in NAD+ salvage within skeletal muscle (65, 91, 92), while NRKs are required for exogenous NR- and NMN-induced NAD+ accumulation albeit with redundancy between NRK1 and NRK2 (91). Indeed, extracellular NMN is converted to NR outside of the myocyte before re-phosphorylation back to NMN and then NAD+ intracellularly (91, 192, 265).

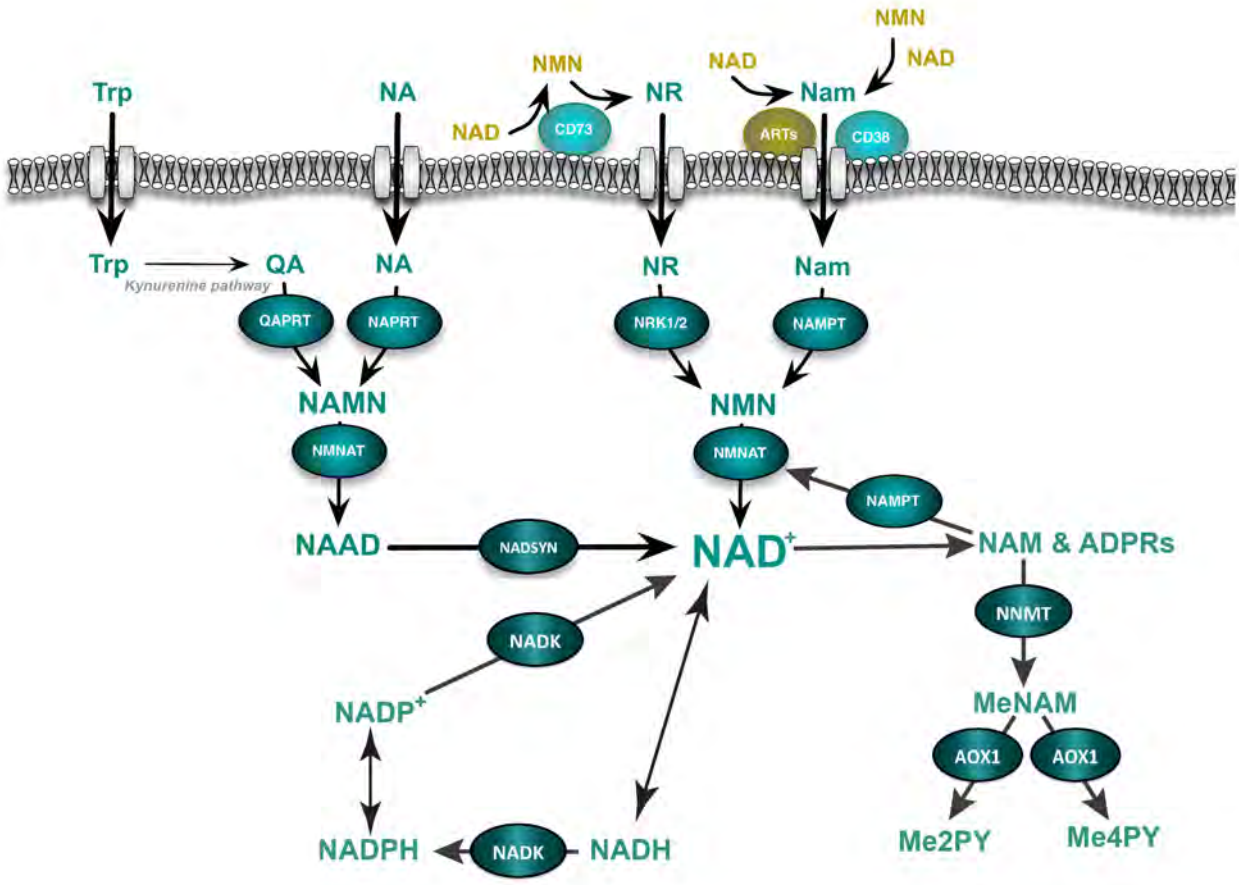

Figure 1.2 NAD$^+$ synthesis and salvage pathways

NAD$^+$ is synthesised from tryptophan via de novo synthesis, from NA via the Preiss-Handler pathway or from NAM and NR via salvage pathways. NAD$^+$ can be reduced to NADH or consumed as a substrate of SIRTs, PARPs and cyclic ADPR synthases forming NAM and ADPR. NAD$^+$ can be re-synthesised from NAM via the salvage pathway or alternatively NAM can be methylated to form MeNAM, preventing NAD$^+$ salvage.

How NAD$^+$/NADH concentrations are altered during exercise is still unclearly defined, primarily due to limitations in dynamic measurement approaches (see review (346)). In trained and untrained rats, low-intensity contractions increase

mitochondrial and cytosolic NAD^+ and the NAD^+/NADH ratio (82). Furthermore, elevations in the NAD^+/NADH ratio occur across a range of contraction intensities (10-100% $\dot{V}O_{2max}$) in canine skeletal muscle (63). Conversely, a series of studies performed by Dr Kent Sahlin and colleagues demonstrated that exercise appears to have the opposite effect on the redox state of human skeletal muscle. Maximal cycling exercise to exhaustion and submaximal isometric contractions (two-thirds maximal voluntary contraction force) alter total NADH concentration ~140% above pre-exercise levels (129, 276) without significant changes in NAD^+ concentration. In contrast, submaximal exercise at 50% $\dot{V}O_{2max}$ decreases total muscle NADH concentrations (158), while continuous exercise at 75% $\dot{V}O_{2max}$ did not alter NADH levels (277). Fluctuations in the ratio of NAD^+/NADH are also affected by exercise intensity, for example, NADH decreased while the cytosolic NAD^+/NADH ratio was unaffected during exercise at 40% $\dot{V}O_{2max}$ but at higher intensities, 75% and 100% $\dot{V}O_{2max}$, NADH increased above pre-exercise values with no changes in NAD^+ concentration (278). Thus it appears that high exercise intensities resulting in limited cellular oxygen availability are required to uncouple the stable inter-conversion between NAD^+ and NADH.

NAD^+ has been identified as an obligatory co-substrate for sirtuin activity (Figure 1.3) (306). Gerhart-Hines and colleagues first demonstrated that increases in NAD^+ increased PGC1α deacetylation in a SIRT1-dependent manner, an effect that was coincidental with increased mRNA expression of proteins involved in mitochondrial fatty acid oxidation (98). However, defining

the *in vivo* role of SIRT1 in mitochondrial biogenesis has been more problematic. For example, SIRT1 gene expression, protein content and deacetylase activity are poorly correlated with skeletal muscle oxidative capacity (48), whilst overall skeletal muscle and nuclear SIRT1 content decreases with exercise training and contraction in humans and rats (117, 118). However, nuclear SIRT1 enzymatic activity is more closely related to exercise and contraction induced mitochondrial biogenesis (117, 118). Furthermore, overexpression of SIRT1 in skeletal muscle results in mitochondrial biogenesis, in a manner that increases transcription of PGC1α and PGC1α target genes as well as PGC1α deacetylation (49). Together these data highlight the functional role of skeletal muscle SIRT1 in mitochondrial biogenesis.

However, skeletal muscle SIRT1 is not required for exercise-induced mitochondrial biogenesis. Muscle-specific loss of SIRT1 deacetylase activity (SIRT1 mKO) does not impair the mitochondrial biogenic response to voluntary wheel running (VWR) in mice (214, 245). Furthermore, post-exercise PGC1α deacetylation and increases in PGC1α gene expression and nuclear protein content were preserved in SIRT1 mKO mice (245). This paradoxical deacetylation of PGC1α was attributed to a reduction in the interaction between PGC1α and the acetyltransferase general control of amino acid synthesis 5 (GCN5) in SIRT1 mKO mice, which would lead to similar net deacetylation of PGC1α despite loss of SIRT1 function (245). It has been previously reported that GCN5 negatively regulates the PGC1α transcriptional pathway through acetylation in cultured hepatic cells (182). In a follow up to the discussed SIRT1

mKO study, Dent *et al* (75) produced a GCN5 mKO mouse and demonstrated that it does not enhance exercise-induced mitochondrial biogenesis, providing further evidence for the *in vivo* redundancy in exercise-induced PGC1α-related mitochondrial biogenesis.

SIRT1 and AMPK activity are thought to be interdependent, following the discovery that AMPK activating stimuli *in vitro* and *in vivo*, including endurance exercise, fasting and AICAR treatment, results in an AMPK-dependent deacetylation of PGC1α and forkhead box protein O1 (FOXO1) (43, 45). Furthermore, SIRT1 is required for *in vitro* effects of AICAR-induced PGC1α deacetylation, PGC1α transcriptional activity and the associated induction of mitochondrial respiration (43). AMPK appears to regulate SIRT1 activity indirectly, through an elevation of cellular NAD^+(43, 45), potentially driven by elevations in β-oxidation (43) or upregulated NAMPT expression (34, 45). Phosphorylation of PGC1α was also necessary for AICAR-induced SIRT1-mediated PGC1α deacetylation independently of alterations in SIRT1 activity or NAD^+ concentrations (43). This provides evidence that the interplay between phosphorylation and acetylation can determine substrate-specific activity.

SIRT1 has also been suggested to regulate AMPK activity through deacetylation and activation of the upstream kinase LKB1 (173). However, the physiological relevance of this to exercise could be questioned, as LKB1 does not increase its activity in response to contraction (279). Indeed, Philp *et al* (245) have demonstrated that muscle-specific loss of SIRT1 deacetylase

activity does not impair endurance exercise induced AMPK phosphorylation and activation. Thus SIRT1 is not required for exercise-induced AMPK activation.

Whilst the majority of sirtuin research in skeletal muscle has centred on SIRT1, the mitochondrially-localised SIRT3 is also of interest (294). SIRT3 is ubiquitously and differentially expressed *in vivo*, enriched in metabolically vigorous tissues such as the brain, heart, liver and skeletal muscle (195, 238). In contrast to SIRT1, SIRT3 protein levels are more abundant in slow-twitch (soleus), compared to fast-twitch (extensor digitorum longus and gastrocnemius) muscles, consistent with tissues with a higher mitochondrial content and oxidative potential (238). SIRT3 protein content increases following exercise training (33, 134, 174, 238), short-term fasting and long-term calorie restriction (238), and is down regulated in mouse models of insulin-resistance (152) and aged human muscle (174). This suggests that SIRT3 is a metabolically flexible protein that may regulate positive effects on mitochondrial oxidative capacity and whole-body metabolism.

Analysis of acetylated substrates in WT and SIRT3 KO muscle identified proteins of complex I, complex III and the ATPase subunit of complex V are SIRT3-specific targets (152). Skeletal muscle specific SIRT3 gain of function has been reported to increase basal energy expenditure and improve endurance capacity, likely due to a phenotypic shift towards oxidative fibres in fast skeletal muscle (188). Beyond activation by NAD^+, it has been suggested that AMPK may regulate SIRT3, as AICAR-mediated increases in SIRT3 and its

downstream substrate superoxide dismutase 2 (MnSOD) were lost in AMPK-α2 kinase dead mice (33). Of interest, SIRT3 can also regulate AMPK activity, as KO of SIRT3 decreases AMPK and CREB phosphorylation and PGC1α expression in response to caloric restriction (238), whilst muscle-specific SIRT3 gain of function mice display elevated AMPK phosphorylation and PPARδ expression (188). Potential mechanisms for this could be through the deacetylation and activation of LKB1 (252) or through alterations in ATP concentrations via interactions with energy modulating proteins (2, 120, 175, 238).

SIRT1 and SIRT3 also deacetylate and activate the transcription factors FOXO1 and FOXO3 in skeletal muscle (26, 43, 45). FOXO1 increases the transcription of pyruvate dehydrogenase kinase 4 (PDK4), lipoprotein lipase (LPL) and increases the membrane localisation of fatty acid translocase (CD36), resulting in a shift towards fatty acid oxidation (22, 64, 96, 156). SIRT3-mediated FOXO3 deacetylation increases FOXO3 binding to mitochondrial DNA (mtDNA) to activate mitochondrial transcription and increase mitochondrial respiration (243). Interestingly, AMPK can also positively regulate FOXO3 activity via phosphorylation (287) and may also mediate SIRT1 and SIRT3 deacetylation of FOXOs (43, 45, 243). During fasting and after exercise, FOXO1 is deacetylated in murine skeletal muscle (45). Furthermore, acute endurance exercise increases the expression of FOXO1 and its transcriptional target thrombospondin 1 (THBS1) (302).

Beyond the regulation of sirtuins, NAD^+ can also modulate cellular metabolism through the PARP enzyme family (15, 16, 39, 59, 253, 291). PARylation is a post-translational modification in which active PARPs catalyse a reaction whereby NAD^+ is cleaved to NAM and ADPR, the latter moiety covalently transferring an ADPR polymer to acceptor proteins (including PARP1 itself via auto-PARylation), building poly-ADPR (PAR) polymers (39, 291). As NAD^+ consumers, PARPs are direct competitors with SIRT1, both of which are nuclear enzymes that require NAD^+ for their catalytic activity (163, 174, 306). PARP1 induction in C2C12 cells leads to a rapid depletion in NAD^+, and subsequent hyperacetylation of PGC1α, indicative of reduced SIRT1 activity (16). Further, *in vitro* inhibition of PARP2 via miR-149, increased NAD^+ levels, enhanced SIRT1 activity and increased PGC1α transcriptional activity (219). Both PARP1 and PARP2 depletion results in higher SIRT1 activity in skeletal muscle *in vivo*, increasing mitochondrial content, improving glucose disposal, insulin sensitivity and protecting from high-fat diet induced obesity (15, 16, 253).

Skeletal muscle contraction concomitantly elevates the activities of PARP1 and SIRT1 in mice (220). Furthermore, in support of PARP1-SIRT1 competition for cellular NAD^+, PARP1 activity increased to a greater extent in aged skeletal muscle; reducing NAD^+ concentrations, impairing PGC1α deacetylation and lowering the expression of mitochondrial biogenic genes, an effect abolished with PARP1 inhibition (220). In human skeletal muscle the regulation of PARPs is an emerging field. Cobley *et al* (59) corroborated the mouse data of Mohamed *et al* (220), demonstrating elevated PARP1 protein content in elderly

skeletal muscle. Furthermore, the effect of acute high-intensity interval exercise (5 x 2 minutes at 80% W_{max}) on PARP1 content seems to differ with age and training status with PARP1 content increasing in young untrained males, whilst decreasing post-exercise in elderly participants (59). In young trained or recreationally active participants, PARP1 protein content does not change acutely following high-intensity (59) or concurrent exercise (304). However, PARP activity (i.e. PARylation) remains to be assessed in exercised human skeletal muscle.

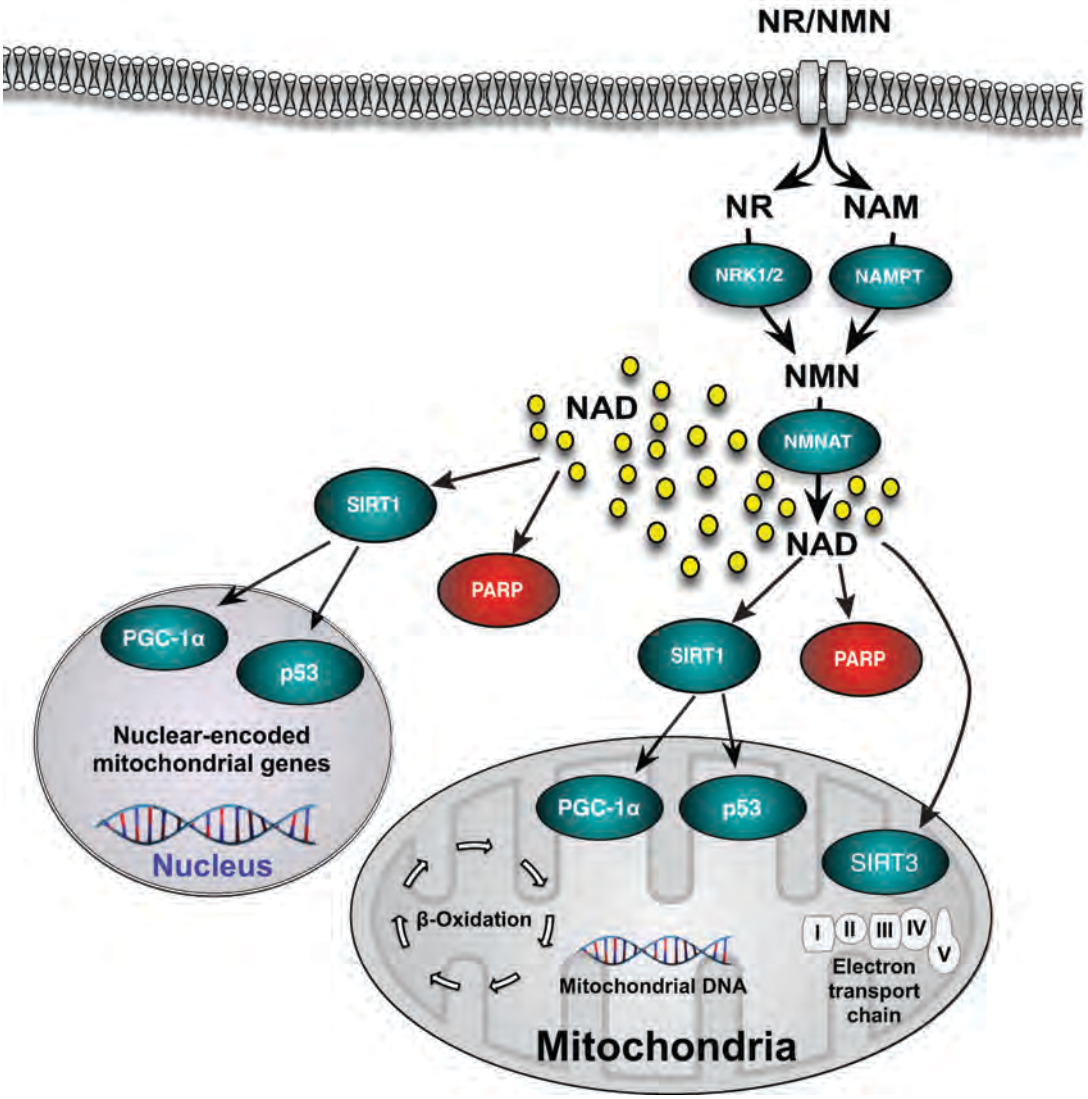

Figure 1.3 NAD^+-related signalling pathways

Increases in cellular NAD^+ concentration can activate SIRTs and PARPs. During exercise SIRT1 deacetylates and activates PGC1α and p53 to promote mitochondrial biogenesis. Activation of the mitochondrially-localised SIRT3 deacetylates and activates multiple proteins of the electron transport chain, increasing respiratory efficiency. PARPs also consume NAD^+ and as such oppose the activities of SIRTs and, thus, mitochondrial biogenesis.

1.4.3 Additional pathways

1.4.3.1 CAMKII

Recently, motor-neuron driven oscillations in cytoplasmic calcium have been identified as central molecular signals to remodel skeletal muscle during and in response to endurance exercise (53, 324, 361). Calmodulin (CaM)-dependent protein kinases (CaMKs) are sensitive to movements in cytosolic Ca^{2+} and become phosphorylated during exercise (Figure 1.4). In human skeletal muscle CaMKII is the predominant isoform and is activated in an intensity-dependent manner (270). Increased activity occurs concomitantly with increased autophosphorylation on threonine 286 (270), with autonomous CaMKII activity and threonine 286 phosphorylation highly correlated in exercised human skeletal muscle (271). CaMKII phosphorylation also appears to be sensitive to the elevated metabolic fluctuations apparent during intermittent versus continuous exercise (62). However, not all human acute endurance exercise studies detect changes in CaMKII phosphorylation (256).

Mechanistically, CaMKII may contribute to exercise-induced mitochondrial biogenesis by indirectly regulating PGC1α transcription through phosphorylation of MEF2 and activating factor 2 (ATF2) either directly or mediated via phosphorylation and activation of p38 mitogen-activated protein kinase (p38 MAPK) (206, 357, 360). These phosphorylation events promote MEF2 or ATF2 assembly on the PGC1α promoter (on MEF2 and CRE binding sites, respectively), augmenting PGC1α transcription (7). ATF2 is required for CaMKII and p38 MAPK induced expression of PGC1α in response to contraction or

caffeine treatment (6, 7, 357), while mutation of the MEF2 and CRE (ATF2 target) binding sites abolishes PGC1α promoter activity during contraction (6). Additionally, CaMKII and AMPK phosphorylation of HDACs induces nuclear export (193, 205, 206, 209, 210), indirectly increasing PGC1α transcription by reducing the ability of HDACs to repress MEF2 activity (6). In exercised human skeletal muscle, HDAC 4/5/7 and ATF2 phosphorylation display a similar time-course and exercise intensity-dependency as CaMKII and AMPK, but not p38 MAPK, phosphorylation (83). Indeed, discordance between CaMKII and p38 MAPK regulation (83, 100) suggests additional decisive stimuli for p38 MAPK regulation (see section 1.4.3.2).

It has also been proposed that CaMKII may phosphorylate and activate the transcription factor CREB, however this evidence mainly arises from studies from other tissues and cell lines (e.g. (296)). In human skeletal muscle, incompatibility between the time course of post-exercise CaMKII^{Thr286} and CREB^{Ser133} phosphorylation (83) and a lack of correlation between endurance training altered CaMKII expression and CREB phosphorylation (269) suggests alternative regulation of CREB. Indeed, in smooth vascular muscle CaMKII inhibition has no effect on CREB^{Ser133} phosphorylation, while CaMKII actually negatively regulates CREB activity through phosphorylation of the inhibitory serine 142 site (194). Nonetheless, the regulation of CREB activity by CaMKII in skeletal muscle remains debated.

1.4.3.2 p38 MAPK

The MAPKs are a ubiquitously expressed family of kinases able to respond to a myriad of cellular stressors. p38 MAPK is able to translate these cellular stresses into a wide variety of adaptive responses including cell proliferation, differentiation and mitochondrial biogenesis (Figure 1.4) (7, 254, 363, 372). In exercising human skeletal muscle, p38 MAPK is phosphorylated and activated (19, 62, 83, 132, 347), independently of exercise intensity (83), and may mediate post-exercise mitochondrial biogenesis (7, 254). However, not all acute endurance exercise studies performed in humans detect changes in p38 MAPK phosphorylation (20, 256).

The importance of p38 MAPK as a signalling moiety in skeletal muscle adaptation to endurance exercise was eloquently demonstrated *in vivo* through muscle-specific isoform deletion in mice (254). Pogozelski and colleagues demonstrated that the loss of the gamma isoform of p38 MAPK eliminates exercise-training induced increases in mitochondrial biogenesis and angiogenesis through the down-regulation of PGC1α and vascular endothelial growth factor (VEGF) (254). Furthermore, in transcripts displaying attenuated acute post-exercise induction there was an enrichment of genes containing the binding sites of NRF2, CREB and ATF2, among others, suggesting the downstream involvement of these proteins in p38 MAPKγ signalling. Conversely, p38 MAPKα and p38 MAPKβ are not required for mitochondrial adaptation to endurance exercise training (254).

As p38 MAPK responds to a myriad of stimuli, it is unsurprising that there are a number of mechanisms of activation. In skeletal muscle, p38 MAPK can be activated in response to AMPK activity (367), circulating factors (260) and mechanical stress (50), as well as elevations in intracellular calcium (357) as described in section 1.4.3.1. However, it remains inconclusive as to which signals are relevant during endurance exercise.

Activation of p38 MAPK by AMPK activators *in vitro* and *in vivo* has suggested that AMPK may be an upstream regulator of p38 MAPK (180, 367). Whilst it is clear that both AMPK and p38 MAPK are activated in a similar time course post-exercise (19, 83), disconnect between the intensity-dependent phosphorylation and activation of AMPK (52, 83) and intensity-independence of p38 MAPK (83) does suggest p38 MAPK is, at least partially, regulated independently of AMPK during exercise. Indeed, AMPK-α2 kinase-dead mice display a robust p38 MAPK phosphorylation in response to exercise (131). Thus it is clear that skeletal muscle p38 MAPK phosphorylation does not require AMPK activity in response to endurance exercise.

Of most promise are circulating factors elevated during exercise. Importantly, Widegren and colleagues have demonstrated that phosphorylation of p38 MAPK is equally elevated in the skeletal muscle of exercising and non-exercising legs in humans undertaking unilateral endurance exercise (347). These data indicate the predominance of systemic activation factors in skeletal muscle p38 MAPK activation during exercise. Cytokines, specifically tumour

necrosis factor α (TNFα), can stimulate p38 MAPK phosphorylation (185) and increase cellular respiration via a p38 MAPK-dependent mechanism (260) in C2C12 myotubes. However, during moderate endurance exercise elevations in IL-1 or TNFα are minimal (318), making it unlikely that these cytokines augment p38 MAPK phosphorylation during exercise. Conversely, circulating NEFAs can be increased during endurance exercise and may activate p38 MAPK through toll-like receptors (TLR) 2 and 4 (371). Single KO of TLR 2 and 4 ablates exercise and heparin (to mimic elevations in circulating NEFA without confounding effects of contraction) induced p38 MAPK phosphorylation (371). However, non-physiological suppression of NEFA availability during exercise does not effect post-exercise PGC1α expression in human skeletal muscle (331) and even elevates basal p38 MAPK phosphorylation, albeit alongside elevations in circulating adrenaline (344). Indeed, elevated adrenaline during exercise (343) represents a potential avenue of p38 MAPK activation, with adrenaline inducing p38 MAPK phosphorylation *in vitro* (343) and *in vivo* (93). Thus while indirect evidence would suggest that circulating factors activate p38 MAPK during exercise, and that this may be reliant on TLR 2 and 4 function, it remains unclear which factors are stimulating this response.

In conjunction with the indirect actions of p38 MAPK on PGC1α expression described in section 1.4.3.1 (via ATF2 and MEF2 phosphorylation), p38 MAPK can also acutely regulate PGC1α protein stability through direct phosphorylation at serine 265 and threonines 263 and 298, resulting in elevated co-transcriptional activation (260). Each of these phosphorylation sites reside in a

repression region of PGC1α, whereby phosphorylation by p38 MAPK releases PGC1α from the transcriptional repressor molecule p160 myb binding protein (p160MBP), providing a mechanism for p38 MAPK mediated increases in PGC1α co-transcriptional activity (85, 166). Co-expression of p160MBP ablates the PGC1α induced increase in mitochondrial respiration and protein expression in C2C12 cells, an effect abolished by the addition of the cytokines TNFα, IL1α and IL1β (85). *In vivo*, p38 MAPK activation in swimming mice is associated with translocation of PGC1α to the nucleus and increased binding of NRF1 and 2 to Cyt-c and cytochrome-c oxidase (COX) IV promoters (358). However, similarly to AMPK, phosphorylation of PGC1α by p38 MAPK has not been substantiated in exercised skeletal muscle, thus the relevance of this potential avenue of regulation remains unclear.

1.4.4 Transcriptional targets

1.4.4.1 PGC1α

PGC1α is a transcriptional co-activator with an important role in maintaining cellular metabolism. Skeletal muscle PGC1α gain-of-function promotes a shift in fibre-type expression towards slow-twitch fibres (187) and increases mitochondrial biogenesis (187, 217, 241), $\dot{V}O_{2max}$ (42, 241) and endurance capacity (42, 241). Conversely, loss-of-function models show the opposite, adverse, phenotype (18, 179, 181). Thus, it is without question that PGC1α is an important metabolic regulator within skeletal muscle.

As has been discussed throughout this review, PGC1α is capable of integrating many cellular signals and co-ordinating transcription of the nuclear and mitochondrial genomes (Figure 1.4). PGC1α can co-ordinate mitochondrial biogenesis through the co-activation of thyroid hormone receptors (TRs) (261), oestrogen-related receptors (ERRs) (290), PPARs (342), NRFs (362), MEF2 (187, 215) and FOXO1 (259), amongst others (90, 240). Co-activation of NRFs increases the transcription of oxidative genes in the nucleus (338, 358, 362) as well as, crucially, the mitochondrial transcription factors TFAM (54, 145, 337, 360) and mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M) (103). Indeed, exercise-induced upregulation of nuclear PGC1α is accompanied by NRF1/2 binding to target gene promoters (358). PGC1α also resides in the mitochondria where it interacts with TFAM and mtDNA (275, 305). It is tempting to speculate that PGC1α may directly activate transcription of the mitochondrial genome through co-activation of TFAM (275). Furthermore, PGC1α is its own transcriptional activator, through MEF2 co-activation, amplifying its own expression and activity (122).

PGC1α regulation via phosphorylation, acetylation and transcription following endurance exercise has been discussed frequently throughout this chapter. Alongside deacetylation by SIRT1 (section 1.4.2), phosphorylation by AMPK (section 1.4.1) and p38 MAPK (section 1.4.3.2), and elevated transcription by ATF2, MEF2 (sections 1.4.1 & 1.4.3.1) and CREB (see section 1.4.4.3), PGC1α activity is substantially governed by its subcellular location. Endurance exercise increases the nuclear (112, 189, 190, 245, 283, 358) and mitochondrial

abundance of PGC1α (275, 305). Post-exercise nuclear PGC1α content is coincidental with upregulation of PGC1α target genes (189, 245, 283, 358), while PGC1α translocation to the mitochondria is concurrent with expression of mitochondrially-encoded genes (275). In line with this, mRNA expression of PGC1α consistently increases following endurance exercise (19, 20, 83, 112, 189, 245, 256) and is intensity-dependent (83, 256). However, PGC1α mRNA induction is similar between sprint-interval or high-intensity interval and continuous exercise (19, 112). PGC1α mRNA expression is also influenced by skeletal muscle glycogen content (20), an unsurprising finding given the influence of AMPK activity on PGC1α regulation.

Mouse and human skeletal muscle express several PGC1α isoforms. Transcription can originate from the canonical promoter to produce PGC1α-a and from alternative promoters to produce PGC1α-b and PGC1α-c (216, 369), although PGC1α-c is lowly expressed in human skeletal muscle (255, 256). Alternative splicing can also generate N-truncated (NT) isoforms of PGC1α (13, 373). At rest, PGC1α expression in human skeletal muscle is regulated only via the canonical promoter (228, 255, 256). However, endurance exercise in humans does not substantially increase PGC1α expression from the canonical promoter (producing PGC1α-a) (255, 256, 299). Instead expression from the alternative promoter (producing PGC1α-b) predominates (255, 256, 364) and confers the intensity-dependence of PGC1α mRNA expression (256).

Despite all the evidence for a central role in mitochondrial biogenesis, PGC1α is not required for exercise-induced mitochondrial biogenesis within skeletal muscle (18, 179, 272). Acute endurance exercise induces comparable increases the mRNA expression of COX5b, a known PGC1α target gene, in PGC1α and WT mice (272). 12 days of VWR in WT and PGC1α mKO mice equally upregulated NRF1, TFAM, COX5a and ATP synthase subunit 5o (ATP5o) (272). Furthermore, no deficits in exercise training induced mitochondrial density or electron transport complex activity were apparent in PGC1α mKO mice (272). The same group have subsequently shown that inducible PGC1α and PGC1β double mKO, thereby reducing the possibility of developmental or PGC1β driven compensation, are also responsive to endurance exercise training (18). In human skeletal muscle, acute PGC1α mRNA induction does not correlate with training-induced mitochondrial biogenesis (109). Thus, although PGC1α is certainly central to many exercise-induced adaptations, other transcriptional processes certainly occur concurrently, independently and/or in concert with PGC1α, and can compensate for a loss of PGC1α.

