# The integrated physiology of glucose homeostasis: regulation by extracellular and intracellular nucleotide sensors

Submitted by

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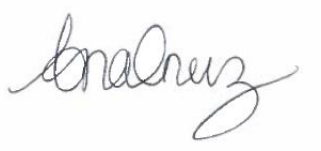
I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.



# Table of contents

# Summary

Physiological glucose levels are maintained by the complex integration of neuroendocrine, hormonal and nutritional signals controlled by multiple tissues in the body. A dysregulation in these mechanisms leads to increasingly prevalent conditions characterised by an inability to regulate blood glucose levels, such as diabetes. Maintaining glycaemia within a target range remains a daily challenge for individuals with both Type 1 and Type 2 diabetes and a better understanding of the pathophysiology of impaired glucose homeostasis in these conditions is still required to identify more effective and targeted therapeutic approaches.

Work in this thesis focused on elucidating the mechanisms by which lipid overflow, be it from increasingly sedentary behaviour or overfeeding, leads to the development of insulin and anabolic resistance in skeletal muscle. Loss of insulin-stimulated glucose clearance by skeletal muscle is a main driver for impaired glucose disposal in Type 2 diabetes and a role for excessive lipid availability in this pathology is well established. Here, muscle cells were treated with high concentrations of a saturated fatty acid and data demonstrated that lipid overflow led to impaired anabolic sensitivity, inflammatory cytokine release and mitochondrial dysfunction. Furthermore, these experiments elucidated a novel role for adenosine tri-phosphate, acting as a signalling molecule, in the regulation of muscle glucose metabolism, identifying insulin and exercise mimetic roles of the nucleotide that could be therapeutically targetable.

This work was translated into humans, where the effect of lipid overflow by high-fat overfeeding was assessed in an experimental model of inactivity-induced insulin and anabolic resistance. Data suggested that two days of disuse (by forearm immobilisation) were sufficient to cause substantial muscle insulin resistance. After 7 days, muscle strength was significantly reduced and anabolic resistance was evident due to decreased forearm balance of potent anabolic amino acids such as leucine. Contrary to the hypothesis, high-fat overfeeding did not accelerate or exacerbate these impairments, suggesting that removal of contraction represents a potent stimulus for loss of substrate demand by muscle, irrespective of energy balance.

Insulin replacement therapy has been the cornerstone of treatment for Type 1 and advanced Type 2 diabetes for over 8 decades. A serious and inadvertent consequence of prolonged insulin therapy is the increased risk of hypoglycaemia. Hypoglycaemia can lead to impaired physiological defences against a decrease in blood glucose and loss of awareness of these changes. AMP-activated protein kinase activators, which are widely used (to target peripheral tissues) as anti-hyperglycaemic agents in Type 2 diabetes have demonstrated central effects that amplify the first defence against hypoglycaemia, or counterregulatory response. Data presented here demonstrated that peripheral administration of a brain permeable AMP-activated protein kinase activator amplified the counterregulatory response to hypoglycaemia by enhancing glucagon levels in healthy rats, without altering fasting blood glucose. This demonstrates important clinical implications for the pharmaceutical use of AMP-activated protein kinase activators as the central roles that regulate blood glucose may supersede the peripheral effects of these compounds, during hypoglycaemia.

Work presented here highlights the complexity of the regulation of glycaemia and discusses the contribution of extracellular and intracellular nucleotides/nucleotide sensors to glucose homeostasis. It can be concluded from this work that strategies to manage or treat diabetes in future should consider the importance of tissue-specific or metabolic status specific actions of the targets of interest.

# List of figures

**Chapter 1 – Introduction**



**Chapter 2 – Materials and methods**



**Chapter 3 – The effect of extracellular ATP signalling on glucose metabolism and fatty acid-induced metabolic dysregulation in skeletal muscle cells**

**Chapter 5 – Regulation of blood glucose by pharmacological activation of AMPK in Sprague-Dawley rats**



**Chapter 6 – General conclusions**

# List of tables

# Publications arising from this thesis

## Chapter 4

Dirks, M. L., Wall, B. T., Otten, B., **Cruz, A. M.,** Dunlop, M. V, Barker, A. R., and Stephens, F. B. *High-fat Overfeeding Does Not Exacerbate Rapid Changes in Forearm Glucose and Fatty Acid Balance During Immobilization.* The Journal of Clinical Endocrinology and Metabolism; 2020. 105(1), 276–289. DOI: 10.1210/clinem/dgz049.

Wall, B.T., **Cruz, A.M.,** Otten, B., Dunlop, M.V., Fulford, J., Porter, C., Abdelrahman, D.R., Stephens, F.B., and Dirks, M.L. *The impact of disuse and high-fat overfeeding on forearm muscle amino acid metabolism in humans*. The Journal of Clinical Endocrinology and Metabolism; 2020. Accepted for publication.

## Chapter 5

Vlachaki Walker, J.M., Robb, J.L., **Cruz**, **A.M**., Malhi, A., Weightman Potter, P.G., Ashford, M.L.J., McCrimmon, R.J., Ellacott, K.L.J., and Beall, C. *AMP-activated protein kinase (AMPK) activator A-769662 increases intracellular calcium and ATP release from astrocytes in an AMPK-independent manner.* Diabetes, obesity & metabolism; 2017. 19 (7): p. 997-1005. DOI: 10.1111/dom.12912

**Cruz, A.M.,** Malekizadeh, Y.M-Vlachaki Walker, J.M., Weightman Potter, P.G., Pye, K., Shaw, S., Ellacott, K.L.J., Beall, C. *Brain permeable AMPK activator R481 raises glycaemia by autonomic nervous system activation and amplifies the counterregulatory response to hypoglycemia in rats*. BioRvix; (2019); DOI:10.1101/749929.

# Author's declarations

All of the work presented in this thesis is a result of experimental work carried out by myself, Ana Miguel Cruz, with invaluable support from research and academic staff and students at the University of Exeter College of Medicine and Health and College of Life and Environmental Science (Sport and Health Sciences).

Work carried out with assistance from others included:

Chapter 3: C2C12 myotube microscope images were taken and processed with assistance from Dr. Holly Hardy.

Chapter 4: Given the nature of the blood sampling method, which requires two paralleled blood samples, these were collected with assistance from Dr. Marlou Dirks and Mrs. Mandy Dunlop. Participant's casts were applied by Dr. Marlou Dirks, Dr. Benjamin Wall or Prof. Francis Stephens (qualified in cast application). Assistance with experimental days was, on occasion, provided by Alistair Monteyne, George Pavis, Mariana Coelho, Sean Kilroe and Tom Jameson.

Chapter 5: Western blotting experiments presented in Figure 5.1.1 were conducted by Dr. Paul Weightman Potter and Dr. Yasaman Malekizadeh. Intracellular ATP assay represented in Figure 5.1.2 was conducted by Ms. Julia Vlachaki Walker. Raw data for acute insulin treatments presented in Figures 5.2B and 5.3 was collected by Dr. Craig Beall and Ms. Julia Vlachaki Walker. These were included in this thesis for completeness.