1.4.4.2 p53

The tumour-suppressor protein and transcription factor p53 has recently been purported to play a role in regulating mitochondrial biogenesis in skeletal muscle. This additional role of p53 is hardly surprising given its ubiquitous regulatory activity in cell-cycle arrest, apoptosis, angiogenesis, DNA repair,

metabolism, signal transduction, translation and transcription (183, 227). In cell culture models of various tissues, p53 is implicated in the transcriptional control of the mitochondrial biogenic proteins PGC1α, TFAM, cytochrome-c assembly protein (SCO2), apoptosis-inducing factor (AIF), mitofusin 2 (MFN2) and DRP1 (11, 184, 202, 239, 312, 341) and is also required for the maintenance of mtDNA copy number (169, 177).

Whole-body KO of p53 reduces endurance capacity and increases lactate production during submaximal exercise, while also impairing the increase in work capacity and $\dot{V}O_{2peak}$ in response to endurance training (239, 281). p53 KO also impairs basal skeletal muscle mitochondrial content, mitochondrial morphology, mtDNA copy number, COX activity, mitochondrial respiratory capacity and apoptosis (239, 281, 284). Thus it is unequivocal that whole-body ablation of p53 impairs skeletal muscle mitochondrial function and endurance capacity. However, contradictory evidence arises when studying the molecular basis of this within skeletal muscle. Basal protein expression of PGC1α in p53 KO mice has been reported to either decrease (281) or remain unchanged (239) compared to WT littermates, although both groups do report a decrease in TFAM and NRF1 mRNA expression (239, 282). Additionally, Saleem and colleagues report p53 KO ablates or impairs exercise-induced upregulation of PGC1α, TFAM, NRF1, complex IV and complex I (282, 283) while, conversely, Park *et al* (239) found no impairment of exercise-induced gene expression on transcriptional regulators (PGC1α, PGC1β, NRF1, NRF2, TFAM), nuclear-encoded genes (Cyt-c, complex IV) or mitochondrially-encoded genes (complex

II, mitochondrially encoded NADH reductase 1 (mtND1)). Ultimately, p53 KO mice display a robust mitochondrial biogenic response to eight weeks of VWR, despite reduced running distance (281). Thus, while the role of p53 in the regulation of acute-exercise induced pro-mitochondrial gene expression remains contradictory and unclear, p53 is unnecessary for chronic mitochondrial adaptations to endurance training.

If p53 is contributing to the regulation of post-exercise mitochondrial biogenesis then alterations in post-translational modifications and/or subcellular location of p53 should occur with exercise. AMPK and p38 MAPK mediated phosphorylation of $p53^{Ser15}$ leads to increased p53 stability, nuclear accumulation and transcriptional activity (10, 37, 154). Skeletal muscle $p53^{Ser15}$ phosphorylation occurs following electrical stimulation in mice (281), continuous and intermittent exercise in humans (19, 20, 112) and can be accentuated following exercise in a glycogen-depleted state (20). This post-exercise phosphorylation occurs in a time course concurrent with alterations in AMPK and p38 MAPK signalling (19, 112, 281). However, p53 phosphorylation following exercise in a glycogen-depleted state occurred in the absence of altered p38 MAPK phosphorylation, but in parallel to an increase in AMPK signalling, suggesting AMPK may be the predominant upstream kinase regulating post-exercise p53 phosphorylation (20).

To date, the effect of acetylation status on p53 activity in skeletal muscle or in relation to mitochondrial biogenesis remains largely unknown. Following

exercise, pan-acetylation of p53 decreases in nuclear fractions of skeletal muscle, indicative of elevated SIRT1 activity (245). In mouse embryonic stem cells, reactive oxygen species (as produced during exercise) trigger p53 deacetylation, translocation of p53 to the mitochondria and subsequent apoptosis in a SIRT1-dependent manner (121). Indeed, p53 has been reported to translocate to the mitochondria following endurance exercise in rodents (274, 283), where it plays a role in mtDNA-stability and apoptosis (274, 281). Within the mitochondria of exercised mice, p53 complexes with polymerase γ 1 (POLG1), TFAM and mtDNA (274, 283) and mediates maintenance of mtDNA stability *in vitro* (274). Furthermore, the attenuation of age-related mtDNA damage and mitochondrial dysfunction by endurance exercise in mtDNA mutator mice is ablated when mtDNA mutator mice have additional p53 mKO (274). This suggests a substantial role of exercise-induced p53 mitochondrial translocation in maintaining mitochondrial integrity in these animals and it would be interesting to know whether this also occurs in healthy models of ageing.

Nuclear content of p53 has also been reported to increase post-exercise (112, 248, 320), accompanied by an increase in PHD finger protein 20 (PHF20), a regulator of p53 stability and transcriptional activity (112). It is attractive to speculate that p53 could then act as a transcription factor. Following fasting in mouse skeletal muscle, p53 releases from inhibitory response elements on the PGC1α promoter and binds instead to an activating region, increasing the expression of PGC1α and antioxidant genes (11). Conversely, it has also been reported that following acute endurance exercise p53 translocates away from

the nucleus (274, 283). The divergent responses measured in the above studies could be explained by the severity of the exercise, as ROS production induces p53 accumulation in mitochondria (121, 274). In the study by Saleem and Hood (283) mice were exercised to exhaustion, a situation that likely involves a large accumulation of oxidative stress (258) and could explain nuclear export and mitochondrial localisation of p53 for mtDNA stability (274) and/or apoptosis (121). Conversely, in less severe models of endurance exercise (112, 248, 320) p53 may translocate to the nucleus, possibly to promote PGC1α expression (11).

p53 also impacts on kinase activity, acting upstream of AMPK, p38 MAPK and CAMKII, therefore influencing nutrient sensing and signal transduction. Saleem *et al* (282) have demonstrated that whole-body knockout of p53 delays post-exercise AMPK signalling; such that no increase in AMPK phosphorylation was apparent immediately post-exercise. Furthermore, CAMKII phosphorylation was delayed and attenuated while p38 MAPK phosphorylation is completely ablated post-exercise (282). Interestingly, PGC1α nuclear translocation was also delayed, possibly consequently to impaired AMPK, p38 MAPK and CAMKII signalling, and may contribute to the apparent attenuation or delay in upregulation of genes involved in mitochondrial biogenesis (282).

Exercise training can upregulate skeletal muscle p53 protein content, in an exercise intensity-dependent manner (111). Following four weeks of sprint interval training (4-10 x 30-s all-out sprints, ~200% W_{max}, 3 times per week), but

not high-intensity interval training (4-7 x 4-min intervals at ~90% W_{max}) or continuous exercise (20-36 minutes at ~65% W_{max}), basal p53 protein increases approximately two-fold. Furthermore, PHF20 also increased following sprint interval training providing a novel potential mechanism of p53 upregulation by exercise training. However, protein content of TFAM, MFN2, DRP1 and AIF, known downstream transcriptional targets of p53, either remained unchanged with training or showed no dependence on exercise intensity and therefore p53 protein content (111). Thus while this study provides interesting evidence of a regulation of skeletal muscle p53 protein content by sprint exercise, the implications of this remain unclear.

1.4.4.3 CREB

It has previously been documented during exercise with normal and low pre-exercise glycogen that the catecholamine adrenaline is increased within the circulation (70, 319, 343). One action of the increased circulating adrenaline is the activation of β-adrenergic receptors and subsequent cAMP accumulation that activates the protein kinase A (PKA) pathway. Endurance exercise has long been known to increase cAMP concentrations in murine skeletal muscle (36, 105, 266). Furthermore, this effect appears intensity-dependent (105).

A number of observations associate β-adrenergic/cAMP/PKA signalling with acute and chronic metabolic regulation of skeletal muscle. Firstly, β-adrenergic receptors are enriched in oxidative type I fibres compared with type II fibres in both rodents and humans (86, 201, 351). Furthermore, induction of cAMP in

muscle cells induces the expression of mitochondrial enzymes (176). Conversely, impairment of β-adrenergic signalling in skeletal muscle impairs PGC1α expression and mitochondrial adaptations to endurance exercise (216, 218, 319, 321). Of particular note, treatment with the β-adrenergic receptor blocker propranolol impairs the improvement in the enzyme activities of cytochrome-c oxidase and 3-hydroxyacyl-CoA dehydrogenase (β-HAD), but not citrate synthase (CS) or succinate dehydrogenase, induced by eight weeks of endurance training in humans (319).

Elevated cellular cAMP concentrations activate PKA (340). Skeletal muscle PKA activity is increased by endurance exercise in mice and rainbow trout (211, 352). In murine skeletal muscle exercise-induced phosphorylation of CREB is ablated by PKA inhibition, supporting a role of cAMP-induced PKA activation in mammalian skeletal muscle (266). Furthermore, in an unbiased phosho-screen using tandem mass spectrometry and annotated kinase-substrate relationships, phosphorylation of seventeen known PKA substrates was enhanced in human skeletal muscle following high-intensity exercise (85-92% W_{max} for ~10 minutes), strongly suggesting elevated PKA activity (132).

The effect of endurance exercise on CREB phosphorylation varies substantially with some studies reporting immediate post-exercise serine 133 phosphorylation (36, 245, 256, 266), dephosphorylation (83), delayed (~3-19h post-exercise) phosphorylation (83) or no change (347, 348). Interestingly in a human unilateral exercise model immediate post-exercise $CREB^{Ser133}$

phosphorylation was elevated in the non-exercising contralateral leg but not in the exercising leg (347). However, this is not a universal finding, which may be related to exercise intensity and/or duration (348). If CREB can be activated in non-exercising muscle and simultaneously remain unchanged in exercising muscle, this would suggest that while humeral factors such as adrenaline stimulate CREB phosphorylation, local (as yet undetermined) contraction or metabolic-mediated factors may suppress $CREB^{Ser133}$ phosphorylation during exercise. Indeed, the trade-off between systemic activators and local suppressors may explain the variation in CREB phosphorylation apparent immediately post-exercise. Sustained elevation of CREB phosphorylation during the post-exercise recovery period (83) may be permissive for continued elevation of PGC1α expression up to eight hours after exercise (339), whilst basal $CREB^{Ser133}$ phosphorylation has been reported to increase with exercise training in mice (238).

In endurance-trained humans, RNA-seq analysis of two participants' response to 65 minutes of endurance exercise at 70% $\dot{V}O_{2max}$ identified the upregulation of CREB-dependent genes, in contrast to a lack of regulation of MEF2-dependent genes (256). qPCR analysis in ten participants confirmed an intensity-dependent effect of exercise on a subset of the CREB-dependent genes, mirroring the intensity-dependence of $CREB^{Ser133}$ phosphorylation apparent in this study. Furthermore, this was concomitant with intensity-dependent expression of exon b1-derived (alternative promoter) PGC1α mRNA four hours post-exercise (256), a promoter region containing a CRE binding site

(369). Thus it could be inferred that exercise-induced activation of CREB may be, at least partially, responsible for the post-exercise expression of PGC1α from the alternative promoter, supporting stimulated muscle cell and exercising rodent data of β-adrenergic/cAMP/PKA/CREB regulation of this splice variant during exercise (216, 321, 345, 369). Nonetheless this evidence is far from conclusive, given the extremely low sample size for the RNA-seq analysis and the possibility that additional CREB-like transcription factors, such as ATF2, may regulate the same transcripts and binding sites.

Phosphorylation of $CREB^{Ser133}$ increases CREB transcriptional activity through association with the histone acetyltransferases CBP and p300 (55, 108, 198), allowing for histone acetylation and polymerase II complex recruitment (159, 232). CREB stimulates gene transcription at promoters containing the CRE binding motif, including PGC1α (122). However, while $CREB^{Ser133}$ phosphorylation appears to be required for CREB activation it may not be sufficient, with phosphorylation at serine 142 by CAMKII, opposing the expression of CREB sensitive genes (194). $CREB^{Ser142}$ phosphorylation is yet to be investigated in skeletal muscle and thus represents an interesting additional level of regulation requiring further study.

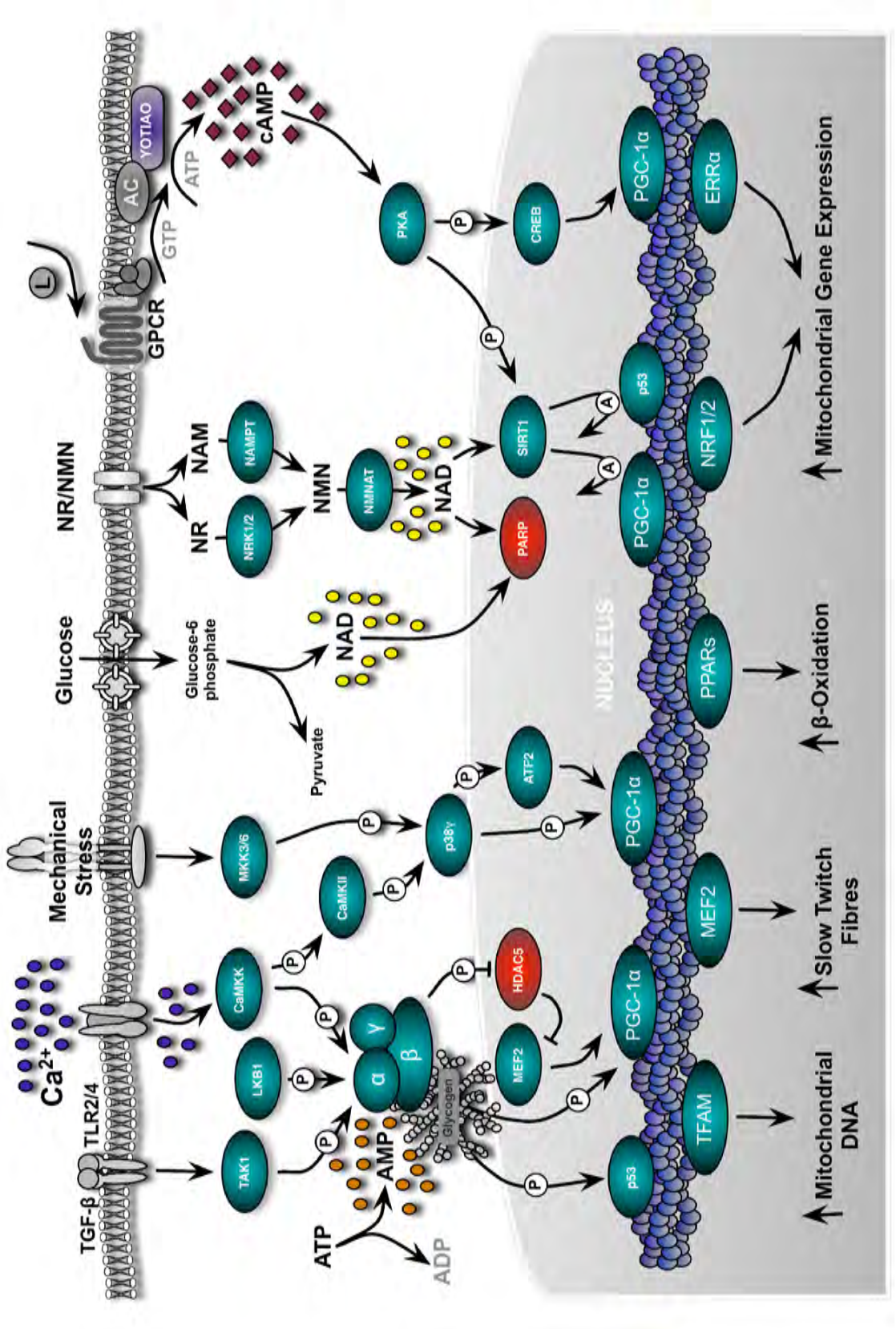

Figure 1.4 Energy- and contractile-sensing pathways orchestrate mitochondrial biogenesis within skeletal muscle

Exercise alters the cellular milieu of skeletal muscle. Alterations in AMP, glycogen, NAD^+, Ca^{2+}, cAMP and activation of membrane receptors activate AMPK, SIRTs, CAMKII, PKA and p38 MAPK during exercise. These interconnected signalling pathways converge on transcription factors and co-activators such as MEF2, ATF2, CREB, p53 and PGC1α to induce mitochondrial biogenesis. PGC1α particularly can co-ordinate the expression of both nuclear and mitochondrial genomes via co-activation and induction of PPARs, ERRs, NRFs, MEF2, CREB and TFAM. Chronic activation of these signalling pathways results in mitochondrial biogenesis and a greater capacity for aerobic ATP production.

1.5 Nutritional strategies to augment skeletal muscle signalling and mitochondrial biogenesis

1.5.1 Energy restriction *per se* increases activity of energy-sensing proteins and mitochondrial biogenesis within skeletal muscle

Skeletal muscle has the flexibility to switch between fuel sources dependent upon energy requirement and availability. In this respect fasting induces elevations in fat oxidation to spare carbohydrate and preserve blood glucose levels for glucose-dependent tissues such as the brain (41, 349, 350).

Like exercise, fasting alters the metabolic environment of skeletal muscle cells. In fasted murine skeletal muscle, elevations in cellular AMP and depletion of glycogen activates AMPK (45, 71, 115, 314) and increased NAD^+ concentrations promote SIRT1-deacetylase activity (45, 98). Together, these induce deacetylation, activation and expression of PGC1α and induction of metabolic and mitochondrial genes (45, 98, 115). However, these effects do not consistently occur in human skeletal muscle, likely due to the higher glycolytic reliance in murine skeletal muscle and therefore greater energetic stress during fasting. In human skeletal muscle, 10-48 hours of fasting does not result in glycogen depletion (80, 203) or increase the phosphorylation of $AMPK^{Thr172}$, ACC^{Ser79} or p38 $MAPK^{Thr180/Tyr182}$ (80, 349, 350), while PGC1α mRNA expression actually decreases 24-48 hours into fasting (80, 349, 350). However, from the time points analysed it cannot be excluded that these markers may show transient activation in the immediate hours following the onset of fasting. Phosphorylation of $CREB^{Ser133}$ does, however, increase with fasting (349). SIRT1 can also be moderately activated by fasting in human skeletal muscle, with Edgett *et al* (80) finding small elevations in SIRT1 mRNA and deacetylation of p53 after 48 hours. Fasting does consistently amplify the expression of PDK4 mRNA in human skeletal muscle (80, 251, 310, 349, 350), suggesting the fasting induced activation of PPARα may be conserved from rodents to humans (161). Furthermore, the mRNA of processes involved in fatty acid metabolism are overexpressed in skeletal muscle following 10 hours of fasting (349).

1.5.2 Exercising in the fasted state alters metabolism and may increase post-exercise skeletal muscle signalling and mitochondrial biogenesis

As has been previously highlighted, performing exercise in a glycogen-depleted state augments $AMPK^{Thr172}$ phosphorylation, AMPK nuclear abundance, AMPKα2 activity, PGC1α nuclear abundance and PGC1α expression (20, 171, 247, 316, 356, 365). Furthermore, performing exercise training in a glycogen-depleted state, via twice-daily training, augments training-induced mitochondrial biogenesis (123, 366) and skeletal muscle lipid metabolism (142). However, the practicality of depleting glycogen and then subsequently exercising could be questioned for implementation in the general population. A more achievable method of increasing energetic stress during exercise may be to undertake endurance exercise following an overnight fast.

Moderate-intensity (50-75% $\dot{V}O_{2peak}$) exercise performed in the fasted state increases systemic fatty acid availability, intramyocellular lipid utilisation and whole-body fat oxidation compared to exercise combined with carbohydrate ingestion (3, 5, 51, 57, 58, 70, 81, 336). Despite this, glycogen utilisation is similar in fed or fasted exercise (5, 70) suggesting that the metabolic effects observed following fasting are not mediated by glycogen availability. Systemic insulin concentrations are lower during fasted versus fed exercise (3, 51, 58, 70), while circulating adrenaline increases during fasted but not fed exercise (3, 70).

As might be expected from a less severe model of energetic stress, the effects of exercising in the fasted state on post-exercise signalling and mitochondrial biogenesis are more modest and less consistent than glycogen-depleted exercise training. Fasted exercise has been demonstrated to augment post-exercise AMPKα2 activity (5), although no differences in AMPK^{Thr172} or ACC^{Ser79} phosphorylation have been reported (5, 70, 293), whilst elevated AMPK^{Thr172} phosphorylation following fed-state exercise may also be apparent (81). Downstream, post-exercise mRNA expression of AMPKα2 and the metabolic genes PDK4, GLUT4 and UCP3, but not PGC1α, are inhibited by glucose ingestion during exercise (57, 58).

Moderate-intensity (65-70% $\dot{V}O_{2peak}$) endurance training performed in the fasted state upregulates activity of the mitochondrial enzymes CS and β-HAD compared to carbohydrate-fed exercise in recreationally active males (313, 334). Conversely, improvements in CS and β-HAD activity are similar when overweight/obese females perform high-intensity interval training in the fed and fasted states (101). However, sex-based differences may occur as fasting has been shown to augment the increase in CS activity following endurance exercise training in males but not females (313). Furthermore, when comparing positive benefits of combining fasting with continuous but not high-intensity interval training (101, 334), it is possible that the duration of exercise and the degree and manner of fuel utilisation is an important consideration in the efficacy of fasted endurance exercise.

To date research investigating the effect of fed versus fasted exercise on post-exercise skeletal muscle signalling responses have, understandably, focussed primarily upon AMPK. However, as has been outlined throughout this review, a multitude of other signalling pathways are potentially sensitive to the elevated energetic stress during fasted exercise and remain to be assessed. Hypothetically, fasted exercise could alter skeletal muscle NAD^+ concentrations and SIRT/PARP signalling, while p38 MAPK and CREB signalling cascades could be modulated by altered circulating FFAs, adrenaline and differential AMPK activation. Thus the potential additive or synergistic effect of combining fasting with exercise on signalling pathways within human skeletal muscle remains incompletely understood.

1.5.3 Niacin elevates skeletal muscle NAD^+ concentrations; promoting sirtuin signalling and mitochondrial biogenesis

The mitochondrial biogenic effects of a myriad of chemical compounds and naturally occurring nutraceuticals, many of which 'mimic' the effects of energy restriction, have been examined in isolation and in combination with endurance exercise. A thorough review of all these compounds is outside the scope of this review (see (67, 73) for comprehensive reviews), instead the effects and efficacy of several forms of niacin (vitamin B3) will be discussed given their ability to modify systemic and skeletal muscle NAD^+ bioavailability.

Niacin is present in many foods and relatively small doses of niacin (~15 mg per day) or tryptophan prevent pellagra. Much higher doses of NA (~2-4 g per day)

are used to treat dyslipidaemia (104). NAM, NA and NR can each increase cellular and mitochondrial NAD^+ concentrations *in vivo*, including within skeletal muscle (44, 328). Increasing cellular NAD^+ via niacin supplementation *in vivo* leads to positive outcomes in models of diabetes (264, 368), ageing (72, 213, 264), obesity (44), vascular dysfunction (72), muscular dystrophy (273) and Alzheimer's disease (196).

Acipimox, a derivative of NA, enhances skeletal muscle mitochondrial function in type II diabetics (333). However, high-dose supplementation of NA can result in painful flushing, while NAM supplementation may cause liver damage (29). NA and NAM supplementation also blunt adipose tissue lipolysis and whole-body fat oxidation through NA binding to niacin receptor 1 (GPR109A) in adipocytes (94, 322, 330, 344). Furthermore, high-doses of NAM actually inhibit SIRTs (23). Conversely, NR treatment does not activate GPR109A, while supplementation increases fat oxidation and metabolic flexibility in mice (44, 297). Interestingly, this is in spite of the main circulatory product of oral NR supplementation in mice being NAM (192). Oral NR supplementation appears to undergo almost complete first-pass metabolism in the murine liver, with exogenous NR-derived myocellular NAD^+ likely formed via extracellular NAM (192). Despite this, elevated systemic NR (4), as well as NAM (328), concentrations have been noted following NR supplementation in humans. Thus NR is emerging as a promising way to boost cellular NAD^+ via high-dose niacin supplementation.

NR treatment *in vitro* and *in vivo* activates skeletal muscle SIRT1 and SIRT3, but not PARP1, resulting in deacetylation of PGC1α and FOXO1 and subsequent mitochondrial biogenesis (44, 47, 162, 273). Accordingly, NR supplementation in mice increases endurance performance, insulin sensitivity and protects against the metabolic consequences of a high-fat diet (44, 273). NR treatment can promote mitochondrial biogenesis and attenuate disease progression in murine models of mitochondrial disease (47, 162), with the effects much more pronounced in models with a metabolic challenge (44, 47, 162). In such conditions, it may be that pervasion of dysfunctional mitochondria simply provides a greater opportunity for improvement. Additionally, NR is likely to be more effective in situations where NAD^+ content is dysregulated such as during high-fat feeding (92) or with mitochondrial myopathy (162). Nonetheless, this highlights the promising role of NR supplementation in the treatment of metabolic and mitochondrial diseases.

To our knowledge no study to date has combined NR supplementation with exercise training. However, elevating skeletal muscle NAD^+ concentration via of overexpression of NAMPT, the rate-limiting enzyme in NAD salvage, augments VWR-induced improvements in endurance capacity in mice (65). This effect is not seen in sedentary mice (65, 92). Thus, elevating NAD^+ concentrations appears more effective in combination with exercise. This lends further support to the hypothesis that elevating NAD^+ concentrations, either genetically or nutritionally, is likely to be more effective during conditions of cellular stress, as substantial NAD^+ turnover occurs during exercise (346).

NR supplementation studies in humans are in their infancy (4, 74, 78, 200, 328). Importantly, NR supplementation in humans has been shown to be safe and does not result in flushing (4, 74, 78, 200, 328). Charles Brenner first demonstrated the bioavailability of 1000 $mg \cdot d^{-1}$ of NR on himself and then on a cohort of participants (328). A single dose of NR can enhance NAD^+ in peripheral blood mononuclear cells (PBMCs) and plasma within five hours and repeated daily dosing stabilises the elevated NAD^+ concentrations (328). Furthermore, in the n=1 experiment the rise in NAD^+ was concomitant with an increase in ADPR, suggestive of increased NAD^+-consuming processes (328). Furthemore, NAD^+ concentration in red blood cells is dose-dependently increased by co-supplementation of NR and pterostilbene, a polyphenol, (500 > 250 > 0 $mg \cdot d^{-1}$ of NR) in healthy elderly participants (74).

Data regarding the effect of NR supplementation on health parameters in humans are beginning to emerge (74, 78, 200). Dellinger and colleagues report that 60-days of 500 $mg \cdot d^{-1}$ NR and 100 $mg \cdot d^{-1}$ of pterostilbene ingestion improved physical function (30-s chair test and 6-min walk test), as well as indications that co-ingestion of NR and pterostilbene could improve liver health (74). Conversely, Martens *et al* (200) report no effect of 6-weeks of 1000 $mg \cdot d^{-1}$ NR supplementation on physical function, VO_{2max} or substrate utilisation during exercise in middle aged and elderly participants, although reductions in blood pressure were apparent. Finally, both Martens *et al* (200) and Dollerup *et al* (78) have demonstrated that NR supplementation for 6 and 12 weeks, respectively,

does not alter body composition, resting substrate utilisation, lipolysis or insulin sensitivity.

1.6 Conclusions

Endurance exercise activates a myriad of interconnecting cellular energy and contractile sensors that converge on downstream transcriptional regulators. The repeated activation of these pathways controls the induction of mitochondrial biogenesis, muscle function and, ultimately, whole-body health. Almost all of these signalling cascades can be targeted by altering the exercise environment, be that via intensity, duration or intermittency of exercise or by altering the nutritional environment (i.e. fasting or nutraceuticals) that exercise is performed in. Particularly, exercising in an energy (or carbohydrate)-restricted state represents an achievable and affordable strategy to maximise post-exercise signalling and, potentially, skeletal muscle adaptations to endurance exercise.

Nonetheless, like any scientific field, the picture is not yet complete. Particularly, this thesis will attempt to further understand the importance and role of p53 in skeletal muscle mitochondrial biogenesis and to elucidate effective nutritional strategies to augment post-exercise skeletal muscle signalling. As discussed, the current role of p53 in skeletal muscle is unclear and confounded by the reliance upon evidence from other tissues and from whole-body p53 KO mice. Thus, we will employ an mKO model of p53 deletion to ascertain the muscle-specific role of p53 in mitochondrial biogenesis. Furthermore, we will investigate how fasting and the fasting mimetic NR influences post-exercise skeletal

muscle signalling. Currently, the mechanisms linking fasted exercise to elevated training-induced mitochondrial biogenesis are unclear. While AMPK appears to be activated to a greater degree in the fasted state, other pathways remain to be examined. Particularly the effect of fasted exercise on sirtuin signalling has yet to be investigated in human skeletal muscle. Additionally, promising evidence for a positive effect of NR supplementation on mitochondrial biogenesis is apparent in cell and rodent models. However, research in human populations remains in the early stages and the potential additive or synergistic effect of NR supplementation and endurance exercise has not been investigated.

1.7 Research Aims

1. Investigate the effect of muscle-specific deletion of p53 on skeletal muscle mitochondrial and metabolic enzyme content and function

2. Investigate how energy restriction in combination with moderate- to high-intensity steady-state exercise can influence post-exercise energy-sensing pathways in humans

3. Investigate how NR supplementation influences metabolism and post-exercise energy-sensing pathways within human skeletal muscle

1.8 References

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1.9 Additional Information

1.9.1 Conflict of Interests

The authors report no conflicts of interest.

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2 SKELETAL MUSCLE FIBRE-SPECIFIC KNOCKOUT OF P53 DOES NOT REDUCE MITOCHONDRIAL CONTENT OR ENZYME ACTIVITY IN MICE

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2.1 Abstract

Tumour protein 53 (p53) has been implicated in the regulation of mitochondrial biogenesis in skeletal muscle, with whole-body p53 knockout mice displaying impairments in basal mitochondrial content, respiratory capacity and enzyme activity. This study aimed to determine the effect of skeletal muscle-specific loss of p53 on mitochondrial content and enzyme activity. Mitochondrial protein content, enzyme activity and mRNA profiles were assessed in skeletal muscle of eight-week-old male muscle fibre-specific p53 knockout mice (p53 mKO) and floxed littermate controls (WT) under basal conditions. p53 mKO and WT mice displayed similar content of electron transport chain proteins I-V and maximal citrate synthase enzyme activity in skeletal muscle. In addition, the content of proteins regulating mitochondrial morphology (MFN2, mitofillin, OPA1, DRP1, FIS1), fatty acid metabolism (β-HAD, ACADM, ACADL, ACADVL), carbohydrate metabolism (HKII, PDH), energy sensing (AMPKα2, AMPKβ2) and gene transcription (NRF1, PGC1α and TFAM) were comparable in p53 mKO and WT mice ($p > 0.05$). Furthermore, p53 mKO mice exhibited normal mRNA profiles of targeted mitochondrial, metabolic and transcriptional proteins ($p > 0.05$). Thus it appears that p53 expression in skeletal muscle fibres is not required to develop or maintain mitochondrial protein content or enzyme function in skeletal muscle under basal conditions.