# List of abbreviations

For the convenience of the reader, listed below, are the commonly used abbreviations in this thesis.

| Abbreviation | Definition |
|---|---|
| $[Ca^{2+}]_i$ | Intracellular calcium concentration |
| 2-DG | 2-deoxy-d-glucose |
| 2DG6P | 2-deoxyglucose-6-phosphate |
| 2-NBDG | 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose |
| 3-HIB | 3-hydroxyisobutyrate |
| 4EBP1/2 | Eukaryotic translation initiation factor binding proteins 1 and 2 |
| 5'-NT | Ecto-5'-nucleotidase |
| 5-BDBD | 5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one |
| AA | Amino acid |
| AAT | Amino acid transporter |
| Ab | Antibody |
| ABC | ATP binding cassette transporter |
| AC | Adenylyl cyclase |
| ACC | Acetyl-CoA carboxylase |
| Ach | Acetylcholine |
| ACLY | ATP citrate lyase |
| ACO2 | Acotinase |
| ACSL | Acyl-CoA synthetases |
| ADA | Adenosine deaminase |
| ADP | Adenosine diphosphate |
| AgRP | Agouti-related peptide |
| AICAR | 5-aminoimisazole-4-carboxamide riboside |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| ANG II | Angiotensin II |
| ANOVA | Analysis of variance |
| ANS | Autonomic nervous system |
| AP | Alkaline phosphatase |
| APS | Ammonium persulfate |
| ARC | Arcuate nucleus |
| AS160 | GTPase-activating protein AKT substrate of 160 kDa |
| AT | Adipose tissue |
| ATP | Adenosine triphosphate |
| ATPγS | Adenosine-5'-*o*-(3-thiotriphosphate) |
| AV | Arterialised-venous |
| AV-V | Arterialised-venous deep-venous |
| BBB | Blood brain barrier |
| BCAA | Branched-chain amino acid |
| BMR | Basal metabolic rate |
| BSA | Bovine serum albumin |
| BzATP | 2' (3')-*O*-4-benzoylbenzoyl-ATP |

| | |
|---|---|
| $Ca^{2+}$ | Calcium |
| CACT | Carnitine acyl-carnitine translocase |
| CALHM | Calcium homeostasis modulator ion channels |
| CAMK | Calcium-calmodulin-dependent protein kinases |
| CaMKKβ | Calcium-calmodulin dependent protein kinase kinase-β |
| cAMP | Cyclic-adenosine monophosphate |
| CASTOR1/2 | Cellular arginine sensor for mammalian target of rapamycin complex 1 |
| CD36 | Cluster of differentiation 36 |
| CD39 | Cluster of differentiation 39 |
| CD73 | Cluster of differentiation 73 |
| CD74 | Cluster of differentiation 74 |
| CD98 | Cluster of differentiation 98 |
| CEACAM1 | Carcinoembryonic antigen-related cell adhesion molecule 1 |
| CGM | Continuous glucose monitoring |
| ChREBP | Carbohydrate response element binding protein |
| Cm | Centimetre |
| CM | Conditioned media |
| CNS | Central nervous system |
| CNT | Concentrative nucleoside transporter |
| Cnx | Connexin hemichannel/gap junction |
| $CO_2$ | Carbon dioxide |
| CoA | Coenzyme A |
| CON | Control |
| CPT1A | Carnitine palmitoyltransferase-1A |
| CPT2 | Carnitine palmitoyltransferase 2 |
| CREB | Cyclic-AMP-response element binding protein |
| CRR | Counterregulatory response |
| CS | Citrate synthase |
| CSII | Continuous subcutaneous insulin infusion |
| Cyt c | Cytochrome C |
| DAG | Diacylglycerol |
| DHAP | Dihydroxyacetone phosphate |
| DIO | Diet-induced obese |
| DMD | Duchenne Muscular Dystrophy |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DMH | Dorsomedial hypothalamus |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPP4 | Dipeptidyl peptidase-4 inhibitors |
| EAA | Essential amino acid |
| eATP | Extracellular ATP |
| EBSS | Earl’s balanced salt solution |
| ECAR | Extracellular acidification rate |
| EDTA | Ethylenediaminetetraacetic acid |
| EE | Estimated energy |
| eEF2 | Eukaryotic elongation factor 2 |
| EGTA | Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |

| | |
|---|---|
| eIF4F | Eukaryotic initiation factor 4F |
| ELISA | Enzyme-linked immunosorbent assay |
| En | Energy |
| E-NPPs | Ectonucleotide pyrophosphatase/phosphodiesterases |
| ENT | Equilibrative nucleoside transporter |
| E-NTPDases | Ecto-nucleoside triphosphate diphosphohydrolases |
| ERK1/2 | Extracellular signal regulated kinase 1/2 |
| ES | Electrical stimulation |
| ETC | Electron transport chain |
| FA | Fatty acid |
| FATBP | Fatty acid binding protein |
| FATBPpm | Plasma membrane fatty acid binding protein |
| $FADH_2$ | Flavin adenine dinucleotide (reduced) |
| FAO | Fatty acid oxidation |
| FASN | Fatty acid synthase |
| FATP | Fatty acid transporter protein |
| FBPase | Fructose-1,6-bisphosphatase |
| FBS | Foetal bovine serum |
| FCCP | Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone |
| FFA | Free fatty acid |
| FGU | Forearm glucose uptake |
| FH | Fumarate |
| FOXO | Fork-head box protein |
| FPG | Fasting plasma glucose |
| g | Gram |
| G | Gauge |
| G6P | Glucose-6-phosphatase |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| GAD | Glutamic acid decarboxylase |
| GAP | GTPase-activating protein |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC-MS | Gas chromatography-mass spectrometry |
| GDM | Gestational Diabetes Mellitus |
| GDP | Guanosine diphosphate |
| GE | Glucose-excited |
| GI | Glucose-inhibited |
| GIP | Gastric inhibitory peptide / Glucose-dependent insulinotropic polypeptide |
| GIR | Glucose infusion rate |
| GIV | Guanine nucleotide-binding protein α subunit-interacting vesicle-associated protein |
| GK | Glucokinase |
| GLP-1 | Glucagon-like peptide 1 |
| GLUT | Glucose transporter |
| GPCR | G-protein coupled receptor |
| GRB10 | Growth factor receptor bound protein 10 |
| GSIS | Glucose stimulated insulin secretion |
| GSK3 | Glycogen synthase kinase 3 |

| | |
|---|---|
| GTP | Guanosine triphosphate |
| GTPase | Guanosine triphosphatase |
| GTT | Glucose tolerance test |
| GWAS | Genome-wide association studies |
| HbA1c | Haemoglobin A1c |
| HCL | Hydrochloric acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HFD | High fat diet |
| HGP | Hepatic glucose production |
| HK | Hexokinase |
| HMB | Hydroxyl-β-methylbutarate |
| HOMA-IR | Homeostatic model assessment for insulin resistance |
| HPMC | Hydroxypropyl methylcellulose |
| HRP | Horseradish peroxidase |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| IA-2 | Islet antigen-2 |
| IAH | Impaired awareness of hypoglycaemia |
| Icv | Intracerebroventricular |
| IDH | Isocitrate dehydrogenase |
| IFN-γ | Interferon-gamma |
| IκB | I kappa B |
| IKK | I kappa B kinase |
| IL | Interleukin |
| IMCL | Intramyocellular lipid |
| INSR | Insulin receptor |
| IP3 | inositol-1,4,5-triphosphate |
| iPAQ | International Physical Activity Questionnaire |
| IR | Insulin resistance |
| IRS | Insulin receptor substrate |
| IS | Insulin secretion |
| iSGLT2 | Sodium glucose co-transporter-2 inhibitors |
| JNK | c-Jun $NH_2$-terminal kinase |
| K | Potassium |
| $K_{ATP}$ | ATP-sensitive potassium channel |
| kDa | Kilo Daltons |
| Kg | Kilogram |
| LAT1 | L-type amino acid transporter 1 |
| LCFA | Long-chain fatty acid |
| LCFA-CoA | Long-chain fatty acyl-Coenzyme A |
| LDH | Lactate dehydrogenase |
| LDL | Low-density lipoprotein |
| Leu | Leucine |
| LHN | Lateral hypothalamic nucleus |
| LKB1 | Liver kinase B1 |
| LPS | Lipopolysaccharide |
| LTB4 | Leukotriene B4 |
| MAFbx | Muscle Atrophy F-Box/atrogin-1 |

| | |
|---|---|
| MAP | Mitogen-activated protein |
| MAP3K4 | Mitogen-activated protein kinase kinase kinase 4 |
| MAPK | Mitogen-activated protein kinase |
| MBH | Mediobasal hypothalamus |
| MDH | Malate dehydrogenase |
| MET | Metabolic equivalent |
| MHz | Megahertz |
| MIF | Macrophage-migration inhibitory factor |
| min | Minute |
| mL | Millilitre |
| mM | Millimolar |
| MODY | Maturity onset diabetes of the young |
| MPB | Muscle protein breakdown |
| MPS | Muscle protein synthesis |
| mRNA | Messenger RNA |
| mTOR | Mammalian target of rapamycin |
| mTORC1 | Mammalian target of rapamycin complex 1 |
| mTORC2 | Mammalian target of rapamycin complex 2 |
| MuRF1 | Muscle-Specific RING-finger protein 1 |
| $Na_3VO_4$ | Sodium orthovanadate |
| NaCl | Sodium chloride |
| NAD | Nicotinamide adenine dinucleotide |
| NAD+ | Nicotinamide adenine dinucleotide (oxidised) |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| NaF | Sodium fluoride |
| NaOH | Sodium hydroxide |
| NaPPi | Sodium pyrophosphate tetrabasic decahydrate |
| NDPK | Nucleoside diphosphate kinase |
| NEAA | Non-essential amino acid |
| NEFA | Non-esterified fatty acids |
| NF-κB | Inhibitor κB (IκB)/nuclear factor κB |
| nM | Nanomolar |
| nm | Nanometre |
| NOX | NAD(P)H oxidase 4 |
| NPY | Neuropeptide Y |
| NPY/AgRP | Neuropeptide Y/Agouti-related peptide |
| NTS | Nucleus of the tractus solaris |
| $O_2$ | Oxygen |
| OAA | Oxaloacetate |
| OCR | Oxygen consumption rate |
| OGDH | α-ketoglutarate dehydrogenase |
| OGTT | Oral glucose tolerance test |
| OXPHOS | Oxidative phosphorylation |
| P1 | Adenosine receptor class |
| P2R | Purinergic P2 receptors |
| P2XR | Purinergic P2 X receptor |
| P2YR | Purinergic P2 Y receptor |
| PAL | Physical activity level |

| | |
|---|---|
| PAT1 | Proton assisted amino acid transporter |
| PBS | Phosphate buffered saline |
| PC | Pyruvate carboxylase |
| PDH | Pyruvate dehydrogenase |
| PDK | Pyruvate dehydrogenase kinase |
| PDK1 | Phosphoinositide-dependent kinase 1 |
| PEP | Phosphoenolpyruvate |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PFK1 | Phosphofructokinase-1 |
| PGC-1α | Peroxisome proliferator-activated receptor γ coactivator-1α |
| Phe | Phenylalanine |
| $P_i$ | Inorganic phosphate |
| PI3K | Phosphoinositide-3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3, 4, 5-tris-phosphate |
| PK | Pyruvate kinase |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PLL | Poly-L-lysine |
| PMSF | Phenylmethylsulfonyl fluoride |
| PNP | Purine nucleoside phosphorylase |
| Pnx | Pannexin hemichannel |
| POMC | Pro-opiomelanocortin |
| PP | Pancreatic polypeptide |
| PP2A | Protein phosphatase 2A |
| PP2C | Protein phosphatase 2C |
| PPADS | Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid |
| PPAR | Peroxisome proliferator-activated receptor |
| PPi | Pyrophosphate |
| PRAS40 | Proline-rich AKT substrate of 40 kDa |
| PTEN | Phosphatase and tension homologue |
| PTPase | Protein tyrosine phosphatase |
| PTP1B | Protein tyrosine phosphatase 1B |
| PUFA | Polyunsaturated fatty acid |
| PVN | Paraventricular nucleus |
| RAC1 | Ras-related C3 botulinum toxin substrate 1 |
| RA-GLP1 | GLP-1 receptor agonists |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RT | Room temperature |
| S473 | Serine 473 |
| S536 | Serine 536 |
| S6 | Substrate 6 |
| S6K | Substrate 6 kinase |
| S79 | Serine 79 |
| SDH | Succinate dehydrogenase |

| | |
|---|---|
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SFA | Saturated fatty acid |
| SLC | Superfamily of solute carriers |
| SM | Skeletal muscle |
| SNAP | Soluble *N*-ethylmaleimide-sensitive factor attachment protein |
| SNARE | Soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor |
| SNAT2 | Sodium-coupled neutral amino acid transporter 2 |
| SOCS | Suppressor of cytokine signalling |
| SREBP1c | Sterol regulatory element binding protein 1c |
| STZ | Streptozotocin |
| T172 | Threonine 172 |
| T1D | Type 1 Diabetes |
| T2D | Type 2 Diabetes |
| T389 | Threonine 389 |
| TBS | Tris buffered saline |
| TBS-T | Tris buffered saline with tween |
| TCA | Tricarboxylic acid cycle |
| TEE | Total energy expenditure |
| TEMED | N,N,N′,N′-Tetramethylethylenediamine |
| TG | Triglyceride |
| Thr202 | Threonine 202 |
| TLR | Toll-like receptor |
| TMB | 3,3,5,5'-tetramethylbenzidine |
| TNF-α | Tumour necrosis factor α |
| TNP-ATP | Trinitrophenyl-ATP |
| Tris | Trisaminomethane |
| TSC2 | Tuberous sclerosis complex 2 |
| Tyr204 | Tyrosine 204 |
| UCP | Uncoupling protein |
| UDP | Uridine diphosphate |
| ULK1 | Unc-51 Like Autophagy Activating Kinase 1 |
| UTP | Uridine triphosphate |
| V | Volt |
| VAB | Vascular access button |
| VGCC | Voltage-gated calcium channels |
| VMH | Ventromedial hypothalamus |
| VMN | Ventromedial nucleus of the hypothalamus |
| VNUT | Vesicular nucleotide transporter |
| Vps34 | Vacuolar protein sorting 34 |
| WAT | White adipose tissue |
| ZMP | AICAR 5’-monophosphate |
| ZnT8 | Zinc transporter 8 protein |
| α-MSH | α-melanocyte stimulating hormone |
| µM | Micromolar |
| µL | Microliter |