2.2 Introduction

Tumour protein p53 (p53) was initially characterised as a tumour suppressor protein (9, 10, 25), serving to regulate cellular metabolism and proliferation (1, 9, 27). More recently, a functional role of p53 for *in vivo* skeletal muscle physiology has been proposed, following observations that p53 can regulate apoptosis (18), atrophy (5), autophagy (19), mitochondrial DNA (mtDNA) stability (17, 20), post-exercise signalling (18, 19), mitochondrial function (12, 18, 19, 26) and endurance performance (12, 18, 26) within skeletal muscle.

Whole-body knockout (KO) of p53 in mice results in a deficient skeletal muscle mitochondrial phenotype (12, 18), displaying reduced mitochondrial mass, mtDNA copy number, cytochrome-c oxidase (COX) enzyme activity and state 3 respiration (12, 18). As a consequence, endurance capacity and voluntary wheel running are also reduced in p53 KO mice (12, 18). In comparison, oncogenic p53 mutations found in the Li-Fraumeni syndrome increase *in vivo* skeletal muscle oxidative phosphorylation in humans (26), while mitochondrial respiration and content of electron transport chain proteins is increased in primary myoblasts from Li-Fraumeni carriers and in mice carrying a p53 R712H polymorphism (26). Thus it is clear that p53 plays an important role in mitochondrial metabolism and function.

Whilst loss of p53 impairs mitochondrial function, importantly, p53 KO mice still respond to endurance exercise training (18). Specifically, p53 KO and WT mice display similar increases in COX activity with training, while trained p53 KO

mice exhibit no difference in electron micrograph determined subsarcolemmal mitochondrial density compared to trained WT mice (18). This suggests that p53 is not essential for endurance exercise induced mitochondrial adaptations (17, 18), and the functional deficits of p53 KO appear to arise in the basal (i.e. non-exercised) state.

Despite the wealth of evidence that p53 is important for whole-body metabolism and skeletal muscle mitochondrial function, determining the importance of p53 specifically in skeletal muscle cannot be ascertained from models of whole-body p53 deletion. In such models, it cannot be excluded that phenotypic differences in skeletal muscle physiology may arise as secondary defects due to dysfunction induced by the loss of p53 in other cell types. Thus to elucidate the role of p53 specifically within skeletal muscle fibres, this study determined the effect of skeletal muscle fibre-specific loss of p53 (mKO) on mitochondrial content and enzyme activity in skeletal muscle.

2.3 Methods

2.3.1 Mouse model development

The development and validation of the p53 mKO mouse has been described previously (5). Briefly, p53 mKO mice were generated by crossing homozygous p53 floxed mice ($p53^{f/f}$; exons 2–10 of the *p53* gene are flanked by *LoxP* restriction sites) with mice expressing cre recombinase under the control of the muscle creatine kinase (MCK) promoter. Control mice (WT) were $p53^{f/f}$ littermates that lack the MCK-cre recombinase transgene. All mice were on a

C57BL/6 background. Eight-week old male mice were used for all experiments. Mice were housed in colony cages at 21°C with 12:12-h light-dark cycles and *ad libitum* access to standard laboratory chow (Harlan-Teklad formula 7913) and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

2.3.2 Tissue collection and preparation

Muscle was obtained from young (8-weeks old), healthy mice under basal conditions. Gastrocnemius, quadriceps and triceps muscle was rapidly dissected and rinsed to remove blood and fur before being snap-frozen in liquid nitrogen. Muscle was powdered using a Cellcrusher tissue pulverizer (Cellcrusher, Co. Cork, Ireland) on dry ice and stored at -80°C prior to analysis.

2.3.3 Immunoblotting

Tissue was homogenised in a 10-fold mass excess of ice-cold sucrose lysis buffer (50 mM tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M triton-X, 25 mM β-glycerophosphate, 1 µM trichostatin A, 10 mM nicotinamide, 1mM 1,4-dithiothreitol, 1% phosphatase inhibitor cocktail 2; Sigma, 1% phosphatase inhibitor cocktail 2; Sigma, 4.8% cOmplete mini protease inhibitor cocktail; Roche) by shaking in a FastPrep 24 5G (MP Biomedicals, Santa Ana, California, USA) at 6.0 $m \cdot s^{-1}$ for 80 s and centrifuging at 4°C and 8000 g for 10 min to remove insoluble material. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules,

California, USA). Samples were boiled at 97°C for 5 min in laemmli sample buffer and an equal volume of protein (20-50 µg) was separated by SDS-PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel. To demonstrate the loss of p53 in mKO skeletal muscle, the triceps muscle from mKO and WT mice were immunoblotted. Gastrocnemius, quadriceps and triceps muscle were all immunoblotted to examine the effect of p53 mKO on the content of mitochondrial, metabolic, signalling and transcriptional proteins. The presented data is from gastrocnemius muscle. Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour before being incubated in primary antibody overnight at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Imaging and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK).

2.3.4 Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBST unless otherwise stated. Antibodies: 3-hydroxyacyl-CoA dehydrogenase (β-HAD;

37673), Mitofillin (110329) and MitoProfile OXPHOS antibody cocktail (110413) from abcam; cytochrome-c (Cyt-c; 556433) from BD Pharmingen; 5' AMP-activated protein kinase alpha (AMPKα; 2603), AMPKβ2 (4148), dynamin-1-like protein (DRP1; 8570), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 2118; 1:5000), eukaryotic elongation factor 2 (eEF2; 2332), hexokinase II (HKII; 2867), mitofusin 2 (MFN2; 9482), pyruvate dehydrogenase (PDH; 2784) and p53 (2524; 1:1000 in 5% non-fat dry milk in TBST) from Cell Signaling Technologies; dynamin-like 120 kDa protein (OPA1; CPA3687) from Cohesion Biosciences; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α; ST1202) from EMD Millipore; citrate synthase (CS; SAB2701077), mitochondrial fission protein 1 (FIS1; HPA017430; 1:500) and mitochondrial transcription factor A (TFAM; SAB1401383) from Sigma-Aldrich; acyl-CoA dehydrogenase medium chain (ACADM; 1:2000), acyl-CoA dehydrogenase long chain (ACADL; 1:5000), acyl-CoA dehydrogenase very long chain (ACADVL; 1:5000) were kind gifts from Prof Jerry Vockley, University of Pittsburgh, USA. Secondary antibodies were used at a concentration of 1:10000 in TBST. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from Cell Signaling Technology; anti-chicken (PA1-28798) was from Thermo Scientific.

2.3.5 Immunofluorescence

Tibialis anterior skeletal muscle was embedded and frozen in tissue freezing medium (Triangle Biomedical, Durham, North Carolina, USA) in nitrogen-cooled isopentane, muscle samples were stored at -80°C. Skeletal muscle cross

sections (7 µM) of WT and mKO tibialis anterior muscle (6 sections per mouse, n = 3 per group) were prepared using a microtome blade (Bright 5040, Bright Instrument Company limited, Huntingdon, England). Muscle sections of WT and mKO were collected onto the same uncoated glass slides and stored at -80°C until future analysis. Samples were thawed and fixed in an acetone:methanol (1:1) solution at -20°C then washed in PBS. Samples were permeabilised in a 0.2% TritonX-100 solution for 10 minutes, then washed in PBS and followed by a 30 minute incubation in 5% normal goat serum (Invitrogen, UK) prepared in 1% BSA. Samples were then incubated in p53 primary antibody (SCBT sc-6243, rabbit, polyclonal; 1:20) prepared in 1% BSA overnight at 4°C. Slides were washed in PBS and incubated for 2 hours in goat anti rabbit IgG Alexa 594 (ThermoFisher Scientific Inc. Waltham, MA, USA; 1:200) secondary antibody. Slides were then stained for dystrophin (DSHB, MANDYS1, mouse, monoclonal; 1:200) for 2 hours, washed in PBS and treated with goat anti mouse IgG2a Alexa 488 (ThermoFisher Scientific Inc. Waltham, MA, USA 1:200) secondary antibody for 2 hours. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI; 1:1000, Sigma-Aldrich, UK), prior to cover slipping with 20µL Mowiol® 4-88 (Sigma-Aldrich, UK). Appropriate secondary antibody only control slides were used to ensure the specificity of the p53 antibody and that no apparent staining of dystrophin was visualized using the 540-580nm excitation filter in which p53 was detected. Slides were visualised using a Nikon E600 widefield microscope with a 40×0.75 numerical aperture objective. Images were captured under three colour filters using a SPOT RT KE colour three shot CCD camera (Diagnostic Instruments Inc., MI, USA), illuminated by a 170 W

Xenon light source. All images were captured using the same exposure time and gain for p53 staining in both WT and mKO muscle sections. A qualitative visual approach was used to indicate the presence or absence of p53.

2.3.6 Enzyme activity assays

Tissue was homogenised in a 10-fold mass excess of ice-cold sucrose muscle homogenisation buffer (24) by shaking in a FastPrep 24 5G (MP Biomedicals) at 6.0 $m \cdot s^{-1}$ for 80 s. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, California, USA). Gastrocnemius, quadriceps and triceps muscle were all analysed for maximal enzyme activity with data presented from triceps muscle. An equal volume of protein (10 µg for CS, 20 µg for β-HAD) was loaded onto 96-well microtiter plates in triplicate. For CS, 10 µL of sample was diluted in 235 µL of reaction buffer (64 mM TRIS pH 8.0, 0.13 mM 5,5-dithio-bis-(2-nitrobenzoic acid), 0.13 mM acetyl CoA). 5 µL of 5 mM oxaloacetate was added to start the reaction and absorbance was read at 412 nm for three minutes in a FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). For β-HAD, 15 µL of sample was diluted in 230 µL of reaction buffer (68 mM TRIS pH 8.0, 270 mM NADH, 270 mM EDTA, 270 mM Triton X-100). 5 µL of 5 mM aceto-acetyl CoA was added to start the reaction and absorbance was read at 340 nm for 12 minutes. Enzyme activity in $nmol \cdot min^{-1} \cdot mg^{-1}$ was determined from absorbance using the equation presented in Spinazzi *et al* (24) corrected for differences in pathlength.

2.3.7 Real time RT-qPCR

RNA was extracted from quadriceps muscle by Tri reagent (Sigma Aldrich, Gillingham, UK) and purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturers instructions. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader. RNA was diluted to 400 ng·μL^{-1} and reverse transcribed to cDNA using the RT^2 First Strand kit (Qiagen, Manchester, UK). RT-qPCR analysis of mRNA content was performed in singleton by using custom designed 384-well RT^2 PCR Profiler Array (Qiagen) and RT^2 SYBR Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection System (Bio-Rad). 84 genes, plus reference genes, were analysed with 43 genes of interest selected *a priori* for analysis in this chapter. 2.8 ng of cDNA was added to each well. The absence of genomic DNA, the efficiency of reverse-transcription and the efficiency of the PCR assay were assessed on each plate and conformed to the manufacturers limits in each case. Relative mRNA expression was determined using the $2^{-\Delta\Delta CQ}$ method (7) with the mean C_Q value for the reference genes *Gapdh, Actb, Hsp90ab1* and *B2m* used as an internal control. Statistical analyses were performed on the $\Delta\Delta$CQ data.

2.3.8 Statistics

Difference between genotypes was determined by independent t-tests using the Statistical Package for the Social Sciences (SPSS) version 22.0. Data are presented as means with 95% confidence intervals. Statistical significance was accepted as $p \leq 0.05$.

2.4 Results

2.4.1 Confirmation of p53 deletion in the p53 mKO mouse model

In agreement with the previous characterisation of the p53 mKO model (5) we observed an ~70% reduction in *Trp53* mRNA (Figure 2.1A), while an ~60% reduction in p53 protein content (Figure 2.1B) in skeletal muscle tissue from p53 mKO mice was also apparent. Immunofluorescence staining of WT and mKO tibialis anterior muscle cross sections indicated an overall reduction in p53 staining and a loss of p53-positive myonuclei (Figure 2.1C). However, non-myofibrillar p53 staining was apparent in both WT and mKO muscle suggesting that the residual p53 apparent in immunoblots of mKO muscle likely reflects expression from non-muscle fibre cells resident within skeletal muscle tissue.

2.4.2 Mitochondrial content and enzyme activity are maintained in p53 mKO

Despite deletion of p53 in skeletal muscle fibres, the content of proteins within the electron transport chain were similar between p53 mKO and WT littermates (Figure 2.2A). Maximal activity of CS (Figure 2.2B), a strong correlate and validated surrogate of mitochondrial content (6), as well as Cyt-c and CS protein content (Figure 2.2A) were also maintained in p53 mKO mice. In addition, proteins involved in the regulation of mitochondrial fission (DRP1, FIS1) and fusion (MFN2, mitofillin, OPA1) were also unaffected by p53 mKO (Figure 2.3). Thus mitochondrial protein content and enzyme activity in skeletal muscle is maintained following muscle fibre-specific deletion of p53.

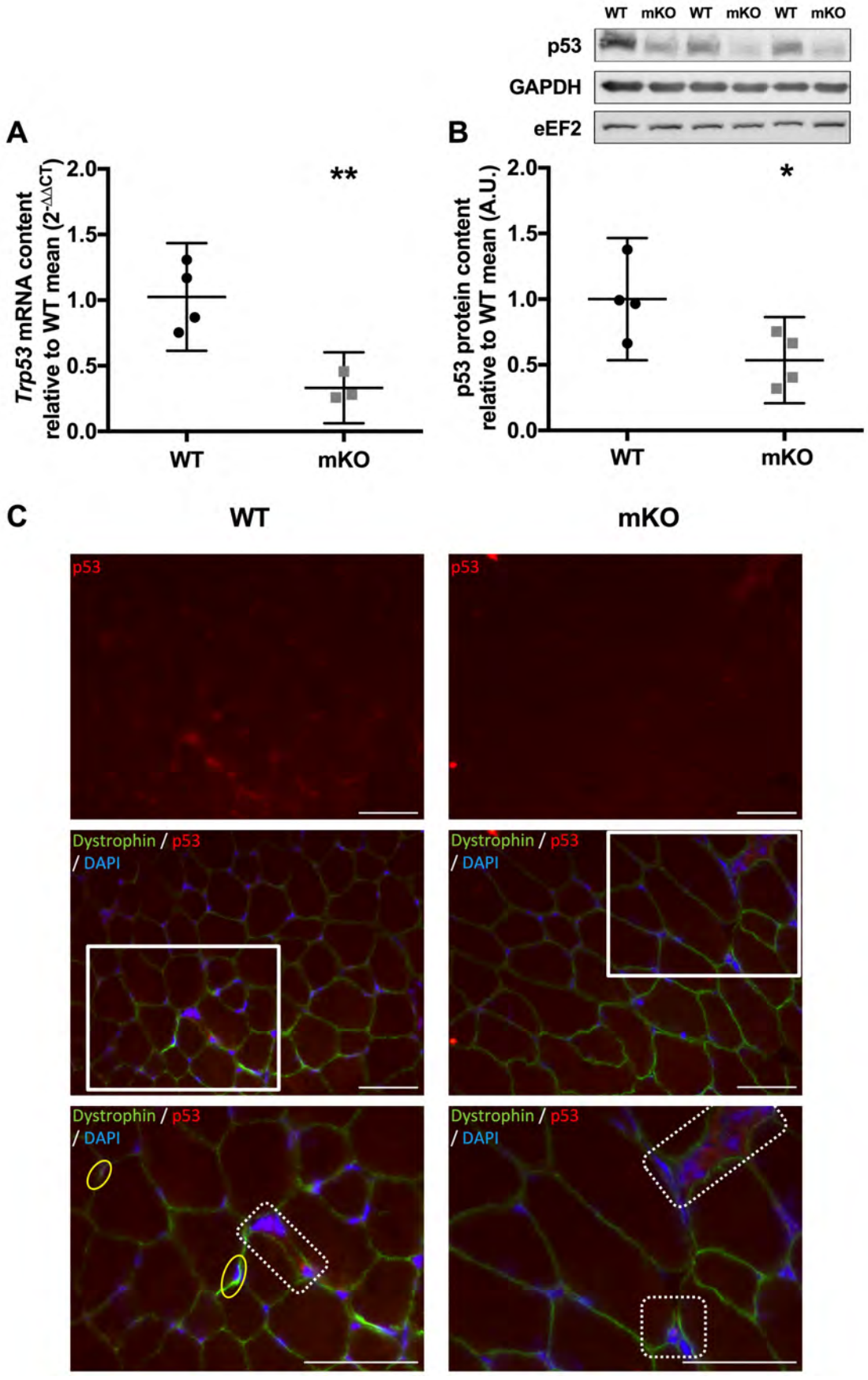

Figure 2.1 Confirmation of p53 deletion in the p53 mKO mouse model

A. *Reduction in Trp53 mRNA expression in quadriceps muscle of p53 mKO (grey squares) vs WT (black circles) mice (n = 3 - 4 per group).* ***B.*** *Reduction in p53 protein content in triceps muscle of p53 mKO mice (n = 4 per group). * p ≤ 0.05 mKO vs WT; ** p ≤ 0.01 mKO vs WT.* ***C.*** *Representative immunofluorescence images for WT (left column) and mKO (right column) tibialis anterior muscle. The top row represents p53 only (red), the middle row shows a composite image of p53 (red), dystrophin (green) and dapi (blue), the bottom row shows an enlarged image of regions highlighted with a white box in the middle row. A reduction in overall staining can be seen in mKO compared to WT. Positive regions of p53 outside of the myofibre are apparent in both WT and mKO muscle and have been highlighted in dotted boxes in the third row. Myonuclei positive for p53 are apparent only in WT muscle and have been highlighted in yellow ovals in the third row. The scaling line in each image represents 50 μM.*

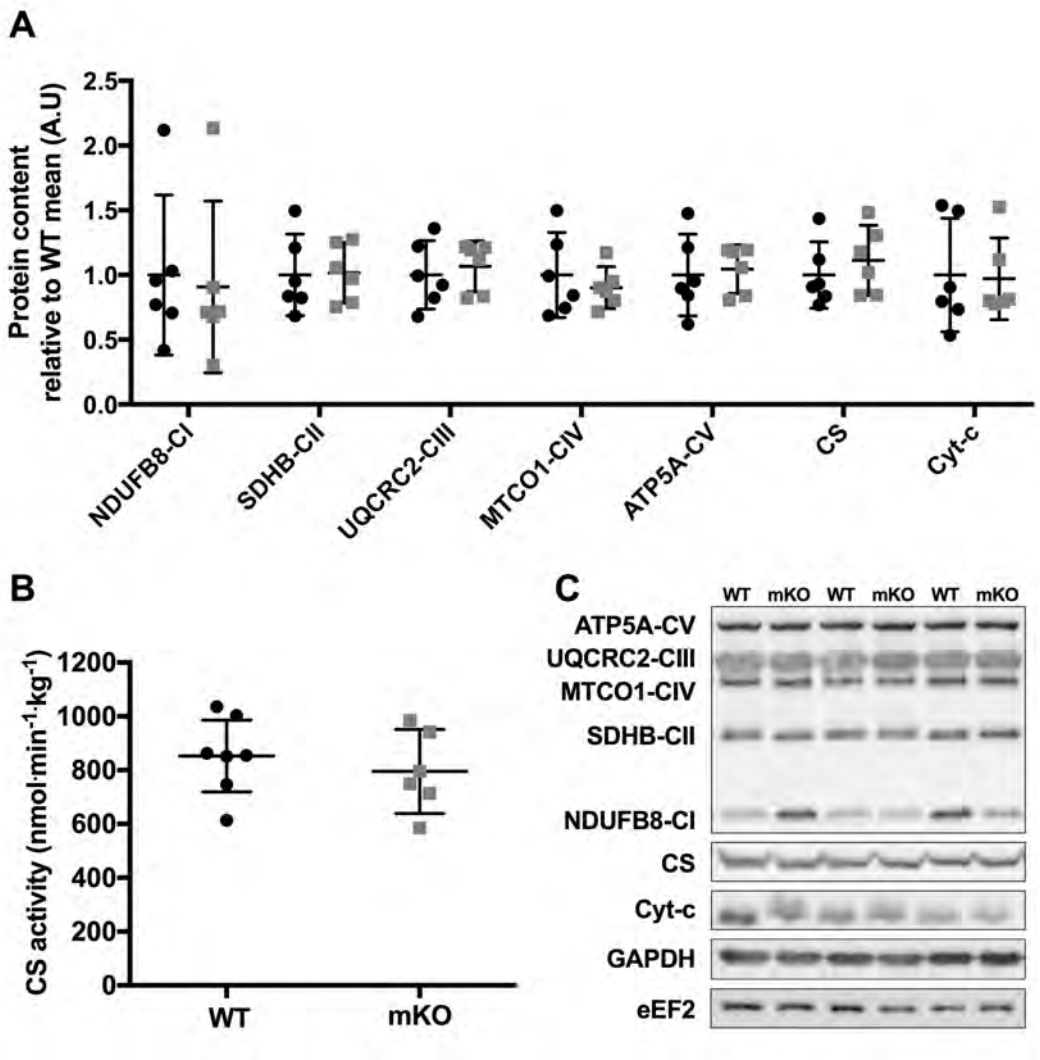

Figure 2.2 Mitochondrial OXPHOS protein content and CS enzyme activity in p53 mKO and WT mice

A. *WT (black circles) and p53 mKO (grey squares) mice display similar protein content of mitochondrial enzymes (complexes I-V, CS and Cyt-c) in gastrocnemius muscle (p > 0.05; n = 6 per group). Similar data are apparent in triceps and quadriceps muscle (see appendices;* Figure 6.1 *&*Figure 6.2*).* ***B.*** *Similar maximal CS enzyme activity in triceps muscle of p53 mKO and WT mice (p > 0.05; n = 6-7 per group). Similar data are apparent in gastrocnemius and quadriceps muscle (see appendices;*Figure 6.3*).* ***C.*** *Representative immunoblot images. Data presented as means ± 95% confidence intervals.*

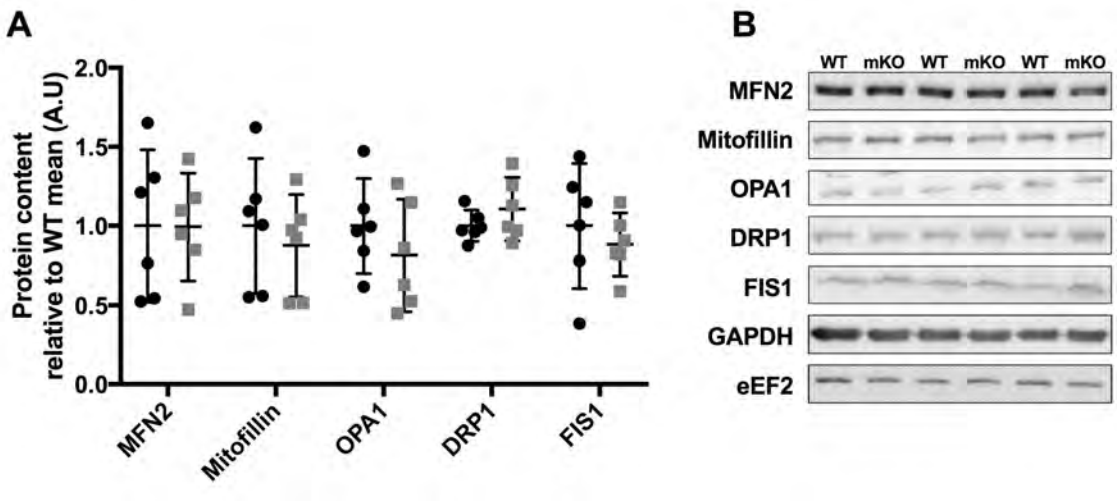

Figure 2.3 Proteins controlling mitochondrial morphology are unchanged in p53 mKO compared to WT mice

A. *WT (black circles) and p53 mKO (grey squares) mice display similar content of proteins regulating mitochondrial fusion (MFN2, mitofillin, OPA1) and fission (DRP1 and FIS1) in gastrocnemius muscle (p > 0.05; n = 6 per group). Similar data are apparent in triceps and quadriceps muscle (see appendices;* Figure 6.1 *&* Figure 6.2*).* ***B.*** *Representative immunoblot images. Data presented as means ± 95% confidence intervals.*

2.4.3 Loss of p53 does not alter regulators of substrate metabolism in skeletal muscle

P53 mKO did not reduce the content of proteins involved in fatty acid transport and metabolism (ACADM, ACADL, ACADVL, β-HAD) or carbohydrate metabolism (HKII, PDH; Figure 2.4A). Furthermore, the maximal activity of the mitochondrial-localised fatty acid metabolic protein β-HAD was unaffected by p53 mKO (Figure 2.4B).

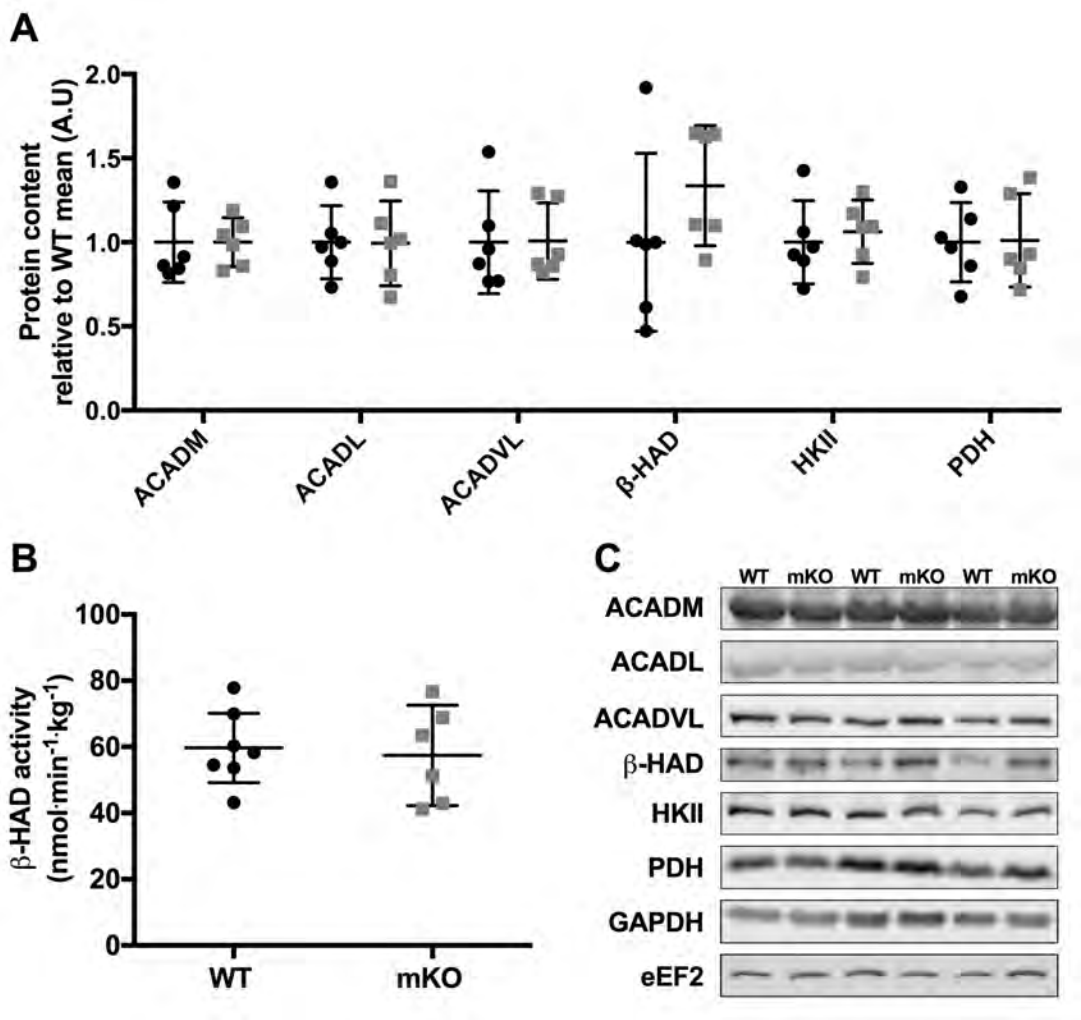

Figure 2.4 Abundance of fat and carbohydrate metabolism proteins are consistent between p53 mKO and WT mice

A. *WT (black circles) and p53 mKO (grey squares) mice display similar content of proteins involved in fatty acid (ACADM, ACADL, ACADVL and β-HAD) and carbohydrate (HKII and PDH) metabolism in gastrocnemius muscle (p > 0.05; n = 6 per group). Similar data are apparent in triceps and quadriceps muscle (see appendices;* Figure 6.1 *&*Figure 6.2*).* ***B.*** *Similar maximal β-HAD enzyme activity in triceps muscle of p53 mKO and WT mice (p > 0.05; n = 6-7 per group). Similar data are apparent in gastrocnemius and quadriceps muscle (see appendices;*Figure 6.3*).* ***C.*** *Representative immunoblot images. Data presented as means ± 95% confidence intervals.*

2.4.4 Proteins controlling energy-sensing and mitochondrial gene expression are unaffected by p53 mKO

Whole-body p53 KO mice exhibit reduced skeletal muscle mRNA and protein expression of TFAM (12) and protein content of PGC1α (18), while p53 has been implicated in the transcriptional control of AMPK subunits (4). Therefore we studied the protein content of PGC1α, TFAM, AMPKα, AMPKβ2 or NRF1 within p53 mKO skeletal muscle. In contrast to whole-body p53 KO mice, p53 mKO does not reduce the protein content of PGC1α, TFAM, AMPKα, AMPKβ2 or NRF1 within skeletal muscle (Figure 2.5A) or the mRNA expression of PGC1α or TFAM (Figure 2.5B).

2.4.5 Gene expression of proteins involved in skeletal muscle function and metabolism are unaltered by p53 mKO

p53 mKO did not affect the mRNA expression of a sub-set of electron transport chain, mitochondrial morphology, mitochondrial transport, carbohydrate and fatty acid metabolism, transcription, angiogenic, or muscle development genes (Figure 2.6).