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*“Nobody realises that some people expend tremendous energy merely to be normal.“ Albert Camus, Notebooks 1942-1951*

# Chapter 1

# General introduction

## 1.1 Diabetes Mellitus

Diabetes is the term given to a collective of metabolic disorders primarily characterised by increased blood glucose, or hyperglycaemia, arising from impaired glucose homeostasis and insulin action. The most recent figures show that 425 million individuals worldwide between the ages of 20 and 79 live with diabetes, with an additional 352 million believed to be at risk of developing the condition (International Diabetes Foundation, 2017). The prevalence is expected to increase further to 9.9 % by the year 2045 raising the number of affected individuals to a staggering 629 million people in the next 25 years (International Diabetes Foundation, 2017). Diabetes places a great physical and emotional burden on individuals and remains a lifelong condition. In addition, it places considerable socioeconomic pressures on individuals and overwhelming costs on global health economies, costing the USA 727 billion USD (in 2017), and the National Health System (UK) £10 billion each year (Diabetes UK, 2014).

Diabetes is primarily classified into Type 2 diabetes (T2D), Type 1 diabetes (T1D) and gestational diabetes mellitus (GDM), referred to as the polygenic disorders, given the role of environmental factors in their aetiology. Other specific types include monogenic diabetes syndrome, including neonatal diabetes and maturity-onset diabetes of the young (MODY), and more recently characterised diseases of the endocrine pancreas (cystic fibrosis and pancreatitis) and drug or chemical-induced diabetes such as that arising from glucocorticoid use in the treatment of HIV/AIDS or after organ transplantation (American Diabetes Association, 2019a).

Diagnostically, hyperglycaemia is assessed by fasting plasma glucose (FPG) levels, glucose tolerance tests in response to 75 g oral anhydrous glucose bolus (OGTT) and glycated haemoglobin A1C (HbA1c) levels. The thresholds for hyperglycaemia are ≥ 7 mmol/L fasting, ≥ 11.1 mmol/L during 2 hour OGTT and HbA1C score ≥48 mmol/moL (Alberti and Zimmet, 1998; American Diabetes Association, 2019a). Hyperglycaemia also causes the symptoms commonly associated with diabetes, such as polyuria, fatigue, nausea, increased thirst and visual impairments (Alberti and Zimmet, 1998).

### 1.1.1 Type 2 diabetes mellitus

T2D is primarily characterised by relative insulin insufficiency and peripheral insulin resistance (IR), leading to hyperglycaemia. Glycaemic status determines disease stage from normal to impaired to diabetic, with gradual increases in blood glucose often occurring several years before diagnosis.

Between 1980 and 2014 the numbers of adults with diabetes increased 4 fold from 180 million to 422 million, an increase partly driven by a rise in the incidence of obesity caused by increasingly sedentary lifestyles and ageing population (International Diabetes Foundation, 2017). T2D accounts for 90-95 % of these cases. Importantly, over 50 % of all cases of T2D could be prevented or delayed by lifestyle interventions, a critical factor to consider as the numbers of people at risk of developing the condition are expected to rise to over 5 million by 2025 in the UK alone (Diabetes UK, 2019).

The aetiology of T2D is not completely understood but well-characterised risk factors for disease development include excess body weight, obesity, physical inactivity, age, and increased consumption of high fat and sugar diets (Chatterjee *et al.,* 2017). Higher frequency is also seen in women with prior GDM and hypertensive and dyslipidaemic individuals. Genetic factors also contribute, as first-degree relatives have increased risk of developing T2D (Zheng *et al.,* 2018). T2D places individuals at a higher risk of developing co-morbidities, particularly cardiovascular complications, which cause 80 % of deaths in T2D. Individuals with T2D are nearly 2.5 times more likely to develop heart attacks, over 2.5 times more likely to experience heart failure and twice as likely to have a stroke, compared to those without diabetes (Diabetes UK, 2019).

### 1.1.2 Type 1 diabetes mellitus

T1D, previously referred to as juvenile-onset or insulin-dependent diabetes, accounts for 8-10 % of all diabetes cases, affecting 400,000 people in the UK, 29,000 of which are children (JDRF, 2018). A doubling of cases in children under 5 and 70 % increase in those younger than 15 years was predicted between 2005-2020 (Patterson *et al.*, 2009) and the incidence is still increasing, especially in the developed world, with the age at onset shifting to a younger age (Van Belle *et al.,* 2011).

Although also characterised by hyperglycaemia, this arises as a consequence of autoimmune pancreatic β-cell dysfunction, resulting in insulin insufficiency and consequent loss of inhibition of glucose production and glucose uptake into tissues. Although the aetiology is not fully elucidated, the condition most likely results from a combination of higher genetic susceptibility and environmental triggers (Van Belle *et al.,* 2011).

Autoreactive T-cells are the primary drivers of β-cell destruction (Pugliese, 2017) resulting in production of autoimmune markers such as glutamic acid decarboxylase (GAD), islet antigen 2 (IA-2), insulin, and zinc transporter 8 protein (ZnT8) (Atkinson and Eisenbarth, 2001), which are used as diagnostic markers of disease. The origin of the autoimmune response remains unclear. Genome-wide association studies identified a genetic risk of T1D (Mehers and Gillespie, 2008; Jerram *et al.,* 2017), supporting the hereditary theory introduced by Harley in 1866 (Harley, 1866). However, as ~50 % of monogenic twins are discordant for T1D, non-genetically determined factors also play an important role in disease aetiology, including enteroviral infection (Filippi and von Herrath, 2008; Richardson *et al.*, 2009) and changes to the microbiome (Zheng *et al.,* 2018).

### 1.2 Glucose homeostasis

Physiological plasma glucose concentration ranges between 4.4-6.1 mmol/L. This is maintained by a balance of glucose clearing processes such as insulin-stimulated glucose uptake into tissues and glucose appearance. Circulating glucose arises from postprandial intestinal absorption (regulated by gastric emptying), from the breakdown of glycogen (polymerised storage form of glucose) during glycogenolysis, and from *de novo* synthesis via gluconeogenesis (Aronoff *et al.*, 2004). The body relies on well-tuned neuroendocrine mechanisms to combat changes to glucose availability such as during hyperglycaemia and hypoglycaemia, to maintain energy balance (Pocai *et al.*, 2005). The pathophysiology underlying impairments in glucose homeostasis, as seen in diabetes, involves a complex interplay between cellular and systemic factors. These are mediated by tissues such as the pancreas, liver, adipose tissue (AT), skeletal muscle (SM) and brain that can sense glucose and act to maintain homeostasis.