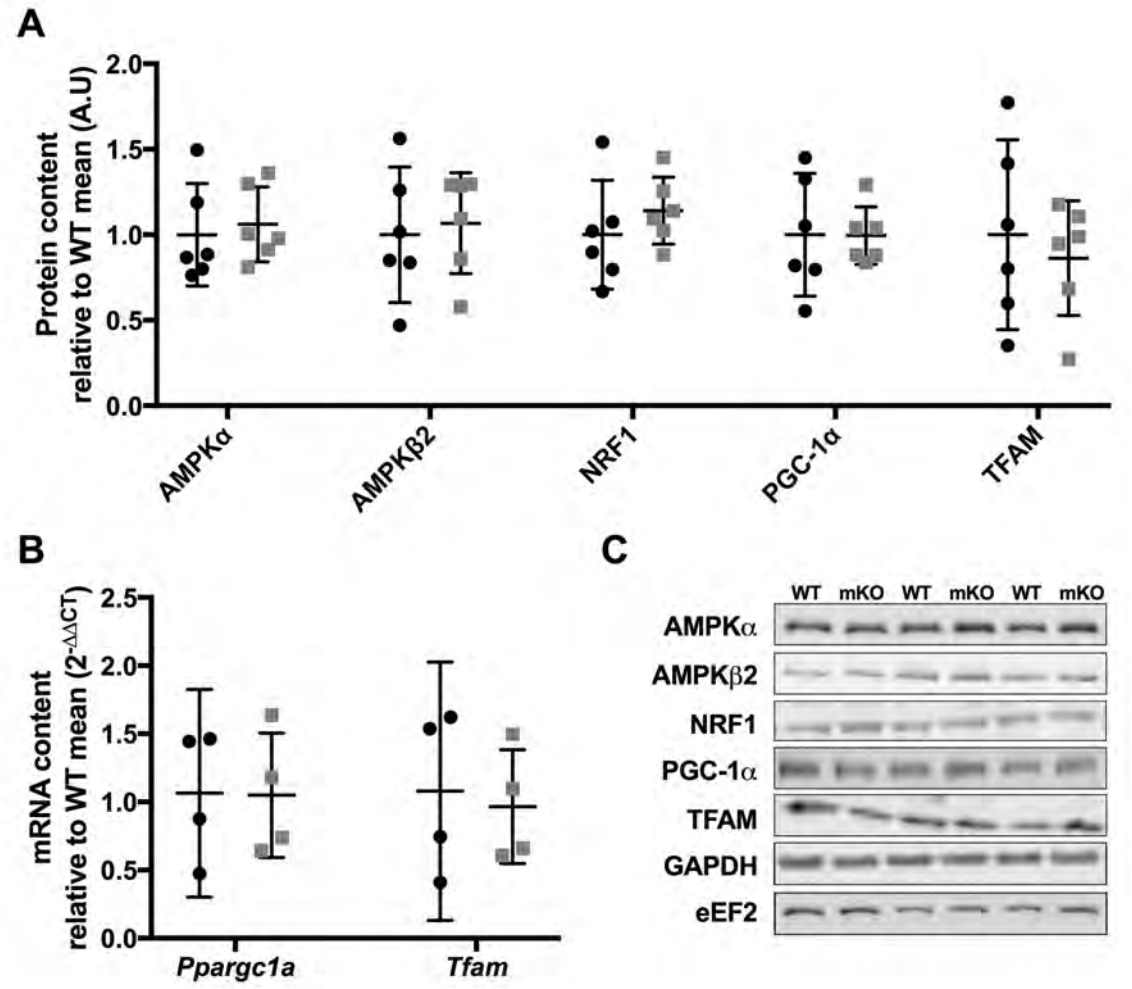

Figure 2.5 Protein content of mitochondrial biogenic signalling and transcriptional proteins in p53 mKO and WT mice

A. *The content of proteins regulating energy sensing (AMPKα, AMPKβ2), nuclear transcription (NRF1 and PGC1α) and mitochondrial transcription (TFAM) are similar in gastrocnemius muscle of WT (black circles) and p53 mKO (grey squares) mice (p > 0.05; n = 6 per group). Similar results were observed in triceps and quadriceps muscle (see appendices;* Figure 6.1 *&*Figure 6.2*).* ***B.*** *The mRNA expression of Pparg1a and Tfam are similar in quadriceps muscle of WT (black circles) and p53 mKO (grey squares) mice (p > 0.05; n = 4 per group).* ***C.*** *Representative immunoblot images. Data presented as means ± 95% confidence intervals.*

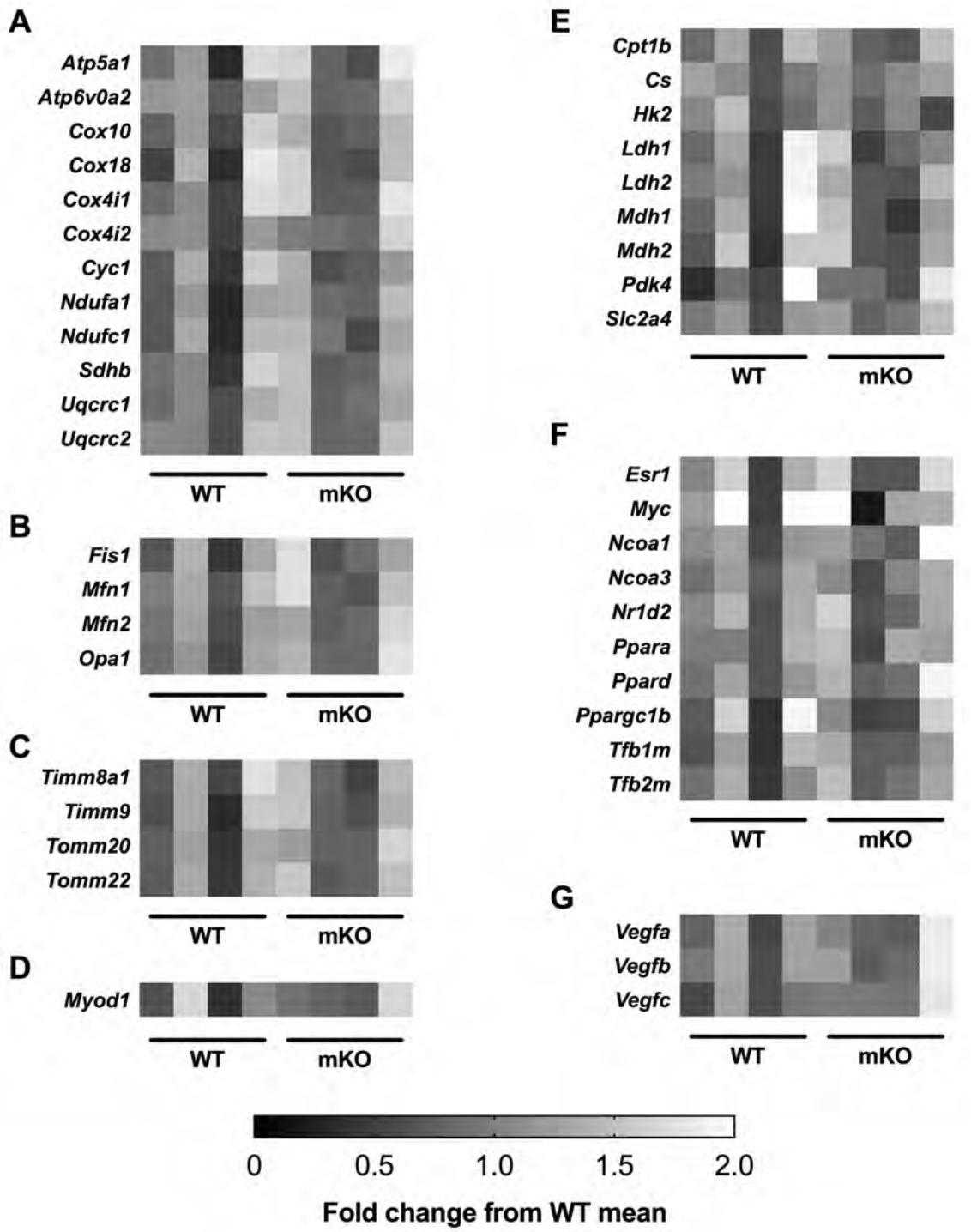

Figure 2.6 p53 mKO and WT mice display similar mRNA profiles

p53 mKO and WT mice display similar mRNA expression of ***A.*** *electron transport chain proteins and proteins involved in regulating* ***B.*** *mitochondrial morphology,* ***C.*** *mitochondrial protein transport,* ***D.*** *muscle development,* ***E.***

carbohydrate and fatty acid metabolism, ***F.*** *transcription and* ***G.*** *angiogenesis. Each column represents the mRNA expression from the quadriceps muscle of a single mouse (p > 0.05; n = 4 per group).*

2.5 Discussion

We report that muscle-specific deletion of p53 does not reduce mitochondrial protein content or enzyme activity within skeletal muscle. This is in contrast to previous research from whole-body p53 KO mice which reported a reduction in mitochondrial mass, mtDNA copy number, COX enzyme activity and state 3 respiration (12, 18, 21). Given these discrepancies, we therefore interpret this data to indicate that the decrement in skeletal muscle mitochondrial function in whole-body p53 KO mice is a secondary consequence of an adverse phenotype of this mouse model, rather than due the loss of p53 *per se* in skeletal muscle fibres.

p53 has been implicated in metabolic control within numerous cell types (1, 9, 27). Indeed, p53 can exert direct or indirect transcriptional control over various metabolic and mitochondrial biogenic proteins in some cell types (e.g. PGC1α (18), TFAM (12, 18), cytochrome-c oxidase subunit 1 (CO1) (11), cytochrome-c assembly protein (SCO2) (9), AMPKβ (4), tp53-inducible glycolysis and apoptosis regulator (TIGAR) (1) and glucose transporter type 4 (GLUT4) (23)). However, our data indicates that in skeletal muscle, p53 is not required to maintain mitochondrial content and function. For example, the maximal activity of CS and the protein content of electron transport chain proteins were

maintained in p53 mKO mice. In addition, the content of proteins controlling mitochondrial morphology, substrate utilisation, energy sensing and transcription were comparable between p53 mKO and WT mice. The muscles examined within this study were, however, all predominantly made up of fast-fibre types and it cannot be excluded that a different phenotype may be apparent in predominantly slow-fibre type muscles. Nonetheless, this data does indicate a normal metabolic phenotype of p53 mKO mice under basal conditions and is consistent with a similar skeletal muscle fibre-type distribution and diameter previously reported in p53 mKO mice (5).

Unimpaired mitochondrial biogenesis in skeletal muscle of p53 mKO mice is in contrast to data published from whole-body p53 KO mice (12, 18, 21). Why whole-body p53 KO mice exhibit mitochondrial defects within skeletal muscle while p53 mKO mice do not is unclear. One explanation for the divergent phenotypes may be differences in the timing of p53 deletion in skeletal muscle between the two models. In the mKO mice examined here cre recombinase was expressed under the MCK promoter and as such p53 would not be deleted until ~13 days into embryonic development (8), whereas p53 is absent throughout the entirety of embryonic development in the germline deletion of the whole-body KO mice. Importantly, p53 is highly expressed in mouse embryos from embryonic day 8.5 to 10.5 (22). During myogenesis, p53 plays an important role in inducing differentiation (2, 14); a period of intense mitochondrial biogenesis (16). $p53^{-/-}$ myoblasts and C2C12 myoblasts treated with dominant-negative p53 inhibitors display impaired myosin heavy chain

induction during differentiation, potentially due to reduced expression of the muscle differentiation controlling proteins retinoblastoma protein (RB) and MCK (2, 14). Although direct evidence for a lack of mitochondrial development during differentiation in $p53^{-/-}$ myoblasts is lacking, mitochondrial deficits may arise in the whole-body KO mice due to a lack of p53 during this developmental period, while conversely p53 is expressed in skeletal muscle of the embryonic p53 mKO mice during this stage. Additionally, a loss of p53 in satellite cells in whole-body KO mice may impair satellite cell differentiation into myocytes (14) during adulthood and therefore the continued regeneration and maintenance of healthy skeletal muscle (15). Thus the loss of p53 in one or multiple cell type(s) other than muscle fibres may contribute to a decrement in mitochondrial function within skeletal muscle.

Several differences are also apparent in the expression of mitochondrial biogenic transcription factors/co-factors between the whole-body KO and mKO models. Park *et al* (12) demonstrated that p53 interacts with the TFAM gene in C2C12 myoblasts and that TFAM expression is reduced in the soleus muscle of whole-body p53 KO mice. Furthermore, PGC1α protein content was decreased in p53 KO mice examined by Saleem *et al* (18), although this contradicts similar PGC1α protein content found between WT and p53 KO mice by Park *et al* (12). In the p53 mKO mice studied here there were no deficits in TFAM or PGC1α protein or mRNA expression, which may explain the normal mitochondrial phenotype exhibited by p53 mKO mice. Alternatively, given the developmental nature of the mKO model, other regulators of mitochondrial biogenesis (13) may

be compensating for the loss of p53 in skeletal muscle, however exploration of all these pathways is outside the scope of this investigation. Thus an inducible muscle-specific knockout model is still required to determine whether p53 is important for maintaining mitochondrial function in mature skeletal muscle.

Finally, the whole-body p53 KO mouse may not be a healthy model. Whole-body p53 KO mice exhibit reduced voluntary wheel running (18), which if recapitulated as lower spontaneous physical activity in non-exercised mice may explain the reduced mitochondrial function. Furthermore, whole-body p53 KO mice develop spontaneous cancers at an early age (3), something that is often concomitant with cachexia and impaired muscle function. Nonetheless, utilising a muscle fibre-specific knockout model allows us, and others (17), to assess p53 function within skeletal muscle without the complications of potential secondary defects associated with loss of p53 in other cell types.

While p53 within skeletal muscle fibres does not appear critical for developing or maintaining mitochondrial content within skeletal muscle of young healthy mice in the basal state, p53 does clearly play a role within skeletal muscle in other physiological contexts. For example, induction of p53 contributes to immobilisation-induced atrophy by orchestrating transcription of *Cdkn1a*/p21 and possibly other genes that promote muscle fibre atrophy, whereas p53 mKO mice are protected from atrophy (5). p53 is also important in maintaining mitochondrial DNA stability and mediates exercise-induced mitochondrial biogenesis in PolG KO mice, a model of accelerated mitochondrial mutations

(17). Thus, it is likely that the importance of p53 may become apparent during ageing. Furthermore, it remains to be determined whether p53 mKO mice respond to endurance exercise training although whole-body p53 KO mice are uninhibited in this respect (18), which would indicate that p53 mKO would also be responsive to endurance exercise training.

Our data indicates that muscle fibre-specific deletion of p53 does not impair mitochondrial protein content or enzyme activity in skeletal muscle. This is evidenced by similar content of proteins within the electron transport chain and proteins regulating mitochondrial morphology, substrate utilisation, energy sensing and transcription between WT and p53 mKO mice. In addition, there were no deficits in the maximal activity of the mitochondrial enzymes CS and β-HAD in p53 mKO mice. While p53 likely has other roles in skeletal muscle physiology, from these data it does not appear that skeletal muscle p53 is necessary for developing or maintaining mitochondrial content in young healthy mice.

2.6 References

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2.7 Additional Information

2.7.1 Conflict of Interests

The authors report no conflicts of interest.

2.7.2 Acknowledgements

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3 POST-EXERCISE SKELETAL MUSCLE SIGNALLING RESPONSES TO MODERATE- TO HIGH-INTENSITY STEADY-STATE EXERCISE IN THE FED OR FASTED STATE

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3.1 Abstract

Exercise performed in the fasted state acutely increases fatty acid availability and utilisation. Furthermore, activation of energy-sensing pathways and fatty acid metabolic genes can be augmented by fasting and fasted exercise. However, whether a similar effect occurs at higher exercise intensities remains poorly understood. This study aimed to assess the effect of fed and fasted exercise upon post-exercise signalling and mRNA responses during moderate- to high-intensity steady-state exercise. Eight male participants (age: 25 ± 2 y, $\dot{V}O_{2peak}$: 47.9 ± 3.8 mL·kg^{-1}·min^{-1}) performed one hour of cycling at 70% W_{max} in the fasted (FAST) state or two hours following ingestion of a carbohydrate-rich mixed-macronutrient breakfast (FED). Muscle biopsies were collected pre-, immediately and three-hours post-exercise from the *medial vastus lateralis*, whilst venous blood samples were collected throughout the trial. Plasma NEFA and glycerol concentrations were elevated during FAST compared to FED, although substrate utilisation during exercise was similar. AMPK^{Thr172} phosphorylation was elevated ~2.5-fold immediately post-exercise and was significantly augmented by ~1.2-1.4-fold during FAST. CREB^{Ser133} phosphorylation was also elevated by ~1.2-1.4-fold during FAST, although CREB^{Ser133} phosphorylation was acutely decreased by ~60% immediately post-exercise. SIRT1 and SIRT 3 activity, assessed by acetylation of p53^{Lys382} and MnSOD^{Lys122}, respectively, were unchanged by fasting or acute exercise. PARP1 protein content decreased by ~25% post-exercise, whereas PARylation of PARP1 was unchanged. mRNA expression of *PDK4* was augmented ~3-4-fold by exercise and ~2-fold elevated throughout FAST, whilst expression of

PPARGC1A mRNA was similarly activated (~10-fold) by exercise in both FED and FAST. In summary, performing moderate- to high-intensity steady-state exercise in the fasted state increases systemic lipid availability, elevates phosphorylation of AMPK^{Thr172} and CREB^{Ser133}, and augments *PDK4* mRNA expression without corresponding increases in whole body fat oxidation and the mRNA expression of *PPARGC1A*.

3.2 Introduction

During endurance exercise, transient perturbations in the cellular and extracellular milieu activate a number of intracellular signalling cascades thought to drive the initial adaptive response to exercise (20). Of note, the exercise-induced activation of AMP-activated protein kinase (AMPK), p38 mitogen activated protein kinase (p38 MAPK), sirtuin 1 (SIRT1) and calcium/calmodulin dependent kinase II (CAMKII) are thought to be central to this response given they activate downstream transcription factors and transcriptional co-activators to initiate mitochondrial biogenesis (4, 5, 13, 19, 30, 42, 71). With regularly repeated exercise bouts, transient upregulation of mRNA and subsequent protein translation manifest as cellular adaptations such as mitochondrial biogenesis (41). Given the associations between skeletal muscle mitochondrial function and the development of insulin resistance (33), the optimisation of the acute post-exercise adaptive response within skeletal muscle holds therapeutic potential.

Performing exercise in the fasted state has the potential to produce favourable metabolic adaptations over and above fed exercise training (61). For example, moderate-intensity (50-75% $\dot{V}O_{2peak}$) exercise, performed in the fasted state, increases fatty acid availability and oxidation compared to exercise combined with carbohydrate ingestion (1, 10, 11, 15). At the cellular level, this response may be mediated in part via an AMPK-dependent mechanism, as skeletal muscle AMPKα2 activity is enhanced during moderate-intensity (60% W_{max}) fasted exercise compared to glucose ingestion (1). Despite this, the optimal

integration of nutrition and exercise to augment adaptive signalling responses remains elusive. For example, AMPK and other exercise-sensitive signalling molecules can be further activated at more vigorous exercise intensities (e.g. ~80% $\dot{V}O_{2peak}$) (19). However, it remains unclear whether this represents a maximal signalling response to moderate- to high-intensity steady-state exercise or whether superimposing fasting-induced nutritional stress can further enhance this. Furthermore, fasting *per se* activates additional exercise-sensitive signalling pathways within skeletal muscle including p38 MAPK (25), although this is not apparent in human skeletal muscle (17), SIRT1 (17) and cAMP response-element binding protein (CREB) (66), which remain to be studied in a fed versus fasted exercise model in humans. Thus the potential additive or synergistic effect of combining fasting with exercise on signalling pathways within human skeletal muscle remains incompletely understood.

Therefore, the purpose of this study was to compare the effect of performing moderate- to high-intensity (70% W_{max}) steady-state exercise in the fasted state or following the consumption of a mixed-macronutrient breakfast on post-exercise signalling and mRNA expression in skeletal muscle. It was hypothesised that fasted exercise would result in an elevated peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) and pyruvate dehydrogenase kinase 4 (*PDK4*) mRNA response over and above that induced by fed exercise and would be associated with augmented $AMPK^{Thr172}$ and $CREB^{Ser133}$ phosphorylation and elevated SIRT1 and SIRT3 deacetylase activity.

3.3 Methods

3.3.1 Participants

Eight recreationally active males (mean ± SD: age, 25 ± 2 years; body mass, 74.6 ± 5.2 kg; peak oxygen uptake ($\dot{V}O_{2peak}$), 47.9 ± 3.8 $mL \cdot kg^{-1} \cdot min^{-1}$; maximal aerobic power (W_{max}), 272 ± 33 W) were recruited to participate. Required sample size was determined *a priori* based on the $AMPK^{Thr172}$ data from Guerra et al (26), which indicated that eight participants was required for $\alpha = 0.05$ and $1-\beta = 0.80$. Participants were fully informed of the study procedures and their right to withdraw before providing written consent to participate. The study was approved by the National Health Service Research Ethics Committee, Black Country, West Midlands, UK.

3.3.2 Pre-testing

After measuring height (Seca 220, Seca, Birmingham, UK) and body mass (Champ II, OHAUS, Griefensee, Switzerland) participants performed a graded exercise test to exhaustion on a cycle ergometer (Lode Excalibur, Groningen, Netherlands). The test began with a five-minute warm-up at 100 W with power increasing by 35 W every three minutes thereafter. Respiratory variables were measured continuously during exercise using a breath-by-breath metabolic cart (Oxycon Pro, Jaeger, CareFusion, Germany), heart rate was monitored throughout (RCX5, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE) were determined using a 6-20 Borg scale during the final 15 seconds of each 3-minute stage (6). $\dot{V}O_{2peak}$ was determined as the

highest rolling 30-second average and was stated as being achieved if the following criteria were met: i) heart rate within 10 beats·min^{-1} of age-predicted maximum and ii) respiratory exchange ratio (RER) > 1.1, or iii) plateau of oxygen consumption despite increasing work-rate. W_{max} was determined as work rate at the last completed stage plus the fraction of time spent in the final non-completed stage multiplied by the increment in work rate (35W).

3.3.3 Experimental trials

Participants performed two experimental trials in a randomised, counter-balanced, crossover design. By necessity of the design (i.e. food intake) it was not possible to blind participants or experimenters. Participants refrained from alcohol for 72 h, caffeine for 24 h and exercise for 48 h prior to each experimental trial. Prior to each experimental trial, participants were provided with a pre-prepared standardised three-day diet (energy contribution: 61% carbohydrate, 18% fat and 21% protein) matched to individual energy intake (mean ± SD: 2688 ± 450 kcal·day^{-1}) determined by a three-day weighed food diary.

Participants arrived at the laboratory at ~8 am following an ~12-hour overnight fast. Upon arrival, participants rested in the supine position for approximately ten minutes before a cannula was inserted into an antecubital forearm vein and a baseline venous blood sample was collected. Participants were then provided with a mixed-macronutrient breakfast (FED) (0.9 g·kg^{-1} body mass (BM) of corn flakes cereal, 3.9 mL·kg^{-1} BM of semi-skimmed milk, 1.1 g·kg^{-1} BM of toasted

wholemeal bread, 0.3 g·kg^{-1} BM of strawberry jam and 3.2 mL·kg^{-1} BM of orange juice (all Sainsbury's, UK); energy intake: 710 ± 49 kcal; macronutrients: 1.75 g·kg^{-1} BM carbohydrate (of which 0.66 g·kg^{-1} BM is sugar), 0.1 g·kg^{-1} BM fat and 0.35 g·kg^{-1} BM protein; energy contribution: 75% carbohydrate, 10% fat and 15% protein), which was consumed within the first 15 minutes of the trial, or participants remained in the fasted state (FAST). The macronutrient composition of the breakfast reflected population trends for relatively greater consumption of carbohydrates, and thus lower fat and protein ingestion, at breakfast time (2, 51, 68). Participants rested for two hours prior to providing a pre-exercise skeletal muscle biopsy from the *medial vastus lateralis*. Participants then cycled for one-hour at 70% W_{max} before a second skeletal muscle biopsy was taken immediately post-exercise (completed within two minutes of exercise cessation). Participants then rested in a supine position prior to a third skeletal muscle biopsy being obtained three-hours post-exercise. In order to minimise discomfort for the participants the first two skeletal muscle biopsies (i.e., pre- and immediately post-exercise) were performed in the same leg with the third biopsy (i.e. 3-hours post-exercise) performed in the contralateral leg. The number of biopsies per leg was counterbalanced in the second trial and the leg receiving the initial biopsy was randomised. A new incision was made for each biopsy at least 2 cm from the previous site. Venous blood was collected throughout rest periods and during exercise. Respiratory variables were measured pre-exercise and at 15-minute intervals throughout exercise, heart rate was monitored continuously throughout exercise and RPE was determined at 15-minute intervals throughout exercise. Carbohydrate and

fat oxidation were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using the moderate-high exercise intensities equation of Jeukendrup and Wallis (31) during exercise and Frayn (22) at rest. Participants drank water *ad libitum* during rest and exercise periods of trial one, with water intake matched during trial two to that consumed during each period of trial one (fluid intake during exercise: 618 ± 341 mL; post-exercise fluid intake: 635 ± 343 mL).

3.3.4 Muscle biopsies

Muscle biopsies were obtained from separate incision sites on the *medial vastus lateralis* under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a Bergström needle adapted with suction. Muscle was rapidly blotted to remove excess blood and flash frozen in liquid nitrogen. Muscle was powdered using a Cellcrusher tissue pulveriser on dry ice and stored at -80°C prior to analysis.

3.3.5 Immunoblotting

Tissue was homogenised in a 10-fold mass excess of ice-cold sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M Triton-X, 25 mM β-glycerophosphate, 1 µM Trichostatin A, 10 mM Nicatinamide, 1mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% cOmplete Mini Protease Inhibitor Cocktail; Roche) by shaking in a FastPrep 24 5G (MP Biomedicals) at 6.0 $m \cdot s^{-1}$ for 80 s and centrifuging at 4°C

and 8000 g for 10 minutes to remove insoluble material. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, California, USA). Samples were boiled at 97°C for 5 min in laemmli sample buffer and an equal volume of protein (20-50 µg) was separated by SDS-PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel. Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour before being incubated in primary antibody overnight at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence HRP substrate detection kit (Millipore, Watford, UK). Imaging and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK).

3.3.6 Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBST unless otherwise stated. Antibodies: acetylated proteins (ab193) and ac-MnSOD^{K122} (ab214675) were purchased from Abcam; AMPKα (2603), p-AMPK^{Thr172} (2535), p-ACC^{Ser79} (3661), CAMKII (3362), p-CAMKII^{Thr268} (12716), CREB (1°: 1:500; 9197), p-CREB^{Ser133} (1°: 1:500; 9191), p38 (9212), p-p38$^{Thr180/Tyr182}$ (4511), poly ADP-ribose polymerase 1 (PARP1; 1°: 1:500; 9542), tumour protein 53 (p53;

2°: 1:2000; 2527) and ac-p53^{K382} (1°: 1:500 in 3% BSA, 2°: 1:2000; 2570) were purchased from Cell Signaling Technology; acetyl-CoA carboxylase (ACC; 05-1098), superoxide dismutase (MnSOD; 1°: 1:2000; 06-984) and poly-ADP-ribose (PAR; 1°: 1:500; MABE1031) were purchased from Merck Millipore. Secondary antibodies were used at a concentration of 1:10000 in TBST unless otherwise stated. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from Cell Signaling Technology.

3.3.7 Real time RT-qPCR

RNA was extracted from ~20 mg of muscle by homogenising in 1 mL of Tri reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-TURRAX homogeniser (IKA, Oxford, UK). Phase separation was achieved by addition of 200 µL of chloroform and centrifugation at 12000 g for 15 minutes. The RNA-containing supernatant was removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturers instructions, which includes a DNase treatment step. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). RNA was diluted to 30 ng·µL^{-1} and reverse transcribed to cDNA in 20 µL volumes using the nanoScript 2 RT kit and oligo(dT) primers (Primerdesign, Southampton, UK) as per the manufacturers instructions. RT-qPCR analysis of mRNA content was performed in triplicate by using Primerdesign custom designed primers for *PPARGC1A* (Accession number: NM_002612.3; Forward

primer: 5'-TTGCTAAACGACTCCGAGAAC-3'; Reverse primer: 5'-GACCCAAACATCATACCCCAAT-3'), *PDK4* (Accession number: NM_013261; Forward primer: 5'-GAGGGACACTCAGGACACTTTAC-3'; Reverse primer: 5'-TGGAGGAAACAAGGGTTCACAC-3') and commercially available *GAPDH* (Primerdesign) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, UK). The qPCR reaction was run as per the manufacturers instructions (Primerdesign) and followed by a melt curve (Applied Biosystems) to ascertain specificity. 2-4 ng of cDNA was added to each well in a 20 µL reaction volume. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression was expressed relative to the expression in the pre-exercise sample during FED for each individual using the $2^{-\Delta\Delta CQ}$ method (36) with the C_q value for *GAPDH* used as an internal control. Statistical analysis was performed on the ΔΔCQ data.

3.3.8 Blood analyses

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA; BD, Oxford, UK) for the collection of plasma. Samples were placed immediately upon ice prior to centrifugation at 1600 g at 4°C for 10 minutes before collection of plasma from the supernatant. Plasma was frozen at -80°C until further analysis. Plasma samples were subsequently analysed on an autoanalyser (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for glucose, lactate, non-esterified fatty acid (NEFA) and glycerol (Randox Laboratories, County Antrim, UK) using commercially available kits.

3.3.9 Statistics

Two-way repeated measures ANOVAs assessed effects of time, treatment and time*treatment interaction effects for all time-course data. Ryan-Holm-Bonferroni multiple comparison corrections were applied *post-hoc* where applicable. Total area under the curve (tAUC) was determined for blood metabolites using the trapezoid method (37). Differences in tAUC for blood metabolites and means for exercising $\dot{V}O_2$, substrate utilisation, heart rate and RPE were assessed using repeated-measures t-tests. Due to issues with blood sampling from one participant, blood analyses are performed with a sample size of 7. All statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0. Data are presented as means with 95% confidence intervals. Statistical significance was accepted as $p \leq 0.05$.

3.4 Results

3.4.1 Substrate availability and utilisation

Plasma NEFA and glycerol concentrations were elevated during FAST (Figure 3.1A & Figure 3.1B), indicative of elevated lipolysis and fatty acid availability. Plasma NEFA concentration displayed main effects for treatment ($p = 0.004$), time ($p = 0.001$) and a treatment*time interaction effect ($p < 0.001$). Plasma NEFA was significantly elevated in FAST compared to FED during exercise (135-165 minutes) and at twenty minutes into recovery (200 minutes; $p < 0.05$). tAUC for plasma NEFA across the experimental period was significantly elevated in FAST ($p = 0.011$). Plasma glycerol concentration showed main

effects for treatment (p = 0.007), time (p < 0.001) and a treatment*time interaction effect (p = 0.015). Plasma glycerol was significantly elevated during exercise (135-180 minutes) in FAST compared to FED (p < 0.05). tAUC for plasma glycerol across the experimental period was significantly higher in FAST (p = 0.006). Plasma glucose displayed a treatment*time interaction effect (p = 0.024), whereby plasma glucose was higher in FAST immediately prior to exercise (120 minutes; Figure 3.1C). However, a lack of treatment effect (p = 0.866) and no difference in tAUC (p = 0.942) shows plasma glucose was similar when the whole trial is considered. Plasma lactate displayed a significant effect for time (p < 0.001), increasing above baseline during exercise (135-180 minutes) and remained so during the first 40 minutes of recovery (180-220 minutes; p < 0.05; Figure 3.1D). No between-treatment differences were apparent for plasma lactate (main effect of treatment; p = 0.774, interaction; p = 0.568, tAUC; p = 0.548). Carbohydrate and fat oxidation during exercise were similar between trials (Table 1). $\dot{V}CO_2$ was significantly lower during FAST exercise (p = 0.045). $\dot{V}O_2$, RER, heart rate and RPE did not differ during exercise between trials (Table 1).

Table 3.1 Physiological responses to 70% W_{max} cycling during FED and FAST

		Time (minutes into exercise)						
		0	15	30	45	60	Mean	*p*
Gas exchange								
$\dot{V}O_2$ (L·min^{-1})	FED	0.41 ± 0.03	2.99 ± 0.23	3.12 ± 0.28	3.13 ± 0.32	3.12 ± 0.30	3.09 ± 0.28	
	FAST	0.46 ± 0.04	2.98 ± 0.21	3.08 ± 0.22	3.08 ± 0.21	3.00 ± 0.23	3.04 ± 0.21	0.537
$\dot{V}CO_2$ (L·min^{-1})	FED	0.35 ± 0.03	2.83 ± 0.21	2.84 ± 0.24	2.82 ± 0.27	2.85 ± 0.26	2.84 ± 0.24	
	FAST	0.38 ± 0.04	2.78 ± 0.19	2.74 ± 0.21	2.70 ± 0.20	2.68 ± 0.20	2.73 ± 0.19	0.045
RER	FED	0.87 ± 0.04	0.95 ± 0.02	0.91 ± 0.03	0.90 ± 0.02	0.91 ± 0.02	0.92 ± 0.02	
	FAST	0.82 ± 0.06	0.93 ± 0.03	0.89 ± 0.05	0.88 ± 0.03	0.89 ± 0.03	0.90 ± 0.03	0.269
Oxidation rates								
Carbohydrate (g·min^{-1})	FED	0.30 ± 0.07	3.07 ± 0.32	2.70 ± 0.26	2.62 ± 0.30	2.76 ± 0.34	2.79 ± 0.27	
	FAST	0.24 ± 0.13	2.87 ± 0.43	2.41 ± 0.43	2.26 ± 0.41	2.40 ± 0.42	2.48 ± 0.39	0.117
Fat (g·min^{-1})	FED	0.09 ± 0.02	0.26 ± 0.11	0.46 ± 0.11	0.50 ± 0.13	0.44 ± 0.14	0.42 ± 0.11	
	FAST	0.14 ± 0.05	0.34 ± 0.17	0.56 ± 0.18	0.62 ± 0.17	0.53 ± 0.20	0.51 ± 0.17	0.361
Intensity								
Heart rate (beats·min^{-1})	FED	67 ± 6	167 ± 4	172 ± 4	174 ± 3	177 ± 4	172 ± 4	
	FAST	60 ± 7	166 ± 5	171 ± 5	174 ± 4	176 ± 3	172 ± 4	0.687
RPE	FED		13 ± 1	14 ± 1	15 ± 1	16 ± 1	15 ± 1	
	FAST		13 ± 2	15 ± 1	16 ± 1	17 ± 1	16 ± 1	0.111

Data presented as means ± 95% confidence intervals (n = 8). Mean values represent the mean of the recorded values during exercise. *p* values represent repeated-measures t-test comparisons between exercising means for FED and FAST.

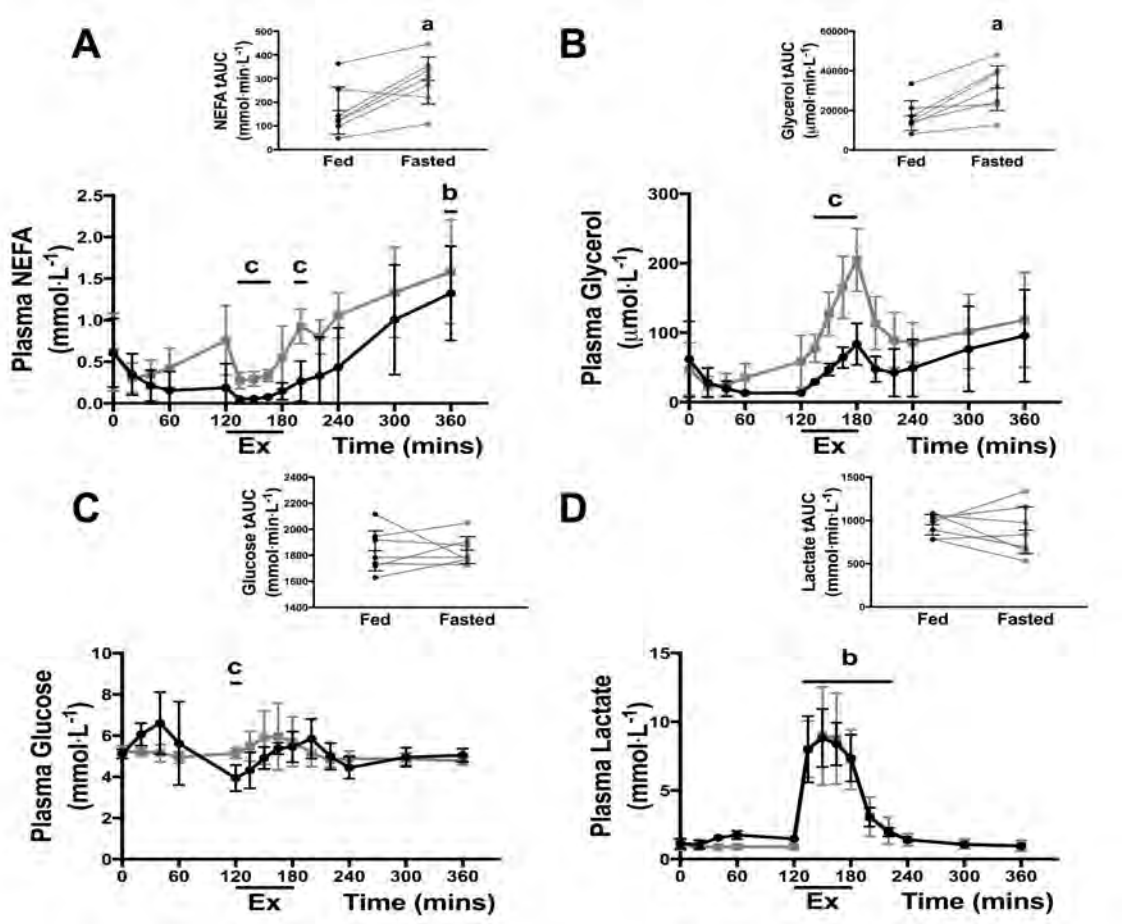

Figure 3.1 Fasting augments NEFA and glycerol availability during endurance exercise

*Time-course and tAUC (inset) for plasma NEFA (**A**), glycerol (**B**), glucose (**C**) and lactate (**D**) in FED (black circles) and FAST (grey squares). Exercise (Ex) was performed between minutes 120 and 180. **a**: main effect of treatment ($p \leq 0.05$); **b**: main effect of time (significantly different to 0 minutes (pre-breakfast); $p \leq 0.05$); **c**: interaction effect (significantly different between FED and FAST; $p \leq 0.05$). Data presented as means ± 95% confidence intervals (n = 7).*

3.4.2 Skeletal muscle signalling

Exercise increased the phosphorylation of AMPK^{Thr172} (main effect of time; $p < 0.001$); increasing ~2.5-fold immediately post-exercise ($p < 0.001$) and remaining ~1.5-fold above baseline three hours post-exercise ($p < 0.001$ vs pre-exercise; Figure 3.2A). Furthermore, AMPK^{Thr172} phosphorylation was elevated during the FAST trial (by ~20% and ~40% at pre- and immediately post-exercise time points, respectively), displaying a main effect for treatment ($p = 0.050$) and a trend towards a treatment*time interaction effect ($p = 0.065$). Phosphorylation of ACC^{Ser79} (Figure 3.2B) increased ~7-fold immediately post-exercise ($p = 0.004$ vs pre-exercise) and remained ~2-fold elevated 3-h post-exercise ($p = 0.016$ vs pre-exercise, main effect of time; $p = 0.002$). Trends towards an effect of treatment ($p = 0.092$) and a treatment*time interaction effect ($p = 0.071$) were also apparent.

FAST also induced an elevation in the phosphorylation of CREB^{Ser133} (main effect of treatment; $p = 0.050$; Figure 3.2C). CREB^{Ser133} phosphorylation also displayed a main effect for time ($p = 0.003$); decreasing in phosphorylation by ~60% immediately post-exercise ($p = 0.001$). No treatment*time interaction effect was apparent (p = 0.667).

Neither p38 MAPK$^{Thr180/Tyr182}$ (main effect of treatment; p = 0.315, time; p = 0.208, interaction; p = 0.105) or CAMKII^{Thr286} (main effect of treatment; p = 0.595, time; p = 0.751, interaction; p = 0.417) phosphorylation were significantly altered by exercise or feeding (Figure 3.2D & Figure 3.2E).

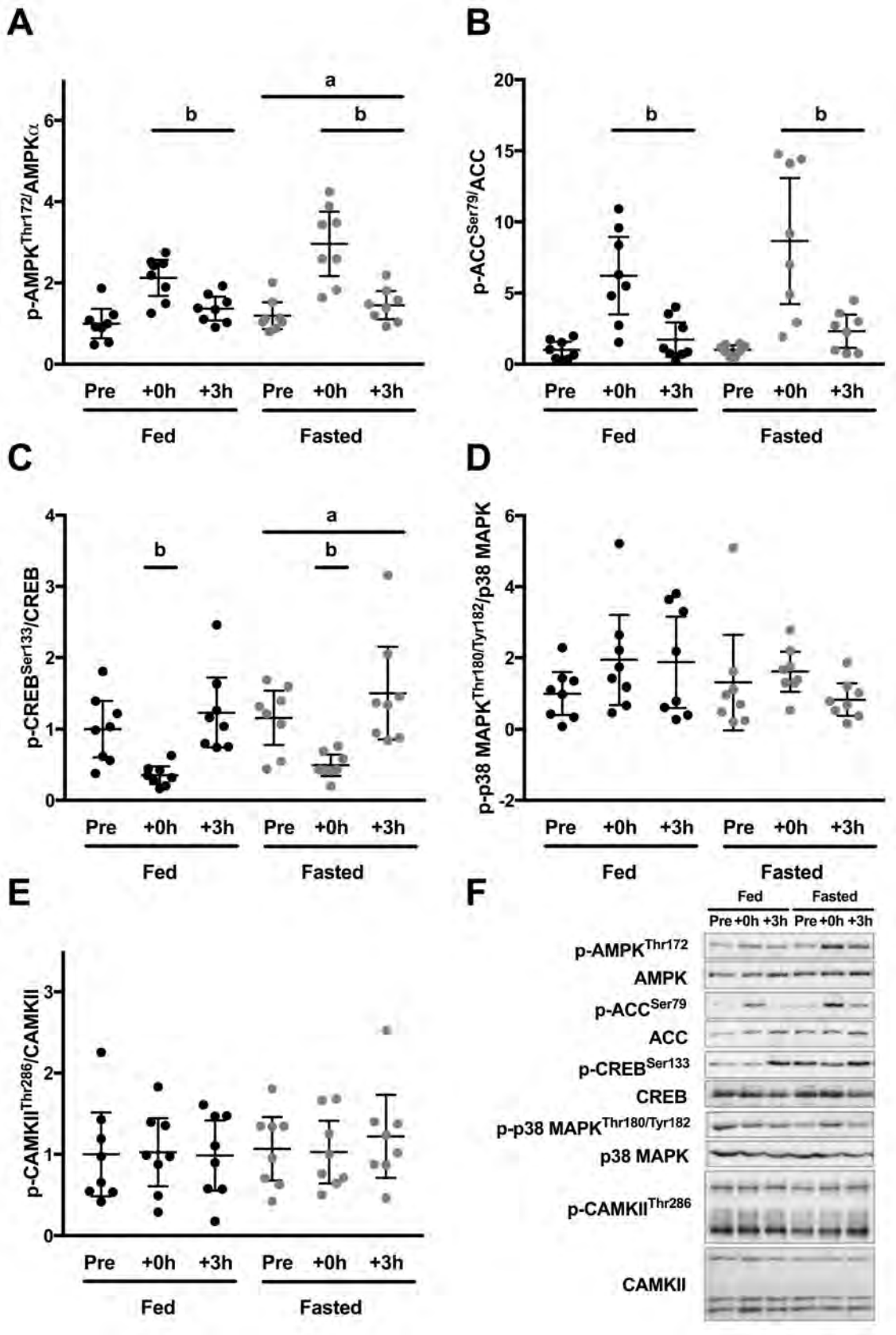

Figure 3.2 Fasting augments the phosphorylation of AMPK^{Thr172} and CREB^{Ser133}

A. *Phosphorylation of AMPK^{Thr172} is increased post-exercise in each trial and is augmented during FAST.* ***B.*** *Phosphorylation of ACC^{Ser79} is increased immediately post-exercise and remains elevated three hours post-exercise in each trial.* ***C.*** *CREB^{Ser133} phosphorylation is augmented during FAST and decreases immediately post-exercise.* ***D.*** *p38 MAPK$^{Thr180/Tyr182}$ and* ***E.*** *CAMKII^{Thr286} remain unchanged throughout the intervention.* ***F.*** *Representative immunoblot images.* ***Pre****: pre-exercise;* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise.* ***a****: main effect of treatment ($p \leq 0.05$);* ***b****: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). All values are presented relative to the group mean for all pre-FED samples. Data presented as means ± 95% confidence intervals (n = 8).*

Global acetylation within skeletal muscle was unaffected by fasting or exercise (main effect of treatment; p = 0.800, time; p = 0.300, interaction; p = 0.166; Figure 3.3A). Furthermore, the acetylation of $p53^{Lys382}$, a SIRT1 deacetylation target (62), was unchanged in response to fasting or exercise (main effect of treatment; p = 0.950, time; p = 0.119, interaction; p = 0.242; Figure 3.3C). In addition, the acetylation of $MnSOD^{Lys122}$, a SIRT3 deacetylation target (57), was unchanged by exercise or fasting as post-hoc analyses revealed no significant differences despite a significant treatment*time interaction effect (main effect of treatment; p = 0.578, time; p = 0.909, interaction; p = 0.024; Figure 3.3D). The protein content of PARP1 (Figure 3.4A), a NAD^+-dependent enzyme that can supresses SIRT1 activity via competition for NAD^+ (3, 38, 48), decreased by ~25% immediately post-exercise (p = 0.029 vs pre-exercise) and remained lower three hours post-exercise (p = 0.034 vs pre-exercise, main effect of time; p = 0.003). There was no effect of fasting on PARP1 protein content (main effect of treatment; p = 0.629, interaction; p = 0.335). Despite a reduction in PARP1 protein content, the content of PARylated PARP1 was unchanged (main effect of treatment; p = 0.490, time; p = 0.776, interaction; p = 0.733; Figure 3.4B).

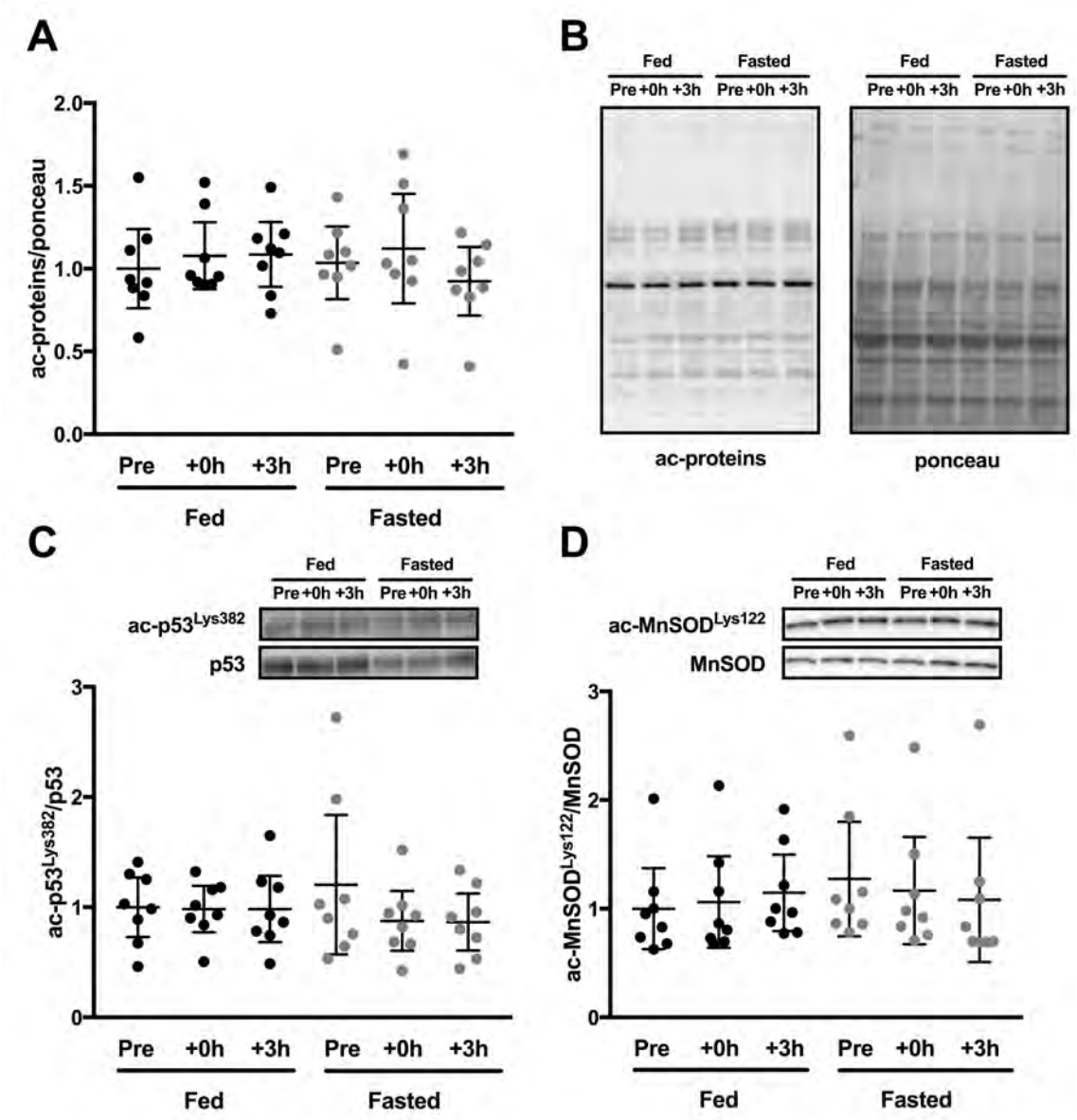

Figure 3.3 Fasting and exercise does not alter sirtuin deacetylase activity following endurance exercise

A. *Global acetylation within skeletal muscle is unaffected by fasting or exercise.* ***B.*** *Representative immunoblot images of global acetylation and Ponceau S stain.* ***C.*** *Acetylation of p53^{Lys382}, a SIRT1 deacetylation site, is unchanged by fasting or endurance exercise.* ***D.*** *Acetylation of MnSOD^{Lys122}, a SIRT3 deacetylation site, is unchanged by fasting or endurance exercise.* ***Pre****: pre-*

*exercise; **+0h**: immediately post-exercise; **+3h**: three hours post-exercise. **b**: main effect of time (significantly different to pre-exercise; p ≤ 0.05). All values are presented relative to the group mean for all pre-FED samples. Data presented as means ± 95% confidence intervals (n = 8).*

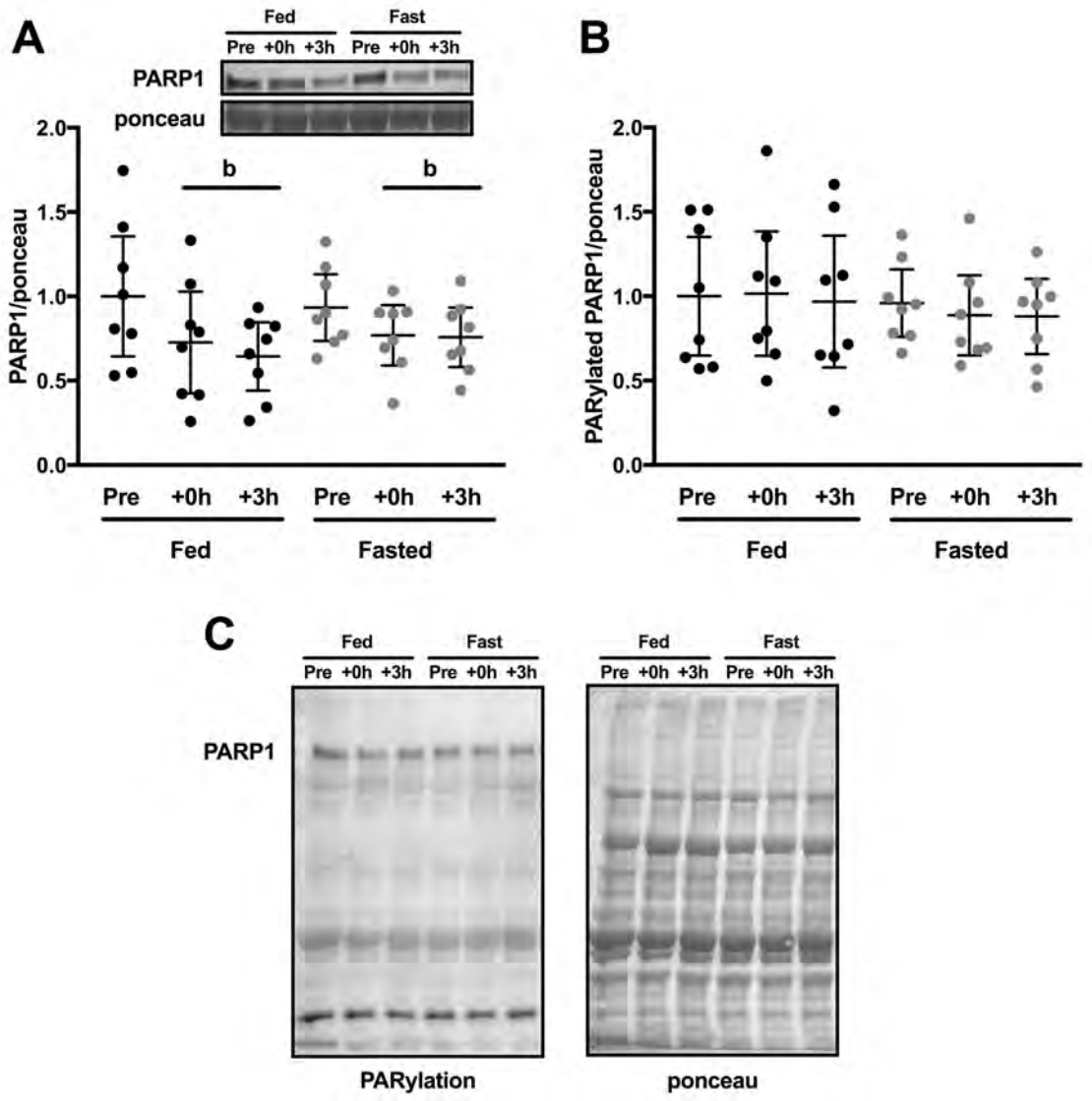

Figure 3.4 Endurance exercise decreases PARP1 protein content, however PARylation is unchanged

***A.** Protein content of PARP1 is decreased immediately post-exercise and remains decreased three hours post-exercise. **B.** Auto-PARylation of PARP1 is*

unaffected by fasting or exercise. ***C.*** *Representative immunoblot images of PARylation and Ponceau S stain.* ***Pre****: pre-exercise;* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise.* ***b****: main effect of time (significantly different to pre-exercise; p ≤ 0.05). All values are presented relative to the group mean for all pre-FED samples. Data presented as means ± 95% confidence intervals (n = 8).*

3.4.1 Metabolic mRNA response

PPARGC1A mRNA expression increased ~10-fold three hours after exercise (main effect of time; $p < 0.000$; $p < 0.001$ vs pre-exercise; Figure 3.5A). *PPARGC1A* mRNA expression was similar in FED and FAST trials (main effect of treatment; $p = 0.863$, interaction; $p = 0.540$).

Expression of *PDK4* increased with time (main effect of time; $p < 0.001$) and was elevated by ~2-3.5-fold throughout the FAST trial (main effect of treatment; $p = 0.001$, Figure 3.5B). There was a trend for a treatment*time interaction effect ($p = 0.083$), whereby at the immediately post-exercise time point *PDK4* expression increased ~2-fold from pre-exercise values only in the FAST trial (FAST immediately post-exercise vs FAST pre-exercise; $p = 0.031$, FED immediately post-exercise vs FED pre-exercise, $p = 0.950$).

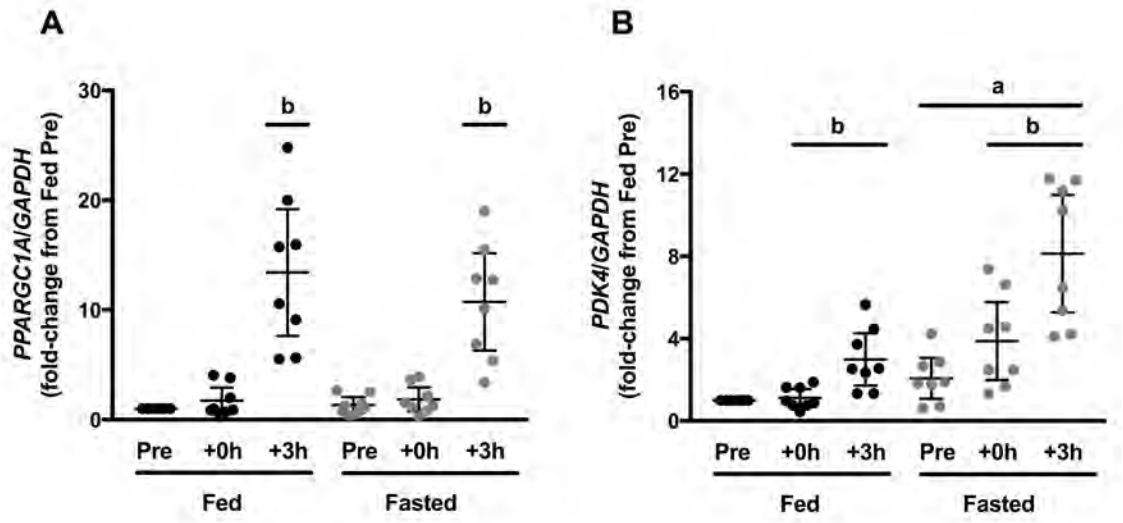

Figure 3.5 Fasting augments *PDK4* mRNA expression, however exercise-induced *PPARGC1A* mRNA expression is similar in fed and fasted states

A. *Resting and exercise-induced PPARGC1A mRNA expression is similar between FED and FAST trials.* ***B.*** *PDK4 mRNA expression is augmented during FAST.* ***Pre****: pre-exercise;* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise.* ***a****: main effect of treatment (p ≤ 0.05);* ***b****: main effect of time (significantly different to pre-exercise; p ≤ 0.05). All values are presented relative to the individual pre-FED value using the* $2^{-\Delta\Delta CQ}$ *method. Data presented as means ± 95% confidence intervals (n = 8).*

3.5 Discussion

Our results build on previous studies (1, 10, 11, 26) to demonstrate that moderate- to high-intensity steady-state endurance exercise (70% W_{max}) performed in the fasted state increases fatty acid availability, augments $AMPK^{Thr172}$ phosphorylation and increases *PDK4* mRNA expression compared to exercise performed following the ingestion of a standard breakfast.

Furthermore, while endurance exercise acutely decreased phosphorylation of $CREB^{Ser133}$, fasting increased the phosphorylation of this activation site. However, despite elevated phosphorylation of AMPK and the transcription factor CREB during fasted exercise, the exercise-induced increases in ACC^{Ser79} phosphorylation, *PPARGC1A* mRNA and whole-body fat oxidation were similar between treatments.

FAST elevated $AMPK^{Thr172}$ phosphorylation throughout the experimental period. Furthermore, a trend (p = 0.065) towards an interaction effect indicates that the exercise-activated $AMPK^{Thr172}$ phosphorylation was likely augmented during fasted exercise. This supports previous research finding elevated AMPKα activity or $AMPK^{Thr172}$ phosphorylation following single-legged moderate-intensity (60% W_{max}) or sprint exercise performed in the fasted state compared to when glucose is consumed (1, 26). However, this is not a universal finding. Similar post-exercise $AMPK^{Thr172}$ phosphorylation following fed and fasted exercise has also been reported (1, 15, 35, 52, 60), while elevated $AMPK^{Thr172}$ phosphorylation following fed exercise can also be apparent (18). It is unclear why these differences in the literature occur, although some of the discrepancies may be due to the amount of carbohydrate ingested in each study. For example, the breakfast provided in the study of Edinburgh *et al* (18), where $AMPK^{Thr172}$ phosphorylationwas augmented in the fed state, included only 65g of carbohydrate. However, the total carbohydrate ingestion in the current study (~130 g) was similar to studies that have demonstrated no effect of fasting on exercise-induced $AMPK^{Thr172}$ phosphorylation (1, 35), although the

pattern of ingestion did differ (i.e. one large dose pre-exercise versus several smaller doses throughout exercise, respectively). Ingestion of a carbohydrate-rich breakfast, albeit one providing more carbohydrate than in the current study, can increase skeletal muscle glycogen content within several hours (9). Thus in the current study, unlike when carbohydrate drinks are provided throughout exercise, exercise may have been commenced with differing levels of skeletal muscle glycogen, which has the potential to influence AMPK phosphorylation and activity within skeletal muscle (5, 34, 43, 55, 70, 72). Furthermore, elevated $AMPK^{Thr172}$ phosphorylation during FAST could be a result of increased allosteric activation by AMP (16, 25, 39, 69). Indeed, skeletal muscle AMP and the AMP/ATP ratio is elevated during exercise performed in the fasted compared to the fed state (35). Nonetheless, it remains inconclusive as to why AMPK phosphorylation is likely enhanced during FAST in the present study.

Consistent with previous literature, fasting and fasted exercise augmented the expression of *PDK4* mRNA (10, 11, 17, 46, 54, 66, 67). However, conversely to continuous glucose ingestion throughout exercise and recovery (10, 11), ingestion of a standard carbohydrate-rich breakfast two-hours prior to exercise did not completely ablate the exercise-induced expression of *PDK4* mRNA. Given that the total carbohydrate and energy intake were similar between this study and those of Civitarese *et al* (10) and Cluberton *et al* (11), this suggests that the timing and frequency of ingestion and/or the type of carbohydrate ingested (i.e. complex starches versus simple sugars) may be important in the regulation of *PDK4* mRNA expression. Substrate availability is known to play an

important role in skeletal muscle *PDK4* mRNA expression (45). In the current study, elevated NEFA availability during FAST may explain activation of *PDK4* mRNA immediately post-exercise (i.e. during exercise) in the FAST trial only. Furthermore, elevating circulating NEFA post-exercise in both trials could explain why *PDK4* mRNA expression is elevated post-exercise following fed exercise in the current study and not when NEFA remains suppressed by continued carbohydrate supplementation (10, 11). Mechanistically, augmented *PDK4* mRNA expression during FAST may be mediated by elevated PPARα activation known to be responsive to exercise, NEFA concentration and energy-stress (40, 44).