### 1.2.1 Glucose metabolism

Glucose homeostasis is maintained by a balance of glucose production, breakdown and interconversion. The processes involved are primarily glucose transport, glycogenolysis, glycogenesis, gluconeogenesis and glycolysis (progressing into oxidative phosphorylation). This thesis primarily touched on the transport and metabolism of glucose in gluconeogenesis and glycolysis so these are described in more detail below. Glycogen handling is outside the scope of this thesis and the reader is referred to a current review for the understanding of glycogen metabolism (Adeva-Andany *et al.*, 2016).

### 1.2.2 Glucose transport

Glucose is sensed and transported via glucose transporters (GLUT), of which there are multiple isoforms with different tissue distributions. For example, GLUT4 translocation to the plasma membrane is regulated by insulin signalling in AT and SM, and the transporter has high affinity for glucose (Km ~ 5 mM), making it a key regulator of postprandial insulin-stimulated glucose uptake and storage (Cushman and Wardzala, 1980). GLUT2 also acts as a glucose sensor and is primarily expressed in pancreatic β-cells and hepatocytes, and it has a low affinity for glucose (Km ~17 mM) ensuring that GLUT2-mediated glucose import occurs during hyperglycaemia (Thorens, 1992). During fasting, when intrahepatic concentrations are high, glucose is exported via GLUT2 to maintain blood glycaemia. GLUT1 has higher affinity for glucose (Km ~ 1-3 mM) and, although ubiquitously expressed, is particularly important in facilitating glucose transport across the blood-brain barrier (BBB), neuronal membranes and erythrocytes (Gorovits and Charron, 2003; Augustin, 2010; Mueckler and Thorens, 2013). GLUT3 is primarily expressed in the brain (also placenta and testes) and has the highest affinity for glucose (Km ~1.5 mM) (Kayano *et al.*, 1988; Simpson *et al.*, 2008).

### 1.2.3 Glycolysis, Krebs cycle & gluconeogenesis

Glycolysis is the cytoplasmic enzymatically controlled process by which glucose is metabolised to pyruvate or lactate following glucose entry into cells. A diagrammatical representation of the molecules and enzymes involved in this process is shown in Figure 1.1.

Glycolytic rate is determined at the level of glucose uptake, glucose phosphorylation by hexokinase (HK) II or IV (glucokinase, GK, in liver and β-cells); and generation of fructose-1,2-biphosphate, catalysed by phosphofructo-1-kinase (PFK1) (Lenzen, 2014). The latter is activated by fructose-2-6-biphosphate whose production is regulated by 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase, which is differentially regulated by nutritional status (Massa *et al.*, 2004). Pyruvate kinase (PK) catalyses the final step to convert phosphoenolpyruvate (PEP) into pyruvate. Pyruvate is then transported into the mitochondria and oxidised to generate acyl groups (oxidative decarboxylation by pyruvate dehydrogenase, PDH) which reacts with Coenzyme A to form acetyl-Coenzyme A (acetyl-CoA). Acetyl-CoA is then used in the Krebs cycle (or citric acid cycle) to generate adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH), reduced flavin adenine dinucleotide ($FADH_2$) and carbon dioxide ($CO_2$). This process (schematic representation in Figure 1.2) harvests high-energy electrons that are re-oxidised in oxidative phosphorylation to maximise ATP. In the absence of oxygen, pyruvate is reduced by lactate dehydrogenase (LDH) into lactate. Conversion of glucose to lactate releases protons and acidifies the extracellular space (Mookerjee *et al.*, 2017). The rate of extracellular acidification is commonly used as a proxy for direct and quantitative assessment of glycolytic rate *in vitro* (Nicholls *et al.*, 2010; Nadanaciva *et al.*, 2012).

Glycolysis is an important regulator of glucose production and insulin secretion (Terrettaz *et al.,* 1986; Rossetti and Giaccari, 1990), stimulates lipogenesis in AT via production of glycerol phosphate (Huo *et al.*, 2010), contributes to hypothalamic control of food intake (Dunn-Meynell *et al.*, 2002; Kang *et al.*, 2006) and plays a role in inflammatory responses (Huo *et al.*, 2010). As such, impairments in the glycolytic pathway are implicated in diabetes pathophysiology, as seen by a hyperglycaemic phenotype in liver-specific knockouts of GK (Postic *et al.*, 1999) and impaired ability of insulin to increase *in vivo* GK activity in obesity-associated IR (Lam *et al.,* 2003).