Elevated activation of AMPK and PDK4 during fasting has the potential to acutely influence substrate metabolism. AMPK phosphorylates ACC thereby increasing fatty acid oxidation (27), while PDK4 phosphorylates PDH-E1α, inactivating the pyruvate dehydrogenase complex (PDC), and thereby inhibiting the decarboxylation of pyruvate to acetyl-CoA and, thus, carbohydrate oxidation (47). However, substrate utilisation during exercise was similar between FED and FAST, despite elevated NEFA availability during FAST. This is likely due to the intensity of exercise dictating reliance upon carbohydrate oxidation and a sufficient endogenous supply of carbohydrate, i.e. muscle and liver glycogen, in the FAST trial. Indeed, plasma glucose concentration was unaffected by FAST, while phosphorylation of ACC^{Ser79} was also similar between treatments. Despite this, it cannot be ruled out that differences in substrate oxidation may arise if the exercise duration was extended, although this would likely require a reduction in

exercise intensity. Overall this supports findings from a recent meta-analysis in which it was determined that fat oxidation was similar between fed and fasted aerobic exercise performed above 70% $\dot{V}O_{2max}$ (63). Nonetheless, elevated plasma NEFA and glycerol concentrations, despite similar rates of fat oxidation, indicate that increased lipolysis during fasted exercise is determined at the adipose tissue, independently of demand from the exercising musculature. In this respect, fasted exercise upregulates the expression of lipolytic genes adipose triglyceride lipase (*PNPLA2*) and hormone sensitive lipase (*LIPE*) in adipose tissue of overweight males (8).

Metabolic adaptations within skeletal muscle are regulated by transcription factors and co-factors, including CREB and PGC1α. Elevated phosphorylation of $CREB^{Ser133}$, purported to increase the transcriptional activity of CREB (23), has been reported within skeletal muscle in the context of fasting (66) and exercise (19, 49). Thus it was examined here whether an additive or synergistic effect of fasted exercise may be apparent on $CREB^{Ser133}$ phosphorylation. Compared to FED, $CREB^{Ser133}$ phosphorylation was elevated throughout the trial during FAST. Whilst, similarly as reported by Egan *et al* (19), exercise reduced phosphorylation of $CREB^{Ser133}$ immediately post-exercise and tended to increase above baseline levels by three-hours post-exercise. However, this is certainly not a universal finding with increased $CREB^{Ser133}$ phosphorylation (49) or no change in phosphorylation (64, 65) also apparent immediately post-exercise. This variation in results could be due to methodological differences in exercise modality and intensity; $CREB^{Ser133}$ phosphorylation decreases to a

larger extent following cycling at 80% $\dot{V}O_{2peak}$ compared to at 40% $\dot{V}O_{2peak}$ (19). Furthermore, the metabolic stress induced from single-legged exercise (64, 65) and two-legged cycling (19, 49) is likely to be different. Interestingly, during unilateral exercise $CREB^{Ser133}$ phosphorylation can be elevated in the non-exercising contralateral leg whilst remaining unchanged in the exercising leg (64). This suggests that while exercise-induced systemic factors may activate skeletal muscle CREB, local contraction-mediated factors may oppose this and even cause dephosphorylation if the intensity is sufficient (19). Circulating catecholamines, which are elevated during fasting and exercise (15), induce cellular cAMP accumulation and activate PKA (28, 50), the upstream kinase of CREB. CREB can also be phosphorylated by AMPK (59), CAMKII (53) and p38 MAPK (56), however differing time-courses of AMPK and CREB phosphorylation and a lack of activation of CAMKII and p38 MAPK in this study suggest other kinases are responsible for post-exercise $CREB^{Ser133}$ phosphorylation. CREB can be dephosphorylated by calcineurin, PP1, PP2A (21) and PTEN (24), although how dephosphorylation of CREB is regulated within skeletal muscle remains unknown. Calcineurin is activated by Ca^{2+} influx (14) and is therefore an attractive candidate to explain CREB dephosphorylation during exercise. Conversely, the activity of PP1 may decrease during contraction (32). Overall it remains unclear why $CREB^{Ser133}$ phosphorylation may decrease during exercise.

Endurance exercise did not alter the activity of the NAD^+-dependent deacetylase SIRT1, as measured by the acetylation of $p53^{Lys382}$ (62). This is in

contrast to evidence in rodent models of exercise whereby SIRT1-mediated deacetylation of p53 is apparent (42). Furthermore, 12 hours of fasting was insufficient to induce $p53^{Lys382}$ deacetylation. Indeed, 48 hours of fasting only reduces $p53^{Lys382}$ acetylation by ~15% (17), thus it appears that substantial energy stress is required to alter SIRT1 activity and p53 acetylation within human skeletal muscle. Comparatively, neither fasting nor exercise altered SIRT3 activity, as measured by the acetylation of $MnSOD^{Lys122}$ (57). This corroborates previous evidence showing SIRT3 is unaffected by single-legged endurance exercise performed in the fasted state (7). As a NAD^+-consumer, PARP1 activity may be opposing activation of SIRT1 (3, 38, 48). Indeed, PARP1 inhibition augments NAD^+ concentrations, PGC1α deacetylation and mitochondrial biogenesis in response to skeletal muscle contraction (38). Thus, we examined the effects of exercise on PARP1 protein content and PARP activity. One hour of 70% W_{max} cycling decreased PARP1 protein content within skeletal muscle. A decrease in PARP1 protein content following high-intensity interval exercise (5 x 2 minutes at 80% W_{max}) has previously been reported in elderly participants, although high intensity interval exercise actually increased PARP1 protein content in young untrained participants (12). The difference in PARP1 response to high-intensity interval training and moderate- to high-intensity steady state exercise in young participants could reflect differences in the cellular stress between the two protocols. Despite decreased content of PARP1, no change in auto-PARylation of PARP1, a measure of PARP1 activity (58), was apparent following fasting or exercise. Thus PARP1 activity, and

presumably PARP1-mediated NAD^+-consumption, was unaffected by endurance exercise.

Despite elevated phosphorylation of the upstream kinase AMPK and transcription factor CREB, *PPARGC1A* mRNA expression was similar between FED and FAST. This corroborates previous data finding similar *PPARGC1A* mRNA expression following exercise performed in the fasted versus glucose-supplemented state (10, 11). Interestingly, this differs from the glycogen-depleted state, where basal and exercise-induced *PPARGC1A* mRNA expression is augmented (5). This indicates that greater metabolic stress is apparent during glycogen-depleted exercise rather than fasting and fasted-exercise. As *PPARGC1A* mRNA expression is, to a degree, self-regulated (29) these data indicate that PGC1α co-transcriptional activity may be similar when exercise is performed in the fed or fasted states. It is intriguing that this is the case despite elevated AMPK and CREB phosphorylation in the fasted state. It could be speculated that the degree of additional AMPK and CREB phosphorylation in FAST is not large enough to augment *PPARGC1A* mRNA expression. Another explanation may be that PGC1α integrates a large number of signals from many converging pathways and as such differential activation of the AMPK pathway without activation of other pathways, e.g. p38 MAPK, CAMKII and SIRT1, is insufficient to alter PGC1α co-transcriptional activity.

Overall, fasting and fasted exercise augments the phosphorylation of $AMPK^{Thr172}$ and the mRNA expression of *PDK4* within human skeletal muscle.

Furthermore, fasting increases the phosphorylation of $CREB^{Ser133}$, although $CREB^{Ser133}$ phosphorylation is acutely decreased during exercise. However, *PPARGC1A* mRNA expression is similar between fed and fasted skeletal muscle in the resting and exercised states. Thus while fasting increases the systemic availability of NEFAs and the expression of genes associated with fatty acid metabolism, greater energy stress is required to augment the expression of the mitochondrial biogenic regulator *PPARGC1A*.

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3.7 Additional Information

3.7.1 Conflict of interests

The authors declare no conflicts of interest.

3.7.2 Acknowledgements

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4 SEVEN DAYS OF NICOTINAMIDE RIBOSIDE SUPPLEMENTATION DOES NOT INFLUENCE WHOLE-BODY OR SKELETAL MUSCLE METABOLISM IN RECREATIONALLY ACTIVE MALES

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4.1 Abstract

Rodent studies have indicated that oral nicotinamide riboside (NR) supplementation can induce sirtuin (SIRT) signalling and mitochondrial biogenesis in skeletal muscle. Furthermore, the safety of NR supplementation has been demonstrated in humans. This study aimed to assess the effect of NR supplementation on resting and exercise-induced sirtuin signalling and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) mRNA expression, as well as substrate utilisation in humans. In a double-blinded, randomised, counter-balanced, crossover design, eight male participants (age: 23 ± 4 years, $\dot{V}O_{2peak}$: 46.5 ± 4.4 $mL \cdot kg^{-1} \cdot min^{-1}$) received one week of NR or cellulose placebo (PLA) supplementation (1000 $mg \cdot d^{-1}$) before performing one hour of cycling at 60% W_{max}. Muscle biopsies were collected prior to supplementation and pre-, immediately and three-hours post-exercise from the *medial vastus lateralis*, whilst venous blood samples were collected throughout the trial. Global acetylation and acetylation of $p53^{Lys382}$, a SIRT1 target, and $MnSOD^{Lys122}$, a SIRT3 deacetylation was unaffected by NR supplementation or exercise. Furthermore, exercise led to an induction of *PPARGC1A* (~5-fold) and *PDK4* (~10-fold) mRNA expression but NR had no effect on this response. There was also no effect of NR supplementation on substrate utilisation at rest or during exercise or on skeletal muscle mitochondrial respiration. However, NR supplementation blunted the exercise-induced activation of skeletal muscle *NNMT* mRNA expression, whereas the mRNA expression of other enzymes involved in NAD^+ metabolism (*NMRK1*, *NAMPT*, *NMNAT1*) were not significantly affected by NR supplementation or

exercise. In summary, one week of NR supplementation at 1000 $mg \cdot d^{-1}$ does not augment sirtuin signalling, *PPARGC1A* mRNA expression or metabolism at rest or during exercise. However, NR supplementation did impair the exercise-induced activation of *NNMT* mRNA within skeletal muscle.

4.2 Introduction

Nicotinamide adenine dinucleotide (NAD^+), including its reduced form NADH, is a redox co-enzyme that shuttles hydride ions between processes of fuel oxidation, as well as within biosynthethic pathways (40). In addition to central roles in these critical metabolic processes, NAD^+ has emerged as a signalling moiety and an obligatory co-substrate for sirtuins (SIRTs), poly ADP-ribose polymerases (PARPs) and cyclic ADP-ribose synthetases (3). Thus NAD^+ is an important substrate in pathways governing metabolic adaptations, DNA repair and apoptosis, among others (3, 40). Given the regulatory role of NAD^+ in lifespan extending processes, it is unsurprising that strategies to elevate cellular NAD^+ are considered as promising therapies. Indeed, elevating cellular NAD^+ *in vivo* leads to positive outcomes in murine models of diabetes (28, 41), ageing (9, 26, 28), obesity (5), vascular dysfunction (9), muscular dystrophy (29) and Alzheimer's disease (24).

The vitamin B3 molecule nicotinamide riboside (NR) has emerged as one strategy to elevate NAD+ in vivo. In rodents, oral NR supplementation increases fat oxidation (at least during the dark, inactive phase) (5), promotes metabolic flexibility (30), improves insulin sensitivity and may improve endurance performance (5), although a trend towards impaired endurance performance has also been noted (20). Mechanistically, *in vitro* and *in vivo,* NR supplementation increases SIRT1 and SIRT3 activities, deacetylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and induces mitochondrial biogenesis (5, 6, 19, 29). Interestingly, and

somewhat unsurprisingly, the effects of NR supplementation are much more pronounced during models of elevated cellular (and thus NAD^+) stress (5, 6, 14, 19, 29). Furthermore, genetically elevating myocellular NAD^+, via muscle-specific overexpression of nicotinamide phosphoribosyltransferase (NAMPT) in young healthy mice, only augments markers of mitochondrial biogenesis when coupled with endurance exercise training (8, 14). Thus, herein, the effect of NR supplementation on cellular signalling in humans is studied in the context of acute endurance exercise, where fluctuation of NAD^+/NADH and NAD^+-consumption have been reported (38).

Studies investigating NR supplementation in humans are in their infancy (1, 10, 11, 25, 34). Importantly, the safety and bioavailability of NR has been demonstrated in humans (10, 11, 25, 34), with NR supplementation reported to improve blood pressure (25), liver health (10) and physical function in the elderly (10), although the latter is not a consistent finding (25). However, despite promising evidence from pre-clinical models (5, 30), no effect of chronic NR supplementation on insulin sensitivity, body composition, lipolysis, $\dot{V}O_{2peak}$ and resting or exercising substrate utilisation is apparent (11, 25). The effect of NR on skeletal muscle signalling at rest and during exercise remains unstudied.

The purpose of this study was to investigate the effects of oral NR supplementation on mitochondrial biogenic signalling and mRNA expression in resting and exercising human skeletal muscle. Furthermore, the effect of NR supplementation on substrate utilisation at rest and during exercise was also

examined. It was hypothesised that NR supplementation would elevate SIRT1 and SIRT3 signalling and increase the exercise-induced expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) mRNA.

4.3 Methods

4.3.1 Participants

Eight recreationally active males (mean ± SD: age, 23 ± 4 years; body mass, 72.4 ± 5.3 kg; peak oxygen uptake ($\dot{V}O_{2peak}$), 46.5 ± 4.4 $mL \cdot kg^{-1} \cdot min^{-1}$; maximal aerobic power (W_{max}), 224 ± 29 W) were recruited to participate. Participants were fully informed of the study procedures and their right to withdraw before providing written consent to participate. The study was pre-approved by the National Health Service Research Ethics Committee, Black Country, West Midlands, UK (17/WM/0321).

4.3.2 Experimental overview

Participants attended the laboratory on five occasions. Prior to the experimental periods, participants attended the laboratory for a pre-testing visit to determine $\dot{V}O_{2peak}$ and W_{max}. The experimental period then consisted of two identical experimental blocks in which participants visited the laboratory before and after a seven-day supplementation period. During the supplementation period participants received either 1000 $mg \cdot d^{-1}$ nicotinamide riboside (NR; Niagen, ChromaDex, Irvine CA, USA) or 1000 $mg \cdot d^{-1}$ of a cellulose placebo (PLA) in a

double-blinded, randomised, counter-balanced, crossover design. Supplements were consumed twice daily such that participants were instructed to consume 500 mg of supplement at ~9 am and ~9 pm each day. A two-week washout period was employed between experimental blocks.

4.3.3 Pre-testing

After measuring height (Seca 220, Seca, Birmingham, UK) and body mass (Champ II, OHAUS, Griefensee, Switzerland) participants performed a graded exercise test to exhaustion on a cycle ergometer (Lode Excalibur, Groningen, Netherlands). The test began at 50 W with power increasing by 25 W every three minutes thereafter. Respiratory variables were measured continuously during exercise using a breath-by-breath metabolic cart (Vyntus CPX, Jaeger, CareFusion, Germany), heart rate was monitored throughout (RCX5, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE) were determined using a 6-20 Borg scale during the final 15 seconds of each 3-minute stage (4). $\dot{V}O_{2peak}$ was determined as the highest rolling 30-second average and was stated as being achieved if the following criteria were met: i) heart rate within 10 beats·min^{-1} of age-predicted maximum and ii) respiratory exchange ratio (RER) > 1.1, or iii) plateau of oxygen consumption despite increasing work-rate. W_{max} was determined as work rate at the last completed stage plus the fraction of time spent in the final non-completed stage multiplied by the increment in work rate (25W).

4.3.4 Experimental trials

Participants refrained from alcohol for 72 h, caffeine for 24 h and exercise for 48 h prior to each experimental trial. For 72 h prior to each experimental trial participants consumed a replicated diet. For the first 48 h of this period participants consumed a diet that replicated their *ad libitum* intake recorded via a weighed food diary prior to the first experimental visit. For the final 24 h prior to each experimental visit participants were provided with a standardised diet (energy: 2271 kcal; macronutrient composition: 63% carbohydrate, 21% fat and 16% protein).

For the pre-supplementation visit, participants arrived at the laboratory at ~8:30 am following an ~12-hour overnight fast. Upon arrival, participants rested in the supine position for approximately five minutes before a venous blood sample was collected via venepuncture from an antecubital forearm vein. A resting skeletal muscle biopsy was then taken from the *medial vastus lateralis*. Participants then consumed the first 500 mg dose of their supplement prior to leaving the laboratory.

For the post-supplementation visit, participants arrived at the laboratory at ~7:30 am following an ~12-hour overnight fast. Participants rested in the supine position for ten minutes prior to a 20-minute measurement of resting metabolic rate under a ventilated hood using the GEMNutrition indirect calorimeter (GEMNutrition, Daresbury, UK). A cannula was then inserted into an antecubital forearm vein and a baseline venous blood sample was collected prior to

providing a pre-exercise skeletal muscle biopsy from the *medial vastus lateralis*. Participants then cycled for one-hour at 60% W_{max} before a second skeletal muscle biopsy was taken immediately post-exercise (completed within two minutes of exercise cessation). Participants then rested in a supine position prior to a third skeletal muscle biopsy being obtained three-hours post-exercise. A new incision was made for each biopsy at least 2 cm from the previous site. Venous blood was collected throughout rest periods and during exercise. Respiratory variables were measured pre-exercise and at 15-minute intervals throughout exercise, heart rate was monitored continuously throughout exercise and RPE was determined at 15-minute intervals throughout exercise. Carbohydrate and fat oxidation were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using the moderate-high exercise intensities equation of Jeukendrup and Wallis (18) during exercise and Frayn (13) at rest. Participants were allowed to drink water *ad libitum* during rest and exercise periods during the visit following the first supplementation period, with water intake during the visit following the second supplementation period matched to the first.

4.3.5 Muscle biopsies

Muscle biopsies were obtained from separate incision sites on the *medial vastus lateralis* under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a Bergström needle adapted with suction. Muscle was rapidly blotted to remove excess blood and was immediately flash frozen in liquid nitrogen. In the case of pre-supplementation and pre-exercise biopsies, an ~20 mg section was removed prior to freezing and placed in ice-cold BIOPS buffer

(2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM DTT, and 50 mM MES) for the immediate measurement of mitochondrial respiration. Frozen muscle was powdered using a Cellcrusher tissue pulverizer on dry ice and stored at -80°C prior to analysis.

4.3.6 High-resolution respirometry

Skeletal muscle fibres were mechanically separated under a light microscope and permeabilised by incubation in BIOPS buffer containing 50 $mg \cdot ml^{-1}$ of saponin for 30 minutes followed by a 15-minute wash in MiR05 buffer (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 $g \cdot L^{-1}$ fatty acid-free bovine serum albumin). Samples were then weighed and analysed in duplicate using an Oroboros O2K (Oroboros Instruments, Innsbruck, Austria). When substantial variability was apparent between duplicates a third sample was run. Data was collected at 37°C in hyperoxygenated (200-400 mM) conditions in MiR05 buffer. The substrate-uncoupler-inhibitor titration performed was as follows: 5 mM pyruvate, 2 mM malate, and 10 mM glutamate was added to measure leak respiration through complex one (CI_L); 5 mM ADP was then added to measure coupled oxidative phosphorylation through complex one (CI_P); 10 mM succinate was then added to measure coupled oxidative phosphorylation through complexes one and two ($CI+II_P$); 10 µM cytochrome-c was added to test outer mitochondrial membrane integrity; titrations of 0.5 µM FCCP until maximal

respiration were then added to measure maximal electron transport chain capacity (CI+II_E); 5 µM antimycin A was then added to measure non-mitochondrial respiration. Respiration was normalised to tissue masses and non-mitochondrial respiration was subtracted to give mass-specific mitochondrial respiration. In all samples the increase in respiration following addition of cytochrome-c was less than 10%, indicating preserved mitochondrial membrane integrity.

4.3.7 Immunoblotting

Tissue was homogenized in a 10-fold mass excess of ice-cold sucrose lysis buffer (50 mM tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M triton-X, 25 mM β-glycerophosphate, 1 µM trichostatin A, 10 mM nicatinamide, 1mM 1,4-dithiothreitol, 1% phosphatase inhibitor Cocktail 2; Sigma, 1% phosphatase inhibitor cocktail 2; Sigma, 4.8% cOmplete mini protease inhibitor cocktail; Roche) using an IKA T10 basic ULTRA-TURRAX homogeniser (IKA, Oxford, UK) followed by shaking at 4°C for 30 minutes and centrifuging at 4°C and 8000 g for 10 minutes to remove insoluble material. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, California, USA). Samples were prepared in laemmli sample buffer, boiled at 97°C for 5 min (with the exception of an aliquot set aside for determination of electron transport chain protein content which remained unboiled) and an equal volume of protein (18-36 µg) was separated by SDS-PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel.

Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour before being incubated in primary antibody overnight at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence HRP substrate detection kit (Millipore, Watford, UK). Imaging and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK).

4.3.8 Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBST unless otherwise stated. Antibodies: acetylated proteins (ab193), ac-MnSOD^{K122} (ab214675) and OXPHOS cocktail (ab110411) were purchased from abcam; AMP-activated protein kinase alpha (AMPKα; 2603), p-AMPK^{Thr172} (2535), p-ACC^{Ser79} (3661), calmodulin dependent kinase II (CAMKII; 3362), p-CAMKII^{Thr268} (12716), cAMP response element binding protein (CREB; 1°: 1:500; 9197), p-CREB^{Ser133} (1°: 1:500; 9191), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; 2118), p38 mitogen activated protein kinase (p38 MAPK; 9212), p-p38 MAPK$^{Thr180/Tyr182}$ (4511), poly ADP-ribose polymerase 1 (PARP1; 1°: 1:500; 9542), tumour protein 53 (p53; 2°: 1:2000; 2527) and ac-

$p53^{K382}$ (1°: 1:500 in 3% BSA, 2°: 1:2000; 2570) were purchased from Cell Signaling Technology; acetyl CoA carboxylase (ACC; 05-1098), superoxide dismutase (MnSOD; 1°: 1:2000; 06-984) and PGC1α (ab3242) and poly-ADP-ribose (PAR; 1°: 1:500; MABE1031) were purchased from Merck Millipore. Secondary antibodies were used at a concentration of 1:10000 in TBST unless otherwise stated. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from Cell Signaling Technology.

4.3.9 Real time RT-qPCR

RNA was extracted from ~20 mg of muscle by homogenising in 1 mL of Tri reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-TURRAX homogeniser (IKA, Oxford, UK). Phase separation was achieved by addition of 200 µL of chloroform and centrifugation at 12000 g for 15 minutes. The RNA-containing supernatant was removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturers instructions, which includes a DNase treatment step. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). RNA was diluted to 30 $ng \cdot \mu L^{-1}$ and reverse transcribed to cDNA in 20 µL volumes using the nanoScript 2 RT kit and oligo(dT) primers (Primerdesign, Southampton, UK) as per the manufacturers instructions. RT-qPCR analysis of mRNA content was performed in triplicate by using Primerdesign custom designed primers (Table 4.1) and commercially available *ACTB*, *B2M* and

GAPDH, (Primerdesign) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, UK). The qPCR reaction was run as per the manufacturers instructions (Primerdesign) and followed by a melt curve (Applied Biosystems) to ascertain specificity. 2-20 ng of cDNA was added to each well in a 20 µL reaction volume. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression is expressed relative to the expression in the pre-exercise sample during FED for each individual using the $2^{-\Delta\Delta CQ}$ method (23) with the geometric mean of C_q values for *ACTB*, *B2M* and *GAPDH* used as an internal control (36). Optimal stability of housekeeper genes was determined using RefFinder (39). Statistical analyses were performed on the ΔΔCQ data.

4.3.1 Blood analyses

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA; BD, Oxford, UK) for the collection of plasma. Samples were placed immediately upon ice prior to centrifugation at 1600 g at 4°C for 10 minutes before collection of plasma from the supernatant. Plasma was frozen at -80°C until further analysis. Plasma samples were subsequently analysed on an autoanalyser (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for glucose, lactate, non-esterified fatty acid (NEFA) and glycerol (Randox Laboratories, County Antrim, UK) using commercially available kits.

Table 4.1 qPCR primer sequences

Gene name	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
NADSYN1	NM_018161	CCAAAAACAGAGGAGCAAGATAC	GGTGTCCGACTCGTAATAATGAT
NAMPT	NM_005746	TTCCCACTACTCCAGCCTAAG	TTTGTGTAAAGGGCAGGTTAATAAA
NAPRT	NM_145201.5	CAGTGAGGTGAATGTCATTGGC	AGGCAACGTCTGCTTCTCG
NMNAT1	NM_022787	AGTCCTTTGCTGTTCCCAATT	AGCACATCCGATTCATAGATAAAC
NMNAT2	NM_015039	ATTGCTGTCTTGTGCTTTGTG	CGTAGCTGGTACTAGATTTTGATAAA
NMNAT3	NM_178177	AGCCTAGATCCTGCCATGAA	GGAGAGATGATACCCTGGATGA
NMRK1	NM_017881.2	GCCAGAGTCTGAGATAGAGACAG	TCCTGGTCTGTTGATACCACAG
NNMT	NM_006169.2	TGCTGTTAGCCTGAGACTCAG	GAGGTGAAGCCTGATTCCATTATG
PDK4	NM_002612.3	GAGGGACACTCAGGACACTTTAC	TGGAGGAAACAAGGGTTCACAC
PPARGC1A	NM_013261	TTGCTAAACGACTCCGAGAAC	GACCCAAACATCATACCCCAAT

4.3.2 Statistics

Two-way repeated measures ANOVAs assessed effects of time, treatment and time*treatment interaction effects for all time-course data. Ryan-Holm-Bonferroni multiple comparison corrections were applied *post-hoc* where applicable. Differences in means for resting and exercising $\dot{V}O_2$, $\dot{V}CO_2$,

respiratory exchange ratio (RER), substrate utilisation, heart rate and RPE were assessed using repeated-measures t-tests. All statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0. Data are presented as means with 95% confidence intervals. Statistical significance was accepted as $p \leq 0.05$.

4.4 Results

4.4.1 Substrate utilisation and systemic availability

Seven days of NR supplementation did not influence resting metabolic rate (PLA: 1859 ± 202 vs NR: 1772 ± 211 kcal·d^{-1}; p = 0.486). Furthermore, substrate utilisation at rest was similar following supplementation of NR or PLA (carbohydrate oxidation: PLA: 0.09 ± 0.04 vs NR: 0.11 ± 0.03 g·min^{-1}; p = 0.446, fat oxidation: PLA: 0.10 ± 0.03 vs NR: 0.09 ± 0.02 g·min^{-1}; p = 0.395, RER: PLA: 0.79 ± 0.04 vs NR: 0.80 ± 0.03; p = 0.563). Carbohydrate and fat oxidation during exercise were also similar between trials (Table 4.2). $\dot{V}O_2$, $\dot{V}CO_2$, RER, heart rate and RPE did not differ between trials during exercise (Table 4.2).

There was no effect of NR on resting or exercising plasma NEFA, glycerol, glucose or lactate (Figure 4.1). Plasma NEFA concentration initially decreased during the first 15 minutes of exercise before returning to pre-exercise values for the remainder of the exercise bout (main effect of treatment; p = 0.891, time; p < 0.001, interaction; p = 0.296). Following exercise (80 minutes) plasma NEFA concentration increased and remained elevated above pre-exercise values from 120 minutes until the end of the trial (240 minutes). Plasma glycerol

concentration increased during exercise and remained elevated above pre-exercise values for the remainder of the trial (main effect of treatment; p = 0.106, time; p < 0.001, interaction; p = 0.720). Plasma glucose was marginally, although significantly, decreased from pre-exercise values at two hours after the cessation of exercise (main effect of treatment; p = 0.175, time; p = 0.010, interaction; p = 0.174). Plasma lactate increased during exercise and remained elevated for the first 20 minutes of recovery (main effect of treatment; p = 0.192, time; p = 0.001, interaction; p = 0.585).

Table 4.2 Physiological responses to 60% W_{max} cycling following supplementation of PLA and NR

		Time (minutes into exercise)					
		15	30	45	60	Mean	*p*
Gas exchange							
$\dot{V}O_2$ (L·min^{-1})	PLA	2.26 ± 0.25	2.28 ± 0.27	2.35 ± 0.27	2.38 ± 0.28	2.31 ± 0.68	
	NR	2.23 ± 0.24	2.27 ± 0.24	2.28 ± 0.26	2.38 ± 0.23	2.29 ± 0.23	0.702
$\dot{V}CO_2$ (L·min^{-1})	PLA	2.11 ± 0.20	2.10 ± 0.24	2.12 ± 0.23	2.13 ± 0.24	2.11 ± 0.22	
	NR	2.09 ± 0.23	2.10 ± 0.23	2.08 ± 0.24	2.17 ± 0.23	2.11 ± 0.23	0.945
RER	PLA	0.94 ± 0.04	0.92 ± 0.04	0.90 ± 0.03	0.90 ± 0.02	0.92 ± 0.03	
	NR	0.94 ± 0.02	0.93 ± 0.02	0.91 ± 0.01	0.91 ± 0.02	0.92 ± 0.01	0.645
Oxidation rates							
Carbohydrate (g·min^{-1})	PLA	2.22 ± 0.26	2.09 ± 0.36	1.96 ± 0.30	1.93 ± 0.26	2.05 ± 0.28	
	NR	2.20 ± 0.27	2.12 ± 0.28	2.00 ± 0.25	2.08 ± 0.34	2.10 ± 0.22	0.720
Fat (g·min^{-1})	PLA	0.25 ± 0.12	0.30 ± 0.14	0.38 ± 0.14	0.41 ± 0.12	0.33 ± 0.13	
	NR	0.22 ± 0.08	0.27 ± 0.07	0.33 ± 0.07	0.35 ± 0.10	0.29 ± 0.06	0.356
Intensity							
Heart rate (beats·min^{-1})	PLA	153 ± 7	160 ± 7	165 ± 8	169 ± 9	162 ± 7	
	NR	154 ± 10	163 ± 9	168 ± 9	172 ± 9	164 ± 9	0.179
RPE	PLA	11 ± 1	13 ± 2	14 ± 2	15 ± 2	13 ± 1	
	NR	11 ± 1	13 ± 1	14 ± 1	15 ± 2	13 ± 1	0.952

Data presented as means ± 95% confidence intervals (n = 8). Mean values represent the mean of the recorded values during exercise. *p* values represent repeated-measures t-test comparisons between exercising means for PLA and NR.