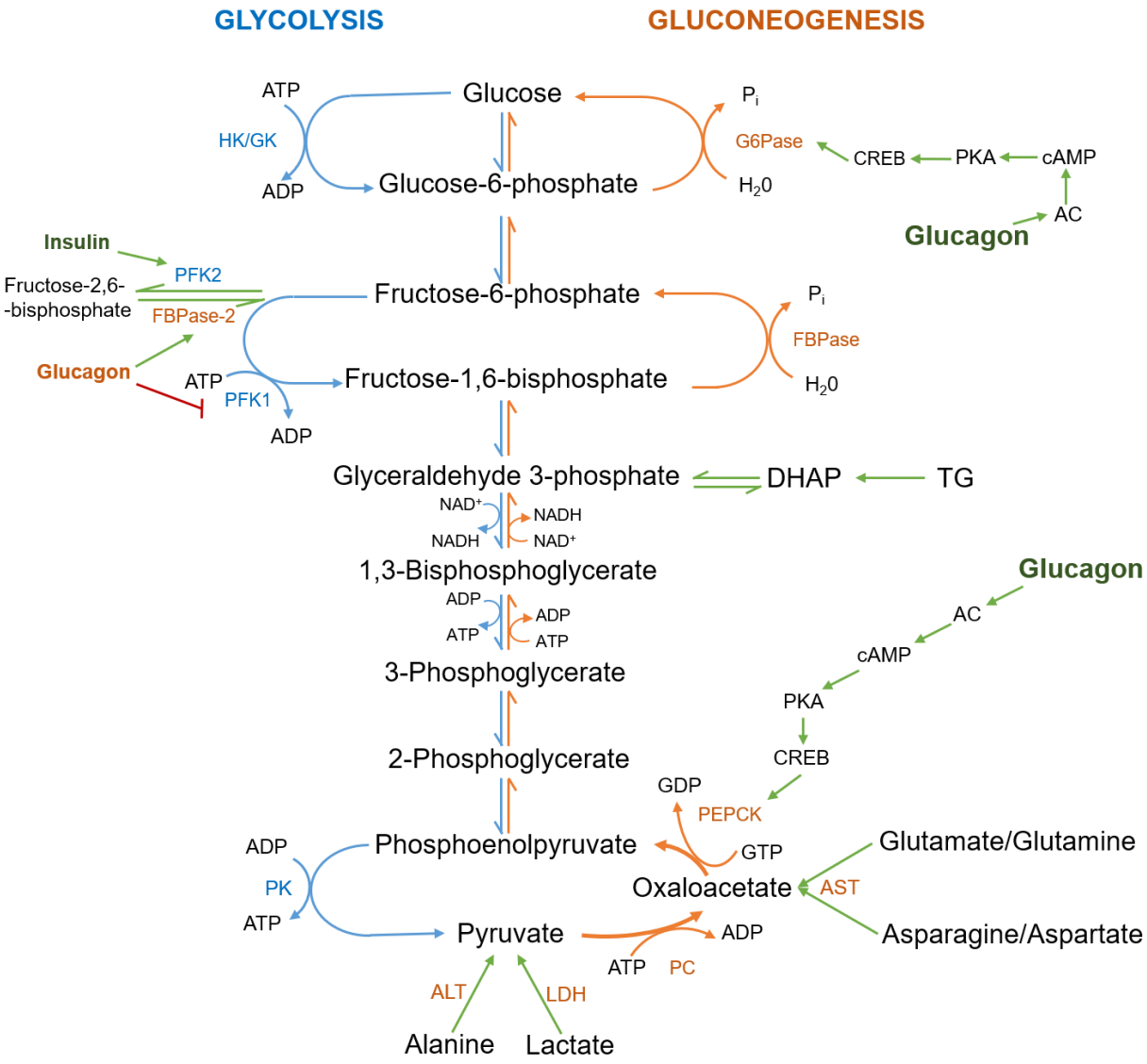



**Figure 1.1 Enzymes and metabolites regulating glycolysis and gluconeogenesis**

Major enzymes and metabolites involved in glycolysis (blue) and gluconeogenesis (orange) including glucogenic amino acids and stimulatory processes (green) and inhibitory processes (red). Enzymes in glycolysis: HK, hexokinase; GK, glucokinase; PFK1, phosphofructokinase-1; PFK2, phosphofructokinase-2; PK, pyruvate kinase. Enzymes in gluconeogenesis: G6Pase, glucose-6-phosphatase; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; FBPase, fructose 1,6-bisphosphatase; FBPase-2, fructose-1,2-bisphosphatase. Glycerol, derived from triglyceride (TG) breakdown is converted to dihydroxyacetone phosphate (DHAP) and subsequently to glyceraldehyde-3-phosphate or fructose-1,6-bisphosphate to enter the gluconeogenic pathway. Glucogenic amino acids such as glutamate/glutamine or asparagine/aspartate are converted into alanine or Krebs cycle intermediates, acting as substrates for gluconeogenesis. AST, aspartate aminotransaminase; ALT, alanine aminotransaminase; LHD, lactate dehydrogenase. Glucagon binds the hepatic glucagon receptor, activating adenylate cyclase (AC) and generates cAMP, which in turn, stimulates cAMP-dependent protein kinase (PKA) which phosphorylates the cAMP-response element binding protein (CREB). In the nucleus, phosphorylated CREB activates the transcription of PEPCK and G6Pase genes.

Gluconeogenesis is the process by which glucose is produced from non-carbohydrate sources such as amino acids (AAs), lactate and glycerol. However, the HK/GK, PFK-1 and PK steps of glycolysis are irreversible and overcome in the gluconeogenic pathway by four enzymes unique to this process, glucose-6-phosphatase (G6P), fructose-1,6-bisphosphatase (FBPase), cytosolic PEP carboxykinase (PEPCK) and pyruvate carboxylase (PC) (Ekberg *et al.*, 1999), as indicated in Figure 1.1. Substrates such as lactate (Cori and Cori, 1929) and alanine (Felig *et al.*, 1970) are first converted into pyruvate, which enters the mitochondria and is carboxylated by PC into oxaloacetate (OAA). OAA is reduced to malate and shuttled to the cytoplasm and converted back to OAA, where it is converted to PEP by PEPCK. There are other glucogenic AAs such as aspartate and glutamate that are first converted into alanine or intermediates of the Krebs cycle for gluconeogenesis (Mallet, Exton and Park, 1969). Glycerol from AT in converted in the liver to dihydroxyacetone phosphate (DHAP) and subsequently into glyceraldehyde-3-phosphate or fructose-1,6,-bisphosphate to enter the pathway (Song *et al.*, 2001). Dysregulation or deficiency in the key regulatory enzymes described above also contributes to the pathogenesis of diabetes.

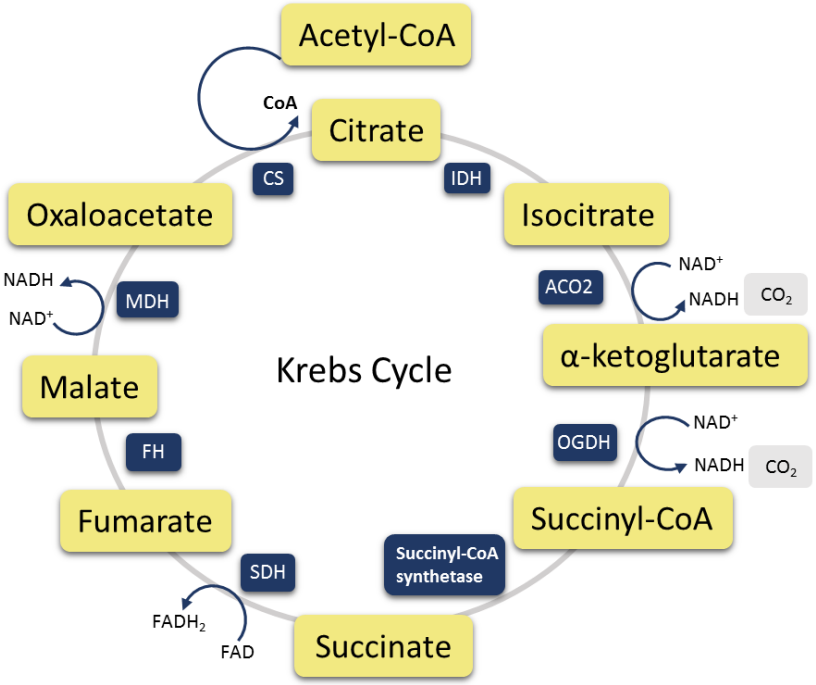



**Figure 1.2. Krebs cycle (TCA/Citric acid cycle)**

Process by which acetyl-CoA derived from carbohydrates, fats or proteins is oxidised to generate ATP and $CO_2$ in the mitochondrial matrix. IDH, Isocitrate dehydrogenase; ACO2, acotinase; OGDH, α-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; FH, fumarate hydratase; MDH, malate dehydrogenase; CS, citrate synthase.