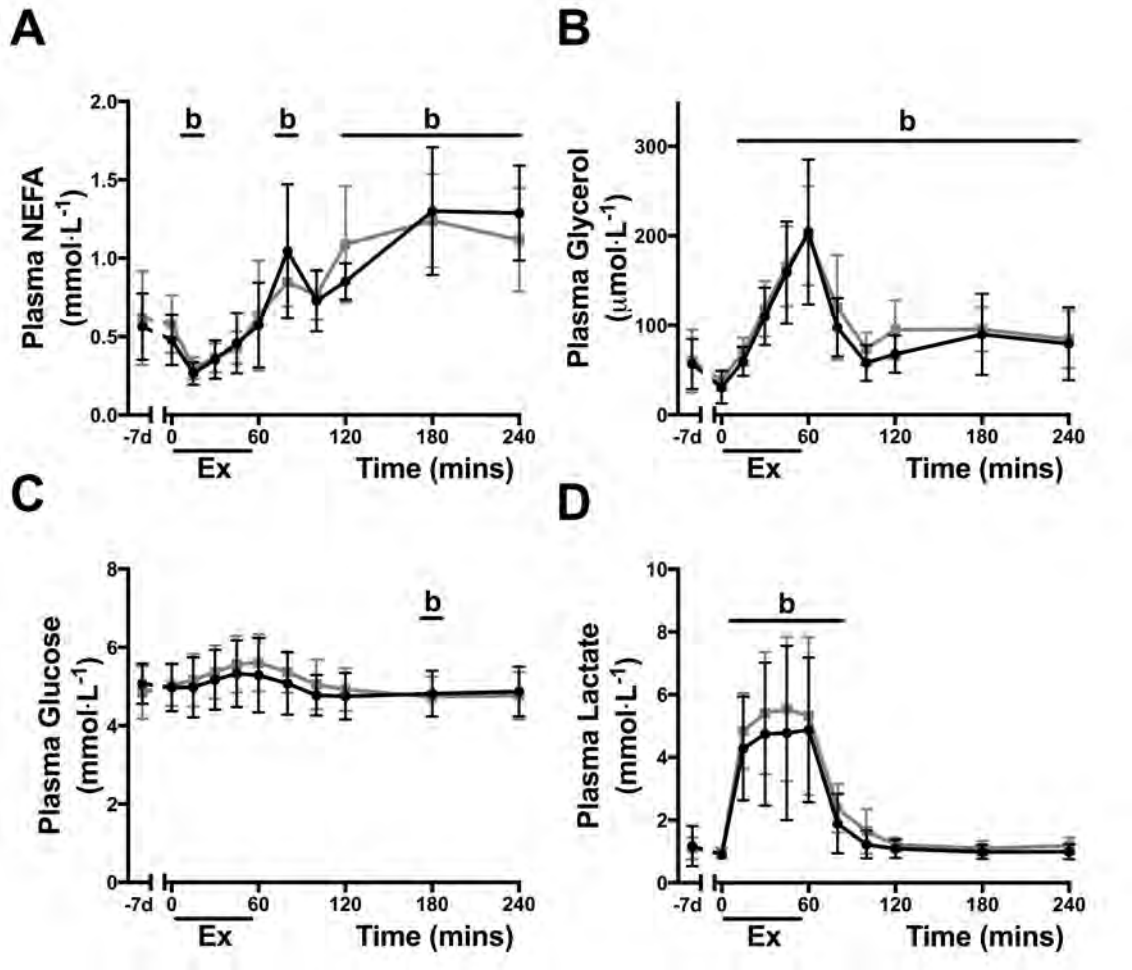

Figure 4.1 NR supplementation does not alter plasma NEFA, glycerol, glucose or lactate at rest or during exercise

*Time-course for plasma NEFA (**A.**), glycerol (**B.**), glucose (**C.**) and lactate (**D**.) in PLA (black) and NR (grey). **b**: main effect of time (significantly different to pre-exercise; p ≤ 0.05. Data presented as means ± 95% confidence intervals (n = 8).*

4.4.2 Skeletal muscle mitochondrial function and protein content

Rates of mitochondrial respiration were similar to those previously reported (15, 16). There were no changes observed in CI_L (main effect of treatment; p = 0.319, time; p = 0.833, interaction; p = 0.588), CI_P (main effect of treatment; p = 0.979, time; p = 0.388, interaction; p = 0.551), $CI+II_P$ (main effect of treatment; p = 0.612, time; p = 0.216, interaction; p = 0.993) or $CI+II_E$ (main effect of treatment; p = 0.657, time; p = 0.190, interaction; p = 0.621) respiration following supplementation of NR or PLA (Figure 4.2A). Furthermore, the content of proteins within each of the five electron transport chain complexes were unchanged following NR or PLA supplementation (Figure 4.2B; $p > 0.05$)

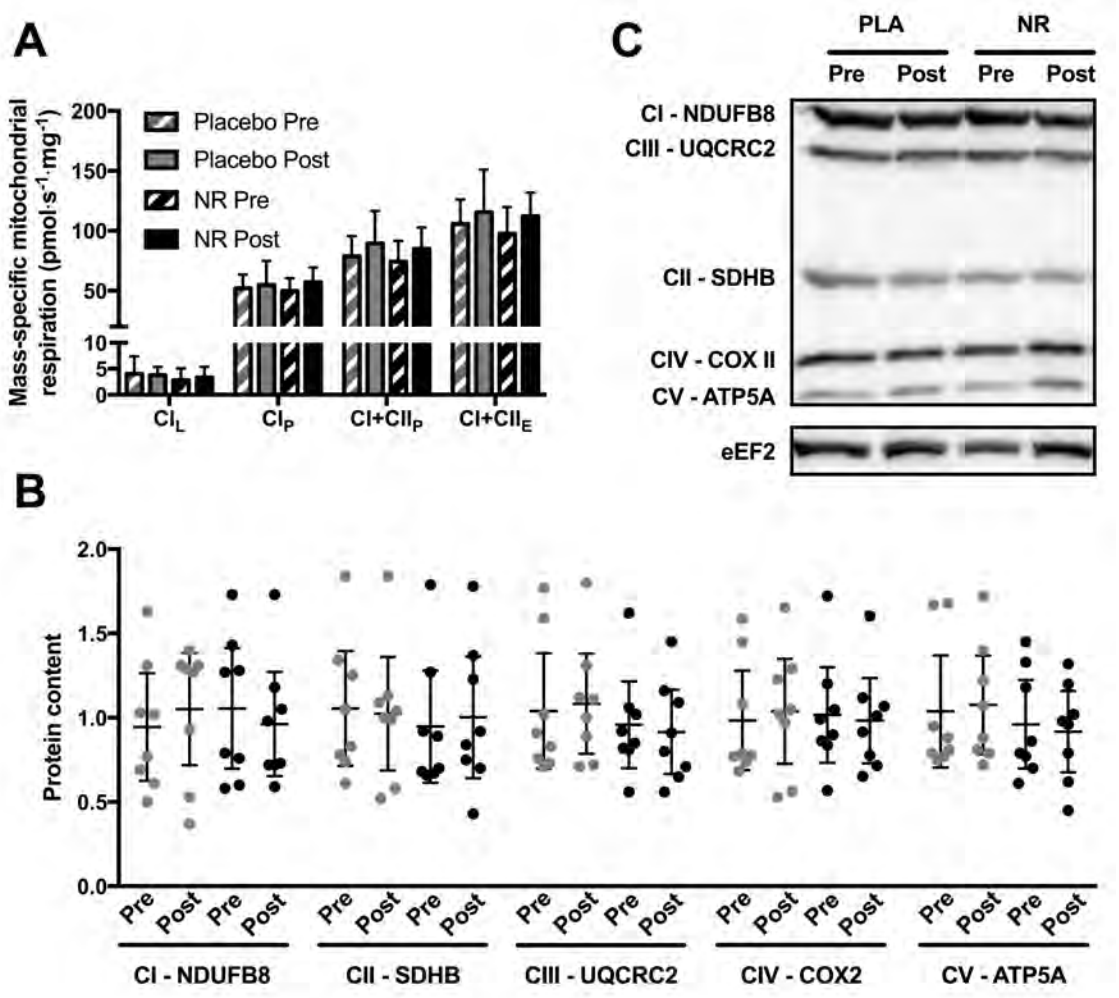

Figure 4.2 Seven days of NR supplementation does not induce mitochondrial biogenesis in skeletal muscle

A. *There were no changes in the mass-specific mitochondrial leak respiration through complex I (CI_L), coupled respiration through complex I (CI_P), coupled respiration through complexes I and II ($CI+II_P$), or maximal electron transport chain capacity ($CI+II_E$) following seven days of NR supplementation ($p > 0.05$).* ***B.*** *Similar content of proteins within the five electron transport chain complexes pre- and post-supplementation of PLA (grey) or NR (black) ($p > 0.05$).* ***C.*** *Representative immunoblot images. Data presented as means ± 95% confidence intervals ($n = 8$).*

4.4.1 Skeletal muscle signalling

Global acetylation within skeletal muscle was unaffected by NR supplementation or exercise (Figure 4.3A; main effect of treatment; p = 0.845, time; p = 0.120, interaction; p = 0.106). Furthermore, the acetylation of $p53^{Lys382}$, a SIRT1 deacetylation target (37), and $MnSOD^{K122}$, a SIRT3 deacetylation target (32), were unchanged throughout the intervention (Figure 4.3C & D; $p53^{Lys382}$: main effect of treatment; p = 0.723, time; p = 0.786, interaction; p = 0.354, $MnSOD^{K122}$: main effect of treatment; p = 0.324, time; p = 0.409, interaction; p = 0.332). The protein content of PARP1 was unaffected by NR supplementation as post-hoc analyses revealed no significant difference despite a significant treatment*time interaction effect (main effect of treatment; p = 0.498, time; p = 0.520, interaction; p = 0.040; Figure 4.4A). Auto-PARylation of PARP1 was also unchanged by NR or exercise (main effect of treatment; p = 0.512, time; p = 0.255, interaction; p = 0.115; Figure 4.4B).

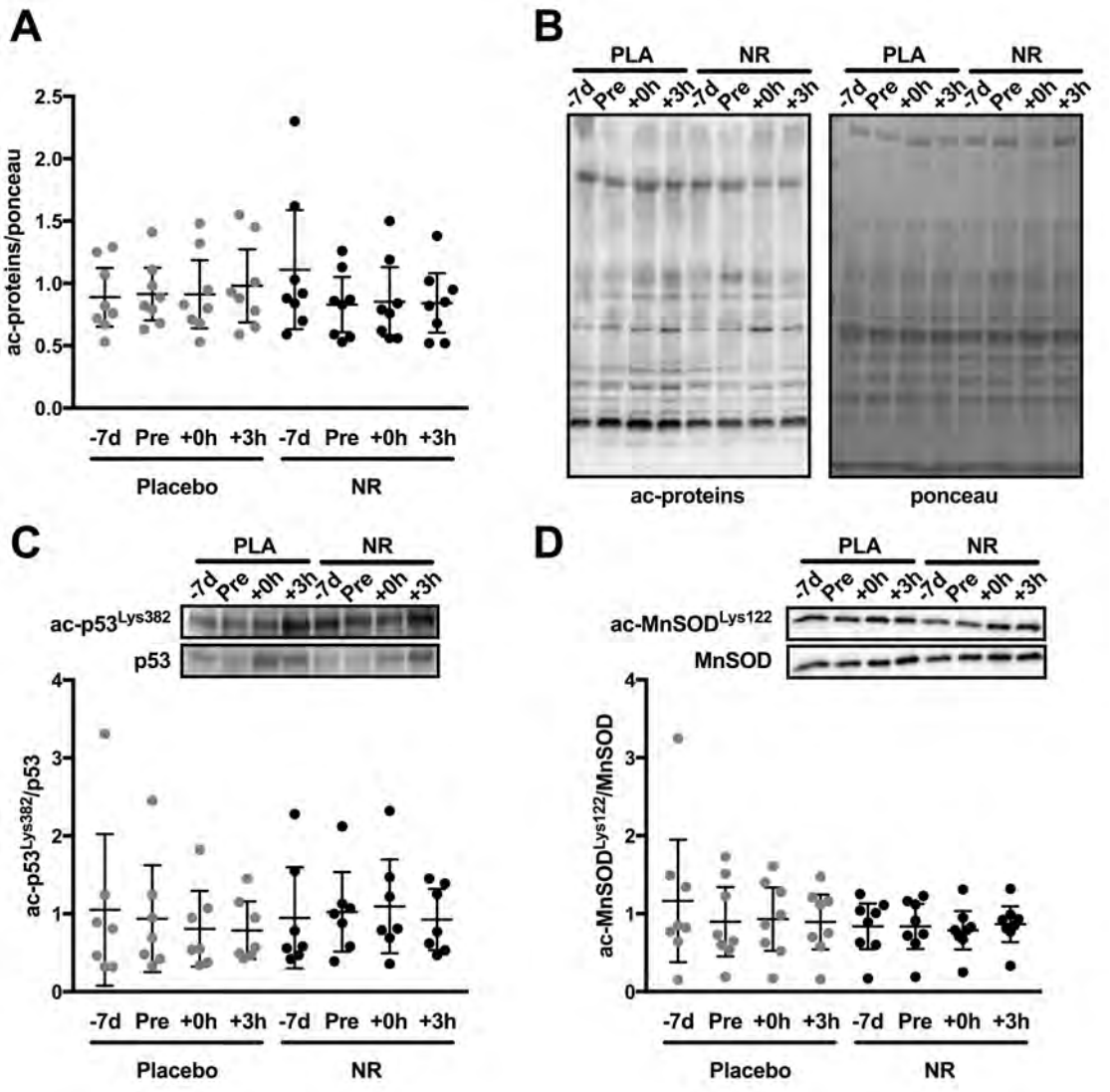

Figure 4.3 Seven days NR supplementation does not influence sirtuin deacetylase activity at rest or following endurance exercise

A. *Global acetylation within skeletal muscle is unaffected by NR supplementation or exercise (n = 8; p > 0.05).* ***B.*** *Representative immunoblot images of global acetylation and Ponceau S stain.* ***C.*** *Acetylation of p53^{Lys382}, a SIRT1 deacetylation site, is unchanged by NR supplementation at rest or following endurance exercise (n = 7; p > 0.05).* ***D.*** *Acetylation of MnSOD^{Lys122}, a SIRT3 deacetylation site, is unchanged by NR supplementation at rest or*

following endurance exercise (n = 8; p > 0.05). ***-7d****: pre-supplementation;* ***Pre****: pre-exercise (post-supplementation);* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise. All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals.*

Exercise increased the phosphorylation of AMPK^{Thr172} (Figure 4.5A, main effect of time; p = 0.002) by ~1.6-fold immediately post-exercise (p = 0.031 vs pre-exercise). There was no effect of treatment (p = 0.216) or a treatment*time interaction effect (p = 0.472). Phosphorylation of ACC^{Ser79} (Figure 4.5B) increased ~4-fold immediately post-exercise (p < 0.001 vs pre-exercise) and remained ~1.4-fold elevated 3-h post-exercise (p = 0.013 vs pre-exercise, main effect of time; p < 0.001). CREB^{Ser133} phosphorylation was unaffected by exercise or NR (main effect of treatment; p = 0.651, time; p = 0.462, interaction; p = 0.810; Figure 4.5C). p38 MAPK$^{Thr180/Tyr182}$ phosphorylation was not significantly affected by exercise or NR (Figure 4.5D), as post-hoc analyses revealed no significant differences despite a treatment*time interaction effect (main effect of treatment; p = 0.124, time; p = 0.942, interaction; p = 0.034). CAMKII^{Thr286} phosphorylation was not altered by exercise or NR (main effect of treatment; p = 0.574, time; p = 0.177, interaction; p = 0.236; Figure 4.5E).

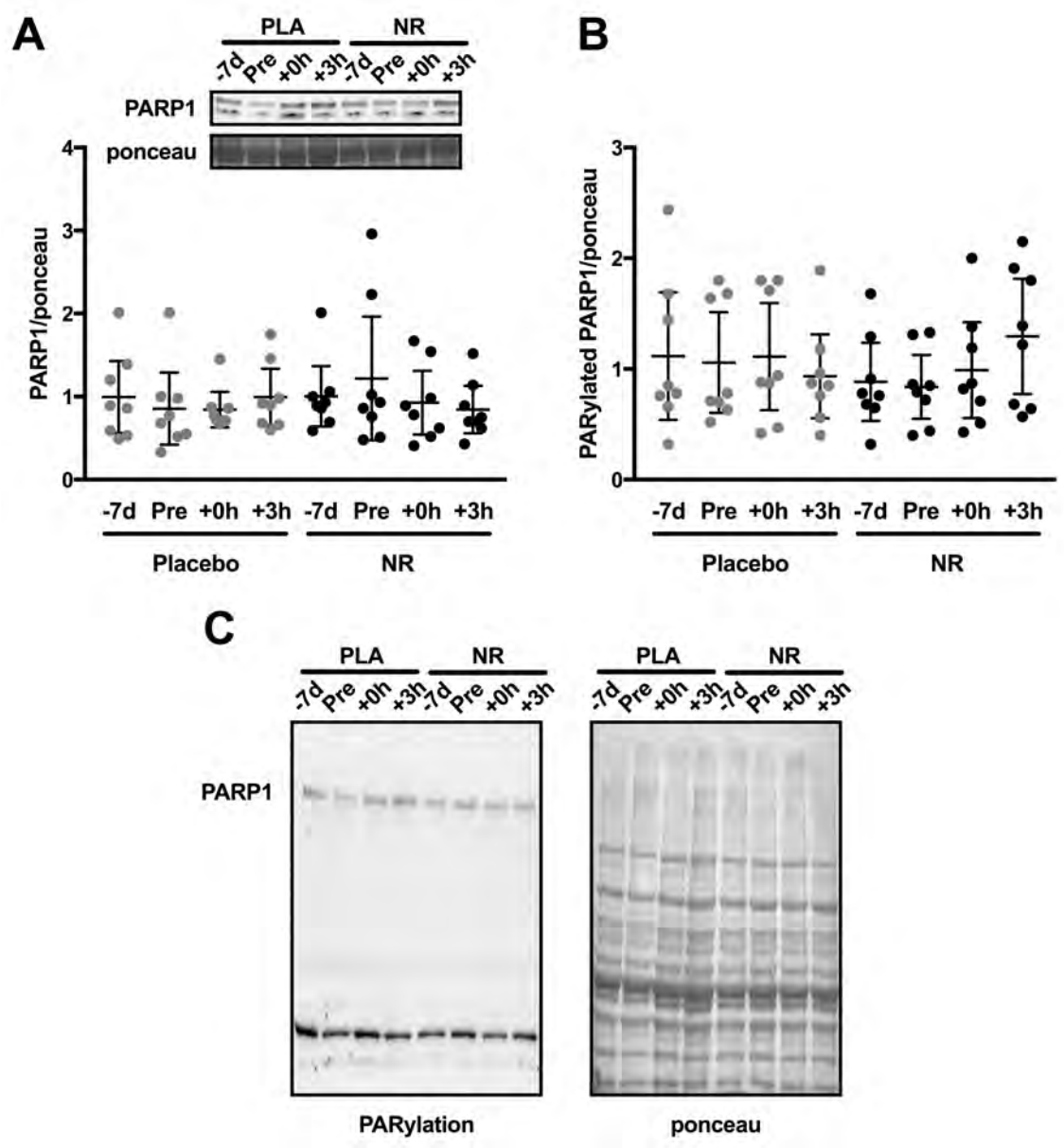

Figure 4.4 Seven days of NR supplementation does not influence PARP1 protein content or PARylation

*PARP1 protein content (**A.**) and auto-PARylation of PARP1 (**B.**) are unaffected by NR supplementation or exercise ($p < 0.05$). **C.** Representative immunoblot images of PARylation and Ponceau S stain. **-7d**: pre-supplementation; **Pre**: pre-exercise; **+0h**: immediately post-exercise; **+3h**: three hours post-exercise. All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals (n = 8).*

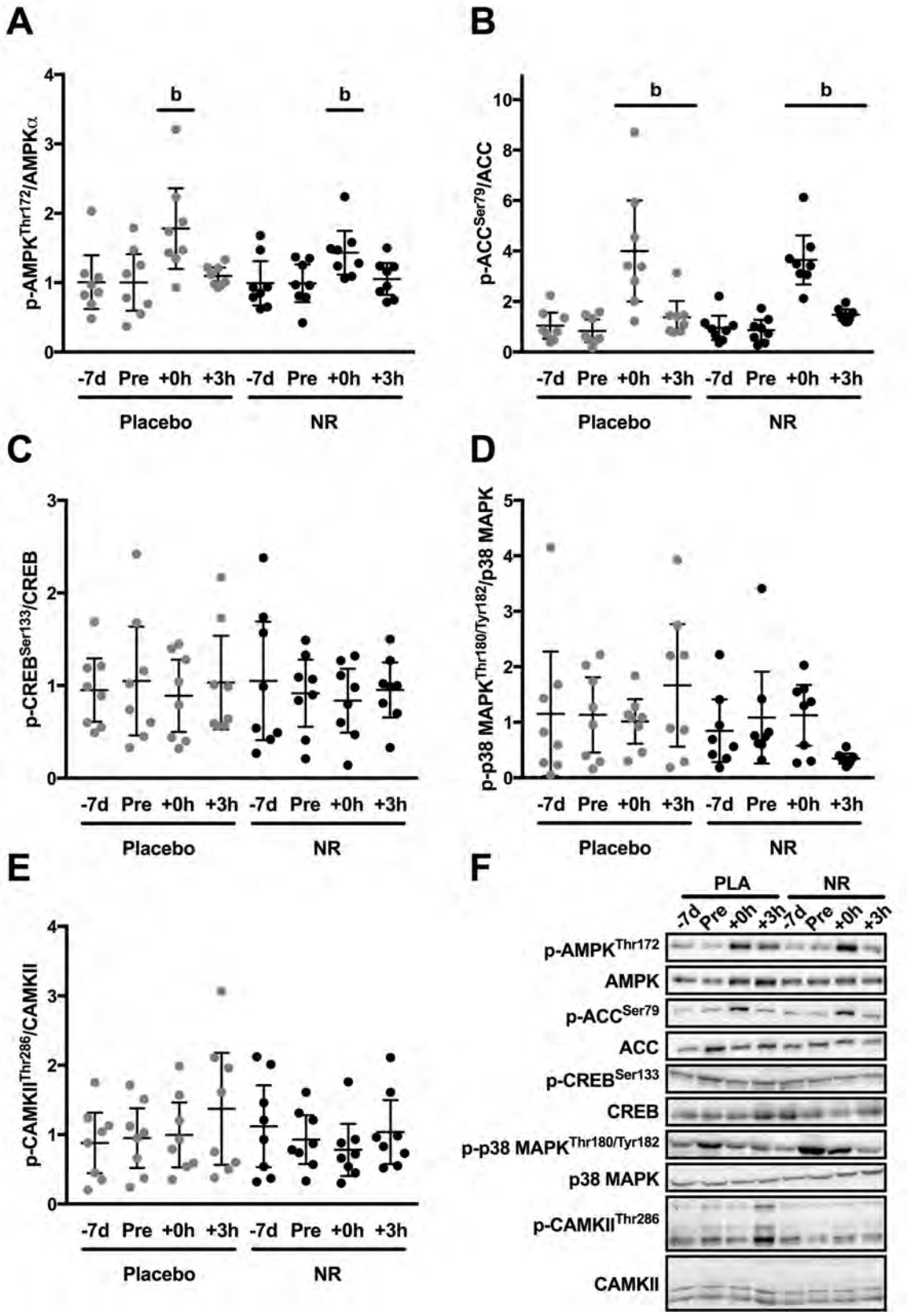

Figure 4.5 Activation of exercise-sensitive signalling pathways following NR supplementation and endurance exercise

A. *Phosphorylation of AMPK^{Thr172} is increased immediately post-exercise in each trial.* ***B.*** *Phosphorylation of ACC^{Ser79} is increased immediately post-exercise and remains elevated three hours post-exercise in each trial.* ***C.*** *CREB^{Ser133},* ***D.*** *p38 MAPK$^{Thr180/Tyr182}$ and* ***E.*** *CAMKII^{Thr286} remain unchanged throughout the intervention.* ***F.*** *Representative immunoblot images.* ***-7d****: pre-supplementation;* ***Pre****: pre-exercise (post-supplementation);* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise.* ***b****: main effect of time (significantly different to pre-exercise; p ≤ 0.05). All values are presented relative to the group mean for all pre-supplementation samples. Data presented as means ± 95% confidence intervals (n = 8).*

4.4.1 Metabolic mRNA response

Seven days of NR supplementation did not alter resting *PPARGC1A* mRNA expression in skeletal muscle (Figure 4.6A). *PPARGC1A* mRNA increased ~5-fold three hours post-exercise (p = 0.025 vs pre-exercise, main effect of time; p = 0.003). Post-exercise *PPARGC1A* mRNA expression was similar in PLA and NR trials (main effect of treatment; p = 0.257, interaction; p = 0.591).

Expression of pyruvate dehydrogenase kinase 4 (*PDK4*; Figure 4.6B) increased post-exercise (main effect of time; p = 0.001) and was ~10-fold elevated three hours post-exercise (p = 0.029 vs pre-exercise). mRNA expression of *PDK4* was similar between PLA and NR trials (main effect of treatment; p = 0.827, interaction; p = 0.521).

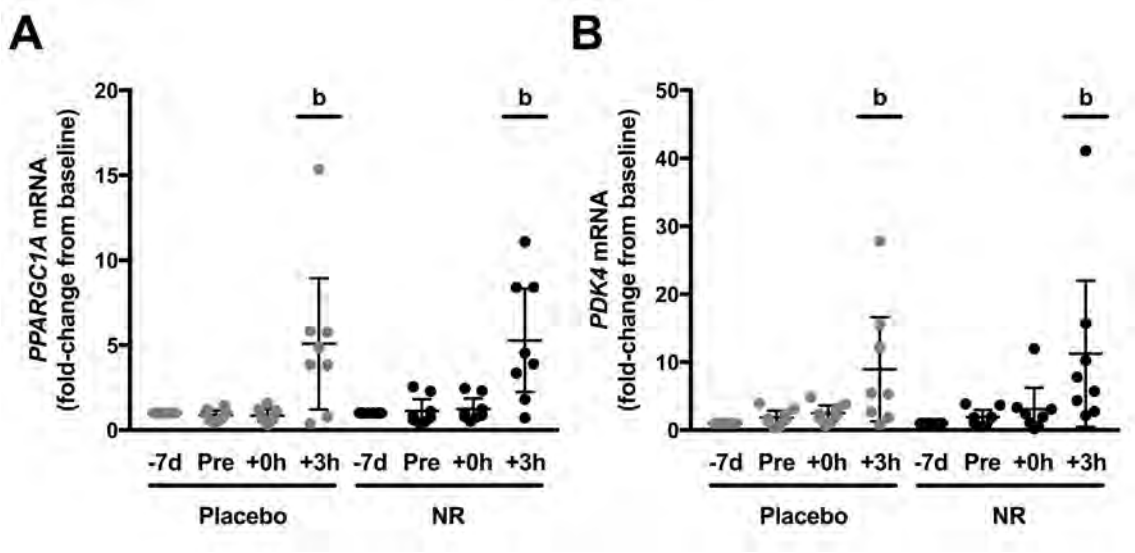

Figure 4.6 Seven days of NR supplementation does not alter resting or exercise-induced *PPARGC1A* or *PDK4* mRNA expression

A. *Resting and exercise-induced PPARGC1A mRNA expression is similar between NR and PLA trials.* ***B.*** *Resting and exercise-induced PDK4 mRNA expression is similar between NR and PLA trials.* ***-7d****: pre-supplementation;* ***Pre****: pre-exercise (post-supplementation);* ***+0h****: immediately post-exercise;* ***+3h****: three hours post-exercise.* ***b****: main effect of time (significantly different to pre-exercise; p ≤ 0.05). All values are presented relative to individual pre-supplementation values for each trial using the* $2^{-\Delta\Delta CQ}$ *method. Data presented as means ± 95% confidence intervals (n = 8).*

4.4.2 mRNA expression of enzymes within the NAD^+ synthesis and salvage pathways

NR supplementation did not alter the mRNA expression of nicotinamide riboside kinase 1 (*NMRK1*; main effect of treatment; $p = 0.432$) within skeletal muscle (Figure 4.7A). *NMRK1* mRNA expression did show a tendency for a main effect of time ($p = 0.071$). There was no treatment*time interaction effect for *NMRK1* mRNA ($p = 0.203$).

mRNA expression of *NAMPT*, the rate limiting enzyme in NAD^+-salvage (8, 12, 14), was unaffected by NR supplementation or exercise (Figure 4.7B; main effect of treatment; $p = 0.303$, time; $p = 0.305$, interaction; $p = 0.442$). Nicotinamide mononucleotide acetyl transferase 1 (*NMNAT1*) mRNA expression was not influenced by NR supplementation (Figure 4.7C), however showed a trend to decrease three hours post-exercise ($p = 0.065$ vs pre-exercise, main effect of time: $p = 0.046$). There was no effect of treatment ($p = 0.482$) nor a treatment*time interaction effect ($p = 0.168$). Of note, *NMNAT1*, the nuclear localised isoform, was the only *NMNAT* isoform that was detectable in our human skeletal muscle samples. mRNA expression of the cytosolic and mitochondrial isoforms, *NMNAT2* and *3* respectively, were indistinguishable from the no template controls (data not shown). The Preiss-Handler enzymes nicotinic acid phosphoribosyltransferase (*NAPRT*) and NAD^+ synthase (*NADSYN1*) were also undetectable (data not shown).

Nicotinamide N-methyltransferase (*NNMT*) increased in expression three-hours post-exercise (Figure 4.7D; $p = 0.010$ vs pre-exercise, main effect of time: $p = 0.001$). However, the post-exercise mRNA expression of *NNMT* was suppressed following NR supplementation (treatment*time interaction: $p = 0.029$), such that the exercise-induced *NNMT* mRNA expression was only increased in the PLA trial (PLA 3h post-exercise vs PLA pre-exercise: $p = 0.010$), while there was also a trend towards a difference between NR and PLA three hours post-exercise ($p = 0.116$). There was no main effect of treatment ($p = 0.148$).

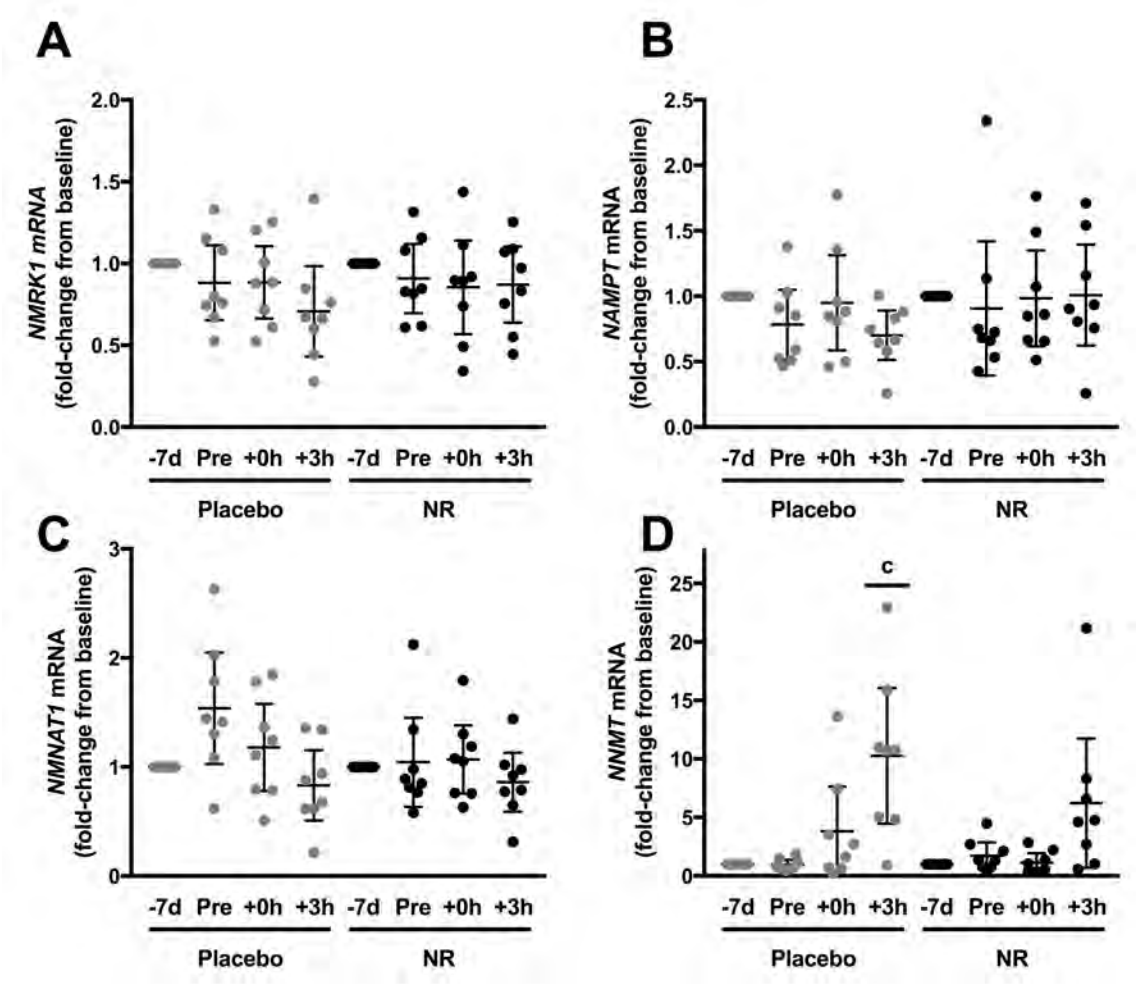

Figure 4.7 mRNA expression of enzymes in the NAD^+ synthesis and salvage pathways within skeletal muscle following NR supplementation and endurance exercise

*The mRNA expression of **A.** NMRK1 and **B.** NAMPT were unaffected by NR supplementation or endurance exercise. **C.** NMNAT1 mRNA expression displayed a tendency to decrease three hours post-exercise (p* = 0.065). ***D.** The mRNA expression of NNMT increased three hours post-exercise in PLA but this was impaired following NR supplementation. **-7d**: pre-supplementation; **Pre**: pre-exercise (post-supplementation); **+0h**: immediately post-exercise; **+3h**: three hours post-exercise. **c**: interaction effect (different to pre-exercise within*

treatment; p ≤ 0.05). All values are presented relative to individual pre-supplementation values for the each trial using the $2^{-\Delta\Delta CQ}$ *method. Data presented as means ± 95% confidence intervals (n = 8).*

4.5 Discussion

Contrary to our hypothesis the activity of the NAD^+-dependent deacetylases SIRT1 and SIRT3 and the mRNA expression of *PPARGC1A* within skeletal muscle were unaffected by NR supplementation. Furthermore, seven days of NR supplementation at 1000 $mg \cdot d^{-1}$ did not alter metabolism at the whole-body level or within skeletal muscle. Finally, and somewhat surprisingly, NR impaired the endurance exercise-induced increases in skeletal muscle expression of *NNMT* mRNA, an enzyme putatively involved in regulating whole-body fatty acid metabolism (31).

Supplementation of 1000 $mg \cdot d^{-1}$ of NR for seven days was insufficient to alter sirtuin activity in human skeletal muscle at rest or following endurance exercise. Acetylation of $p53^{Lys382}$, a SIRT1 deacetylation target (37), and $MnSOD^{Lys122}$, a SIRT3 deacetylation target (32), as well as global acetylation were unchanged throughout the intervention. Furthermore, the basal and exercise-induced expression of *PPARGC1A* mRNA was similar between NR and PLA trials, indicative of similar PGC1α activity (17). In rodents supplemented with NR for periods of 4-16 weeks skeletal muscle SIRT1 and SIRT3 are activated and mitochondrial biogenesis is induced (5, 6, 19, 29). However, on the whole, it does appear that sirtuin activity is more tightly regulated in human rather than

rodent skeletal muscle. For example, endurance exercise induces p53 deacetylation in mice (27) but not in humans (Chapter 3). Thus, given the lower metabolic rate and likely slower NAD^+-turnover in human compared to rodent skeletal muscle, the NAD^+-metabolome and consequent SIRT signalling may be less sensitive to NR supplementation in human skeletal muscle. Furthermore, PARP1 protein content and activity, as assessed by PARP1 auto-PARylation (33) was not significantly induced by exercise or NR supplementation in the current study. This is in partial contrast to data from chapter 3, whereby PARP1 protein content was decreased post-exercise, suggesting that exercise intensity (i.e. 70% W_{max} in chapter 3 vs 60% W_{max} in the current chapter) is an important modulator of PARP1 protein content. However, this does support rodent data showing that NR supplementation does not alter basal PARylation within skeletal muscle (5, 29).

NR supplementation for one week did not alter circulating substrate availability or whole-body substrate utilisation either at rest or during 60% W_{max} cycling in healthy recreationally active males. This is in accordance with recent reports where NR supplementation of 1000 $mg \cdot d^{-1}$ for six weeks and 2000 $mg \cdot d^{-1}$ for 12 weeks had no effect on resting energy expenditure, substrate utilisation or fasting concentrations of glucose or NEFA (11, 25). Furthermore, six weeks of NR supplementation does not alter RER during an incremental exercise test in elderly males (25). However, these data are in contrast to rodent studies, which have demonstrated that NR supplementation can increase metabolic flexibility (30) and fat oxidation during the inactive phase (5), which occurs alongside

induced mitochondrial biogenesis (5). Changes in substrate utilisation with NR supplementation may, therefore, be a physiological outcome of mitochondrial biogenesis. Indeed, in the current study no changes in skeletal muscle mitochondrial respiration or content of electron transport chain proteins were apparent, which perhaps is unsurprising given the relatively short supplementation period. Nonetheless, just two weeks of 750 $mg \cdot d^{-1}$ supplementation of acipimox, a nicotinic acid (NA)-derivative and thus an NAD^+ precursor, increases skeletal muscle mitochondrial respiratory capacity in type II diabetics (35). However, whether the mitochondrial biogenic effects of acipimox in this population are derived from alterations in skeletal muscle NAD^+-metabolism or the confounding effects of NA-induced alterations in whole-body NEFA metabolism remains unclear (35). Studies employing longer duration NR supplementation in basal and exercising conditions that investigate the effect of NR supplementation on mitochondrial biogenesis in humans are warranted.

Our data indicates that exercise and NR alter the NAD^+-consumption/salvage machinery within skeletal muscle. The mRNA expression of *NNMT*, a methyltransferase of nicotinamide (NAM) that produces methylated NAM (MeNAM) and prevents NAD^+-salvage (2), is increased following endurance exercise although this was impaired by NR supplementation. Previous studies have also shown an increase in skeletal muscle *NNMT* mRNA and/or protein expression following endurance exercise training in rats (21) and four days of energy restriction in humans (31). Ström *et al* (31) went on to demonstrate elevated skeletal muscle *NNMT* mRNA expression coincided with an increase

in circulating MeNAM. In addition, plasma MeNAM concentrations are increased following a single bout of endurance exercise in mice, an effect that could only be partially explained by increased NNMT activity in the liver (7). MeNAM can be secreted from human primary myotubes and can induce lipolysis in rat primary adipocytes (31). However, despite elevations in circulating MeNAM during NR supplementation in humans (34), whole-body fatty acid availability at rest, during exercise and during the post-exercise recovery period are unaffected by NR supplementation. The reduction in exercise-induced skeletal muscle *NNMT* mRNA expression following NR supplementation is a particularly surprising finding given elevated plasma MeNAM concentrations during NR supplementation (34). However, as the liver is the major NR-consuming organ (22) and hepatic NNMT activity is high (7) it is likely that the source of additional NR-derived circulating MeNAM is the liver, and it is possible that a negative feedback loop could be preventing additional activation of NNMT in skeletal muscle. The chronic effects of NR on impairing exercise-induced *NNMT* mRNA expression on skeletal muscle NNMT content and activity, and whole-body and skeletal muscle fatty acid metabolism, warrant further investigation.

A current limitation of this study is that the NAD^+-metabolome in skeletal muscle and blood was not analysed. A lack of alteration of metabolism, skeletal muscle mitochondrial biogenesis or sirtuin activity in the current study raises the question of whether this supplementation protocol sufficiently altered skeletal muscle NAD^+ concentrations, at least prior to and/or during exercise. Seven days of 1000 $mg \cdot d^{-1}$ NR supplementation was chosen as this dose and duration

was deemed safe and is sufficient to alter the NAD^+-metabolome in plasma and PBMCs of human participants (34). Furthermore, one week of NR supplementation increases skeletal muscle NAD^+ concentration in mice (5). Nonetheless, the effect and time course of NR supplementation on the skeletal muscle NAD^+-metabolome in humans remains unstudied and a skeletal muscle bioavailability study is warranted. Despite this, a suppression of *NNMT* mRNA expression three hours post-exercise in the NR trial does indicate that, at least at this time point, the skeletal muscle NAD^+-metabolome may be different between NR and PLA trials. Analysis of the NAD^+-metabolome in skeletal muscle and plasma would be particularly informative moving forward.

Overall, 1000 $mg \cdot d^{-1}$ of NR supplementation for seven days does not alter mitochondrial biogenic signalling, including sirtuin activity and *PPARGC1A* mRNA expression, in resting or exercised human skeletal muscle. Furthermore, one week of NR supplementation did not influence substrate metabolism at rest or during 60% W_{max} cycling. Despite this, NR supplementation did reduce the exercise-induced expression of *NNMT* mRNA in skeletal muscle, which plays a putative role in whole-body fatty acid metabolism.

4.6 References

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4.7 Additional information

4.7.1 Conflict of interests

ChromaDex provided nicotinamide riboside and placebo supplements free of charge under a material transfer agreement with the University of Birmingham. The University of Birmingham did not receive any financial support from ChromaDex for the completion of this trial. The authors declare no other conflicts of interest.

4.7.2 Acknowledgements

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5 GENERAL DISCUSSION

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5.1 Introduction

Repeated activation of endurance exercise-sensitive signalling pathways induces metabolic adaptations, including mitochondrial biogenesis, within skeletal muscle (23). Ultimately, these adaptations contribute to the improvement and/or maintenance of whole-body health. Despite this, the optimal activation of these pathways remains incompletely understood. Therefore, the overarching aim of this thesis was to expand upon our understanding of the regulation of post-exercise signalling pathways within skeletal muscle, with specific focus on the activation of energy-sensitive signalling pathways. Furthermore, how fasting and nicotinamide riboside (NR), a nicotinamide adenine dinucleotide (NAD^+) donor influence metabolism was of particular interest.

To address these questions this thesis utilised a translational approach incorporating a rodent model to examine the skeletal muscle-specific role of tumour protein p53 (p53), a putative mitochondrial biogenic regulator, followed by two human physiology studies investigating the influence of nutrition on exercise-sensitive signalling pathways. This chapter will summarise, synthesise and further contextualise the data contained within this thesis.

5.2 Role of p53 in skeletal muscle mitochondrial biogenesis

P53 is purported to be an important regulator of mitochondrial biogenesis within skeletal muscle. Whole-body knockout (KO) of p53 impairs mitochondrial content and function in skeletal muscle (22, 26, 27). However, whether this

resulted from a loss of p53 in skeletal muscle *per se* remained unclear. Here we demonstrated that mice containing a muscle-specific knockout (mKO) of p53 did not exhibit mitochondrial defects (Chapter 2). Thus we concluded that the mitochondrial dysfunction in whole-body p53 KO mice was not due to a loss of p53 in developed skeletal muscle fibres. Nonetheless, this does not preclude p53 a role in mitochondrial biogenesis, as compensatory mechanisms may be at play, or within the post-exercise adaptive response. Particularly, p53 undergoes post-translational modifications in response to endurance exercise (5, 6, 18, 24, 26) and fasting (15), indicative of a functional regulation of p53 by energy stress and exercise. Thus we continued to study p53, namely its acetylation status, in the context of endurance exercise. In contrast to exercise in mice (24) and prolonged (48 hour) fasting in humans (15), $p53^{Lys382}$ acetylation was unresponsive to an ~12 hour fast, one-week of NR supplementation or exercise performed in fed, fasted or NR supplemented states. Therefore, it appears that greater metabolic stress, as apparent in near-exhaustive exercise in mice or prolonged fasting in humans, is required to alter p53 acetylation in human skeletal muscle.

5.3 Manipulating nutrition to influence metabolism

During moderate-intensity exercise (50-75% $\dot{V}O_{2peak}$), fasting augments fatty acid availability and fat oxidation (1, 2, 9-11, 13, 16, 32). However, at higher exercise intensities, substrate utilisation is similar between fed and fasted exercise, despite differential systemic fatty acid availability (Chapter 3)(32). NR also has the potential to augment fatty acid metabolism (7, 28), although a

recent report suggests this does not occur in resting humans (14). Our data corroborates Dollerup *et al* (14), showing that NR supplementation (albeit for a shorter duration) does not alter substrate utilisation or fatty acid availability at rest and advances this published work to demonstrate that this is also the case during steady-state endurance exercise. Furthermore, skeletal muscle mitochondrial respiration is unaffected by one week of 1000 $mg \cdot d^{-1}$ NR supplementation (Chapter 4).

5.4 Manipulating nutrition to influence skeletal muscle signalling

Increased activation of AMP-activated protein kinase (AMPK) and fatty acid oxidative mRNA transcripts in response to fasted exercise has been previously reported (2, 10, 11, 13, 19). Here we show that during moderate- to high steady state exercise (70% W_{max}) fasting and fasted-exercise augment $AMPK^{Thr172}$ phosphorylation and pyruvate dehydrogenase kinase 4 (*PDK4*) mRNA expression. Furthermore, we provide support for previous studies that suggest that fasting elevates cAMP response-element binding protein $(CREB)^{Ser133}$ phosphorylation (33). Despite elevated $AMPK^{Thr172}$ and $CREB^{Ser133}$ phosphorylation, the downstream exercise-induced activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) mRNA expression was unaffected by fasting. Previous literature has also demonstrated similar exercise-induced activation of *PPARGC1A* mRNA in fed and fasted states (10, 11). Taken together, these data suggest that fasting and fasted exercise can readily influence the fatty acid metabolic machinery, however greater energy stress is required to alter the regulation of the

mitochondrial biogenic regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). Indeed, a similar effect is apparent with chronic exercise training. Fasted exercise training in males augments training-induced increases in fatty acid metabolic protein content, 3-hydroxyacyl-CoA dehydrogenase (β-HAD) enzyme activity, maximal fatty acid oxidation rate and intramyocellular lipid breakdown during fasted exercise (12, 31). However, the effect of fasting on surrogate markers of exercise-induced mitochondrial biogenesis is more inconsistent (12, 29, 31).

Sirtuin 1 (SIRT1) activity, as measured by the acetylation of $p53^{Lys382}$, was unaltered by 12 hours of fasting and exercise performed in the fasted, fed and NR supplemented states. Elevated SIRT1 mRNA expression alongside p53 deacetylation has been reported following 48 hours of fasting in human skeletal muscle (15), whilst exercise (24) and NR supplementation induces SIRT1-related deacetylation in rodents (7, 8, 20). SIRT1 deacetylates and activates PGC1α (17). However, an induction of *PPARGC1A* mRNA in spite of a lack of exercise-induced activation of SIRT1 in chapters 3 and 4 provides further evidence for the potential redundancy of SIRT1 in exercise-induced PGC1α activation (24). Furthermore, the concomitant lack of alteration in SIRT1 and SIRT3 activity and global acetylation across the nutritional and exercise interventions within Chapters 3 and 4 suggests that a substantial degree of metabolic stress is required to alter acetylation and sirtuin signalling in human skeletal muscle. In addition, for the first time in exercised human skeletal muscle, we analysed the activity of PARP1, which competes with SIRTs for

NAD^+ (3, 21, 25). PARP1 protein content decreased following cycling at 70% W_{max} but not 60% W_{max}, indicating that exercise intensity, and the associated cellular stress, is an important modulator of PARP1 protein content. However, PARylation, and therefore PARP activity, was unaffected by exercising, fasting or NR supplementation.

5.5 Recommendations for practice

Fasted exercise increases fatty acid availability, fat oxidation (at moderate exercise intensities) and mRNA transcripts involved in fatty acid utilisation within skeletal muscle. If the individual aim is to increase the capacity for fat oxidation then fasted exercise training represents a good strategy (4). Conversely, training in the fed-state promotes carbohydrate oxidation (12) and may be beneficial in maintaining glucose control (16). Thus there appears to be benefits of both fed and fasted exercise, with the choice of the nutritional strategy dependent upon the desired outcome. It is also noteworthy to highlight our observation of a statistical trend for an elevated rating of perceived exertion (RPE) during fasted exercise and anecdotally participants certainly suggested that they found the fasted exercise harder. Ultimately, any exercise is better than no exercise so in many cases the recommendation for most individuals should be to exercise in a way that they enjoy or at least can best tolerate.

From the available evidence and the data presented herein, we conclude that it is premature to recommend NR supplementation to improve metabolic health or augment endurance exercise adaptations in humans, despite promising

evidence from rodent models. Optimal NR supplementation strategies to increase NAD^+ concentration in skeletal muscle (or other tissues) are still unknown. Additionally, the apparent effect of impairing nicotinamide N-methyltransferase (*NNMT*) mRNA expression, which may be involved in regulating whole-body fat metabolism (30), further highlights the need for additional research.

5.6 Limitations

As with all research, the studies contained within this thesis should be considered within their context and as such extrapolation should be considered with care. For example, analysis of mitochondrial content and enzyme activity in p53 mKO mice (Chapter 2) was undertaken in young healthy mice and does not rule out the potential for an important role of skeletal muscle p53 for mitochondrial function with ageing, other deleterious conditions or during endurance exercise training. The human physiology studies within chapters 3 and 4 are limited by sample size due to the onerous nature of performing such studies. Furthermore, these studies were only performed in young healthy males, therefore age and/or sex-based differences cannot be excluded. Analyses in these studies were also restricted by tissue quantities and feasibility of experimental techniques. Indeed, the semi-quantitative nature of the immunoblotting may limit the sensitivity of these analyses. A proteomic methodology would likely provide greater sensitivity, whilst this ‘untargeted’ approach would also provide a broader insight. Finally, the human physiology studies are acute in nature, with limited time points, and as such provide only a

snapshot in time. Long-duration training studies are ultimately required to understand the true adaptive nature of these interventions.

5.7 Future research

Future studies should examine the muscle-specific role of p53 during additional physiological stressors such as endurance exercise training and ageing. In addition, the role and regulation of post-translational modifications of p53 and how these relate to the function of p53 within skeletal muscle still need to be clearly defined. The effects of fasting, exercise and NR on the NAD^+-metabolome is particularly intriguing, especially in the context of the interaction between exercise and NR in the regulation of NNMT and the metabolic role of methylated nicotinamide (MeNAM). Further analysis of samples collected during these PhD studies will hopefully add another piece to this complex puzzle. NR supplementation in humans remains a promising strategy but research investigating the optimal supplementation strategy in terms of dose, frequency and duration of supplementation is required. It is also to be determined whether NR supplementation can augment endurance-training adaptations. Finally, it should be examined whether similar or divergent effects of these studies are apparent in different populations (e.g. females or the elderly).

5.8 Conclusions

Throughout this thesis we have investigated the regulation of exercise-sensitive signalling pathways. In particular, we demonstrate that skeletal muscle p53 is not required for developing or maintaining mitochondrial function in young

healthy mice; that fasting and fasted exercise induces the phosphorylation of $CREB^{Ser133}$ and $AMPK^{Thr172}$ while augmenting the mRNA expression of *PDK4* but not *PPARGC1A*; and one week of NR supplementation at 1000 $mg \cdot d^{-1}$ does not alter skeletal muscle mitochondrial respiration and whole-body substrate utilisation at rest or during endurance exercise, while SIRT1 and SIRT3 activity and *PPARGC1A* mRNA expression at rest and following endurance-exercise are also unaffected by NR supplementation. An unexpected finding of this thesis is that supplementation of NR may suppress post-exercise induction of skeletal muscle *NNMT* mRNA expression, an enzyme putatively involved in regulating whole-body fatty acid metabolism (30). Overall, this thesis contributes novel data to the understanding of metabolism and skeletal muscle signalling following endurance exercise and how nutrition and endurance exercise could be integrated to optimise specific adaptations.

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6 APPENDICES

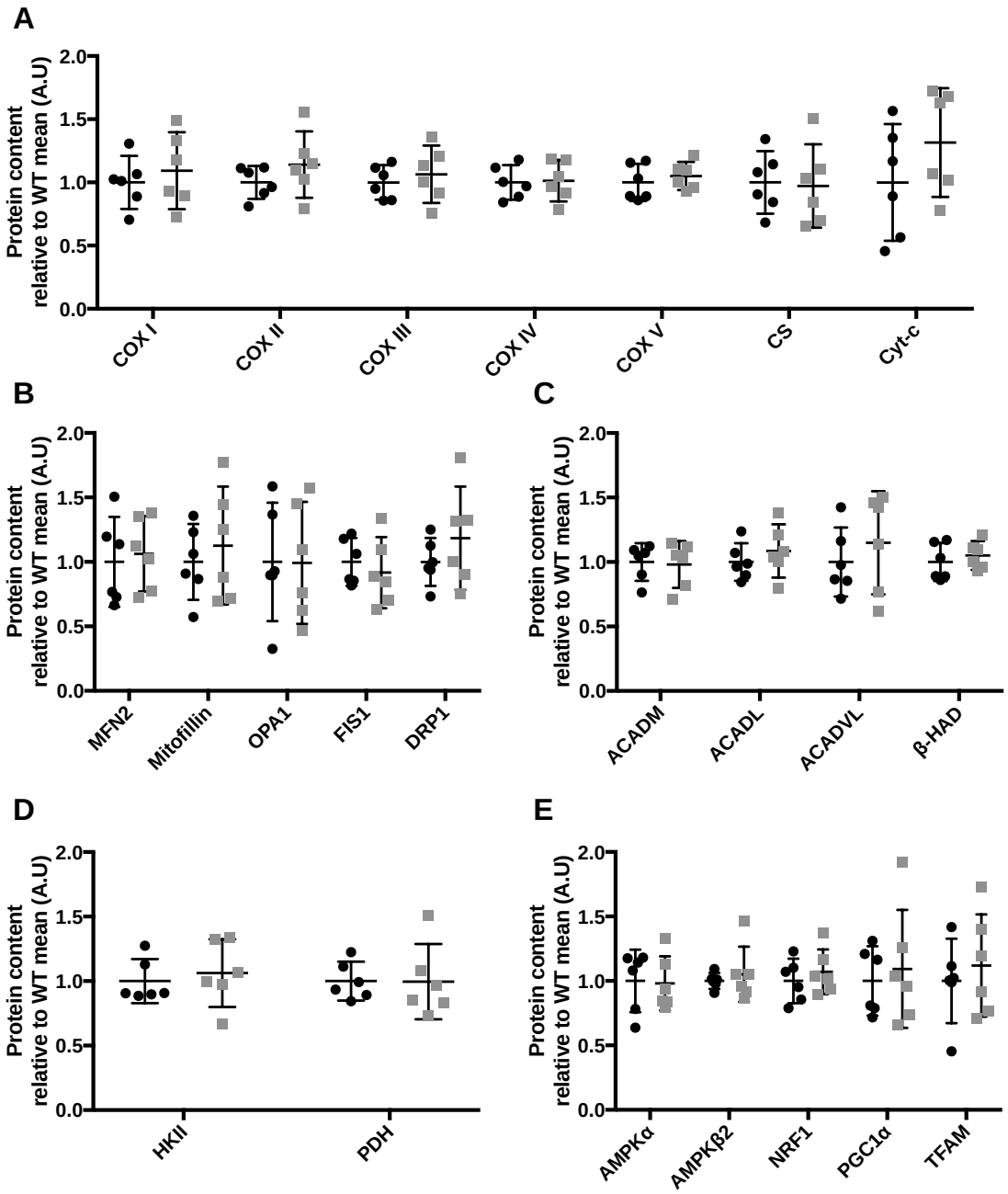

Figure 6.1 Abundance of selected proteins are similar in the quadriceps muscle of p53 mKO and WT mice

WT (black circles) and p53 mKO (grey squares) mice display similar content of ***A.*** *mitochondrial proteins and proteins involved in* ***B.*** *mitochondrial morphology,* ***C.*** *fatty acid metabolism,* ***D.*** *carbohydrate metabolism and* ***E.*** *mitochondrial*

biogenic signalling and transcription within quadriceps muscle ($p > 0.05$; $n = 6$ per group).

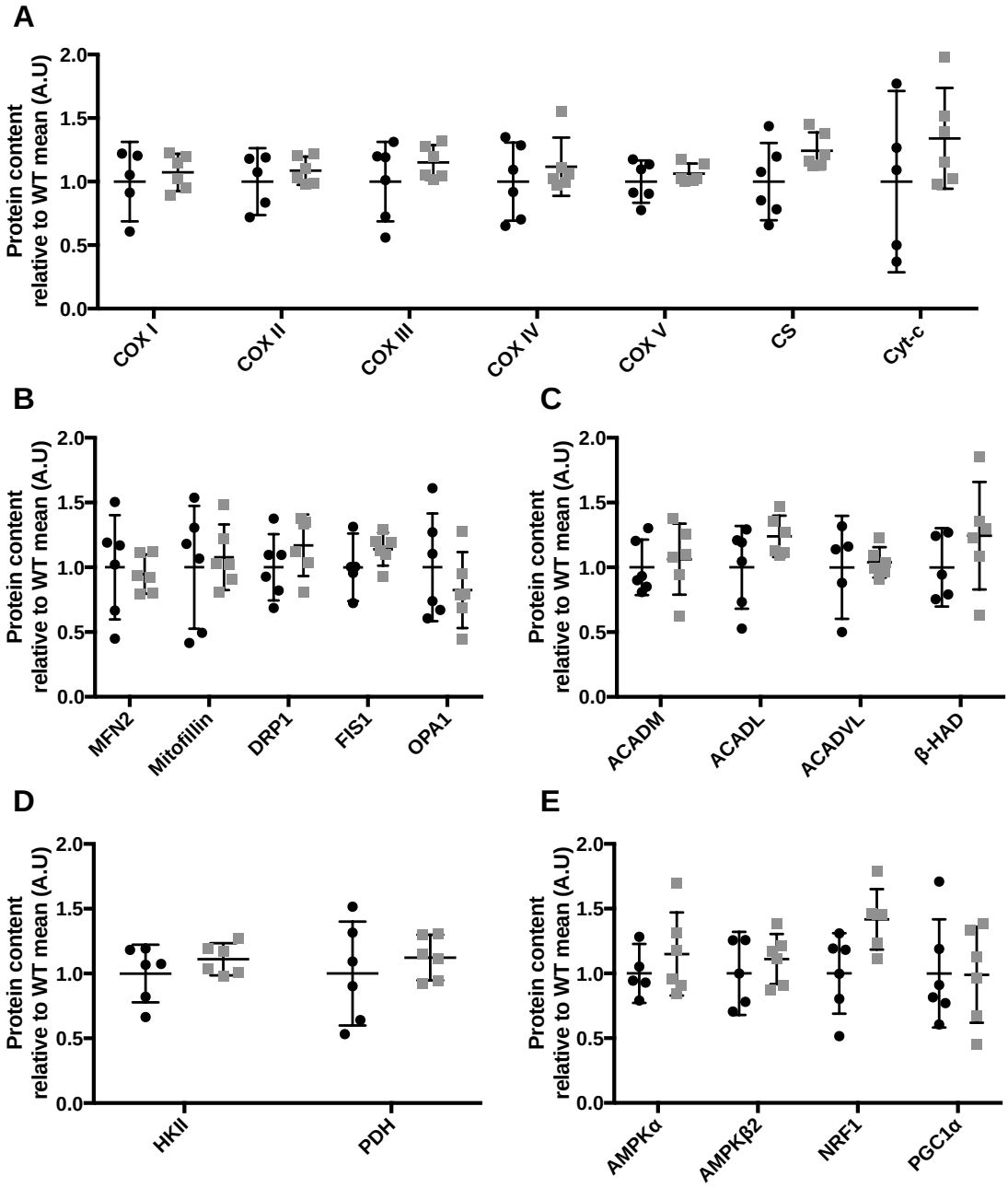

Figure 6.2 Abundance of selected proteins are similar in the triceps muscle of p53 mKO and WT mice

WT (black circles) and p53 mKO (grey squares) mice display similar content of A. mitochondrial proteins and proteins involved in B. mitochondrial morphology, C. fatty acid metabolism, D. carbohydrate metabolism and E. mitochondrial

biogenic signalling and transcription within quadriceps muscle ($p > 0.05$; $n = 5$-6 per group).

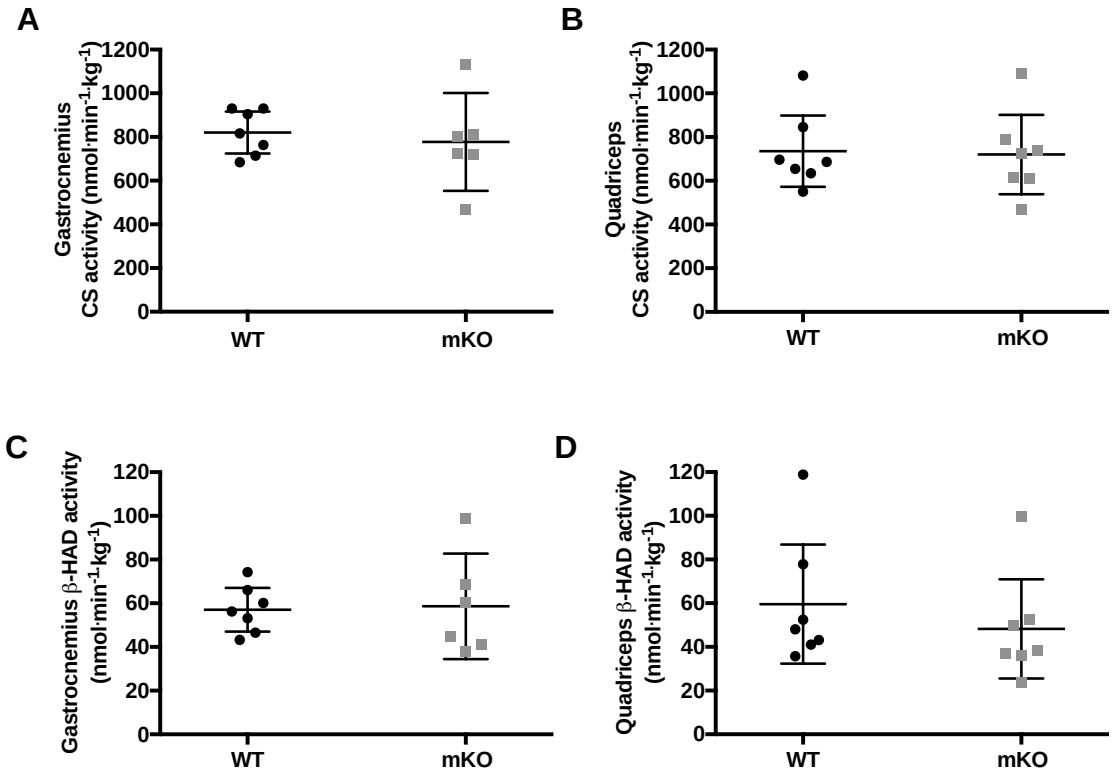

Figure 6.3 Similar CS and β-HAD activity is apparent between p53 mKO and WT mice in both gastrocnemius and quadriceps muscle

*WT (black circles) and p53 mKO (grey squares) mice display similar activity of CS in **A.** gastrocnemius and **B.** quadriceps muscle (p > 0.05; n = 6 per group). Similar activities of β-HAD are apparent in **C.** gastrocnemius and **D.** quadriceps muscle (p > 0.05; n = 6 per group).*