### 1.2.4 Oxidative phosphorylation (OXPHOS)

In OXPHOS, the final stage of cellular respiration in eukaryotic cells, the electrons released by re-oxidation of by-products of the Krebs cycle create an electron-motive force that is converted into a proton-motive force as electrons flow through the electron transport chain (ETC) across the inner mitochondrial membrane. Electrons ($e^-$) originate from NADH entering complex I (NADH ubiquinone reductase), succinate conversion to fumarate at complex II (succinate dehydrogenase) and oxidation of $FADH_2$ (Slack, 2013). Electron flow is then directed by reduced ubiquinone which is processed through complex III, and $e^-$ passed to cytochrome c and directed to complex IV (cytochrome c oxidase) (Jastroch *et al.*, 2010). These complexes generate protons to create a proton gradient across the mitochondrial membrane which is used to drive ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate ($P_i$) at complex V (ATP synthase, $F_0F1$-ATPase). ATP synthesis is coupled (partly mediated by uncoupling proteins, UCPs) to this proton gradient and offset by proton leak (flow of protons in the opposite direction) (Jastroch *et al.*, 2010). The ETC also generates significant ROS, such as superoxide, so that mitochondrial oxygen consumption is associated with oxidative damage by oxygen radicals (Balaban *et al.,* 2005). A schematic representation of the complexes involved in this pathway is represented in Figure 1.3.

Mitochondrial dysfunction contributes to the pathophysiology of IR and is seen in both T1D and T2D as mitochondrial biogenesis, number, morphology and fission/fusion dynamics become dysfunctional (Sivitz and Yorek, 2010). The mechanisms by which mitochondrial dysfunction contributes to IR are described in more detail below.

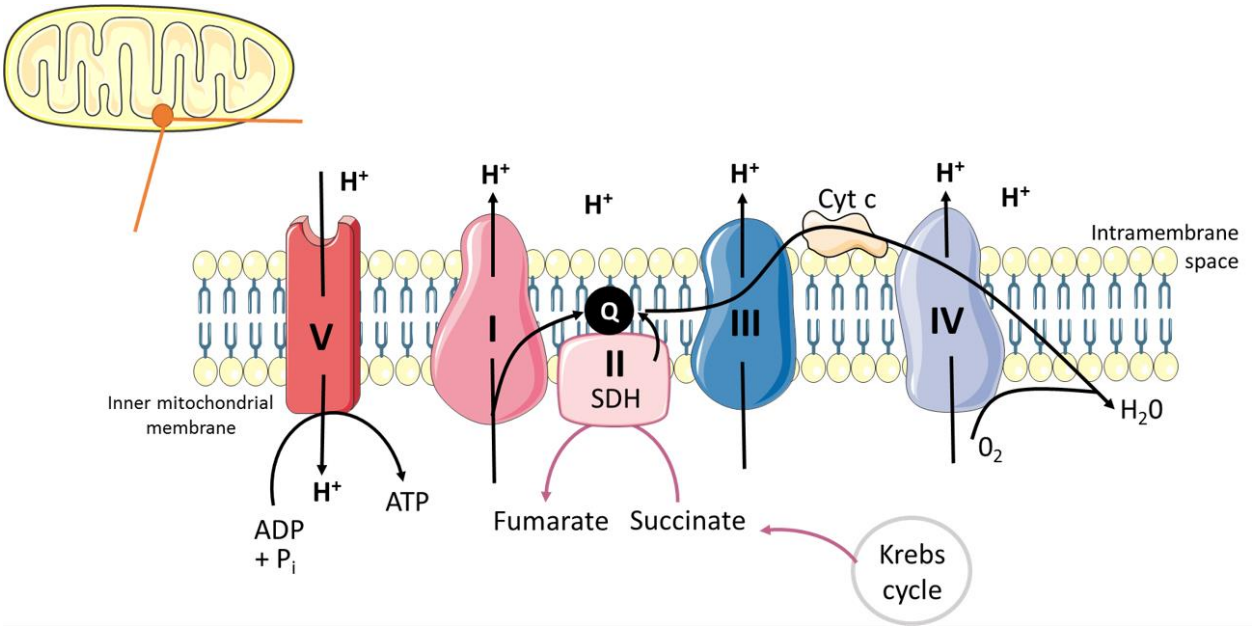



**Figure 1.3. Electron transport chain and oxidative phosphorylation**

Complexes involved in ETC and OXPHOS across the inner mitochondrial membrane. Cyt c, cytochrome C; Complex I, NADH dehydrogenase; Complex II, SDH, succinate dehydrogenase; Complex III, ubiquinol-cytochrome C reductase; Complex IV, cytochrome-c oxidase; Complex V, $H^+$-transporting ATPase.

### 1.2.5 Pancreas

Pancreatic endocrine cells cluster in islets of Langerhans which account for 1-2% of pancreatic mass (Chandra and Liddle, 2009). These cells produce and secrete insulin, amylin and C-peptide (β-cell); glucagon (α-cell); somatostatin (δ-cell); ghrelin (ε-cell) and pancreatic polypeptide (PP cells) (Jouvet and Estall, 2017).

In response to elevated plasma glucose, AAs and fatty acids (FAs) following a meal, the β-cells synthesise and secrete insulin, a process referred to as glucose-stimulated insulin secretion (GSIS) (Ashcroft *et al.*, 1994). Insulin acts in target tissues to promote glucose disposal, glycogenesis (Miller and Larner, 1973; Sibrowski and Seitz, 1984), lipogenesis (Walton and Etherton, 1986; McTernan *et al.*, 2002) and protein synthesis (Biolo *et al.*, 1995), representing one of the most important anabolic stimuli in the body. Elevated blood glucose causes glucose to enter the β-cell via facilitated diffusion through GLUT2 on the cell surface and glucose is metabolised intracellularly through glycolysis and OXPHOS, resulting in the generation of ATP and increased ATP/ ADP ratio. This causes closure of the ATP sensitive potassium (K) channel ($K_{ATP}$) and subsequent decrease in the outward-directed $K^+$ current, causing membrane

depolarisation and influx of calcium ions ($Ca^{2+}$) through L-type voltage-gated calcium channels (VGCC). The increase in intracellular calcium concentration ($[Ca^{2+}]_i$) results in the translocation and fusion of insulin-containing large dense-core vesicles to the plasma membrane and insulin secretion (IS) into the circulation (Komatsu *et al.*, 2013). Insulin granules contain an equimolar amount of insulin and C-peptide, and as C-peptide has a slower metabolic clearance than insulin, it is often used as a marker of endogenous insulin secretion (Jones and Hattersley, 2013). Although genetically and functionally similar (identical catalytic GK activity) (De Vos *et al.*, 1995; Ferrer *et al.,* 1995; McCulloch *et al.*, 2011), rodent and human islets differentially express GLUT2 and GLUT1 and GLUT3, respectively, which results in differing glucose-sensing properties. The higher affinity of GLUT1 and GLUT3 transporters leads to insulin secretion at lower glucose concentrations in humans compared to mice (Henquin *et al.,* 2006) and coincides with the differences in basal glucose concentrations between species (4-7 mM in man (Aronoff *et al.*, 2004) compared to 7-14 mM in mice (Togashi *et al.*, 2016).

GSIS is bi-phasic, occurring via an initial transient “first-phase response” followed by a gradually developing secondary secretion (Curry *et al.,* 1968). First phase GSIS occurs from vesicles already present at the plasma membrane in response to increased nutrient availability (MacDonald, Joseph and Rorsman, 2005). Diabetes is associated with the selective loss of first phase IS (White, Shaw and Taylor, 2016). Second phase IS requires metabolisable secretagogues (glucose or lipids), *de novo* vesicle biosynthesis and sustained maintenance of vesicular transport to the plasma membrane (Gembal *et al.,* 1992; Henquin, 2000). Vesicular transport and membrane fusion are partly regulated by the superfamily of the soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor proteins (SNAREs) (Jewell *et al.,* 2010).

Importantly, in a pre-diabetic state, β-cells expand to compensate for higher insulin requirements (Chen *et al.*, 2017). Over time, however, β-cell compensation progressively fails to meet insulin requirements. This occurs in conjunction with enhanced mitochondrial ROS production (Newsholme *et al.*, 2016) driven by higher circulating glucose and lipid levels, leading to glucolipotoxicity, inflammation, and ultimately β-cell fatigue and death (Cnop *et*

*al.*, 2005; Hotamisligil and Erbay, 2008). In contrast, blood glucose decreases during fasting. Low blood glucose, or hypoglycaemia, leads to suppression of IS (from healthy β-cells) and subsequent stimulation of glucagon from pancreatic α-cells (discussed further under counterregulation). This increases hepatic and renal gluconeogenesis to increase blood glucose levels (Orci *et al.*, 1975; Gerich *et al.*, 1979). Glucagon secretion is also stimulated by sympathetic and parasympathetic nerves of the autonomic nervous system (ANS) activated by hypoglycaemia-sensing neurones in the hepatoportal vein (Hevener, Bergman and Donovan, 1997; Burcelin, Dolci and Thorens, 2000) and central nervous system (CNS) (Marty *et al.,* 2007; Lamy *et al.*, 2014). Conversely, during hyperglycaemia, insulin (Ostenson, 1979) and somatostatin (Starke *et al.,* 1987) suppress glucagon secretion. A state of hyperglucagonaemia is characteristically seen in diabetes, where hyperglycaemia occurs in response to lack of postprandial inhibition of glucagon secretion (Baron *et al.*, 1987), and this is not suppressed by exogenous insulin administration in T1D (Dinneen *et al.*, 1995).

### 1.2.6 Liver

Hepatic glucose production (HGP) accounts for approximately 90 % of endogenous glucose production (Ekberg *et al.*, 1999), and is maintained by glucose-raising (gluconeogenesis, glycogenolysis) and glucose-lowering (glycogen synthesis, glycolysis) processes (Rui, 2014). The liver is also a main site for lipid biosynthesis, maintaining lipid fluxes between meals (Nguyen *et al.*, 2008). Systemic regulation of glucose homeostasis by the liver primarily arises from an initial increase in glycogenolysis and subsequent onset of gluconeogenesis, as glycogen stores become depleted (during prolonged fasting) (Röder *et al.*, 2016). Obligate glucose-consuming tissues such as the brain rely on HGP during fasting whereas, postprandially, the liver suppresses glycogenolysis and gluconeogenesis and promotes glucose uptake (Moore *et al.*, 2012).

HGP is hormonally regulated by insulin, which directly enhances glycogen synthesis (maximal during hyperglycaemia and hyperinsulinaemia) (Al-Khalili *et al.*, 2005) and acutely suppresses adipose lipolysis, reducing supply of FAs and glycerol for gluconeogenesis (Chakrabarti *et al.*, 2013). Glucagon suppresses hepatic glycolysis by inhibiting PFK-1 (Pilkis *et al.*, 1982) while promoting

gluconeogenesis in a cyclic-adenosine monophosphate (cAMP)-protein kinase A (PKA) and cAMP-response element binding protein (CREB)-mediated manner, enhancing activation of PEPCK and G6P (Gonzalez and Montminy, 1989).

In T2D, impaired hepatic glucose metabolism is primarily a consequence of lipid-induced IR, enhanced glucagon levels and excessive lipolysis. The mechanisms by which these factors regulate gluconeogenesis are beyond the scope of this thesis but, for reference, were comprehensively reviewed by Peterson and colleagues (Petersen *et al.,* 2017).

### 1.2.7 Adipose tissue

AT, namely white adipose tissue (WAT) plays an important metabolic role in its ability to store excess fat in the form of triglycerides (TG) through lipogenesis (Trayhurn, 2007). In instances of negative energy balance, energy expenditure requirements or when storage exceeds adipocyte capacity, these TG are broken down into free FAs (FFAs) and glycerol via lipolysis (Lafontan and Langin, 2009). These are then released into the circulation and utilised by muscle, liver and other organs to maintain energy balance.

Postprandial glucose uptake into AT accounts for ~10-15 % of total body glucose disposal. However, alterations in adiposity play a critical role in impaired glucose homeostasis. Anti-hyperglycaemic mediators released by AT are endocrine in nature, such as adipokines leptin and adiponectin (Lago *et al.*, 2009). Leptin is particularly important in repressing orexigenic pathways in the hypothalamus to suppress food intake. Leptin also enhances muscle and liver insulin sensitivity (Amitani *et al.*, 2013), and reduces intramyocellular lipid (IMCL) accumulation by activation of AMP-activated protein kinase (AMPK) (Minokoshi *et al.,* 2012). AT also responds to insulin by decreasing lipolysis while enhancing FA and TG synthesis from lipids and glucose (Dimitriadis *et al.*, 2011).

Paradoxically, AT releases blood glucose-raising mediators such as resistin, cytokines tumour necrosis factor α (TNF-α) (Hotamisligil, 1999) and interleukin 6 (IL-6) (Rotter *et al.,* 2003) and excessive non-esterified FAs (NEFAs) (Roden *et al.*, 1996). The involvement of these markers in the pathogenesis of IR is explored below.

### 1.2.7.1 Lipogenesis

Lipogenesis is the process by which FFAs and subsequently TGs (esterified), are synthesised from acetyl-CoA subunits produced during glycolysis and occurs primarily in AT and liver (Coleman and Lee, 2004) (and at a smaller scale in SM (Hollands and Cawthorne, 1981). The flow of carbons from glucose to fatty acids occurs via a coordinated series of enzymatic reactions summarised in Figure 1.4. Glucose, via glycolysis and the Krebs cycle, is converted to citrate, which is transported to the cytoplasm and is acted on by ATP-citrate lyase (ACLY) to release acetyl-CoA (Watson *et al.,* 1969). The resulting acetyl-CoA is irreversibly converted to malonyl-CoA under the regulated catalytic activity of acetyl-CoA carboxylase 1 (ACC1), marking the first step in fatty acid synthesis (Bianchi *et al.*, 1990). Synthesis of saturated long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH is then catalysed by fatty acid synthase (FASN) (Wakil, 1989). Repeated condensation, reduction, dehydration and reduction of acetyl-CoA and malonyl-CoA bound to the acyl-carrier protein domain of FASN generates (initially) a 16 carbon-chain fatty acid, referred to as palmitic acid (or palmitate). Palmitate is subsequently elongated and desaturated to generate different carbon-chained complex fatty acids such as palmitoleic acid, stearic acid and oleic acid.

Lipogenesis is regulated by allosteric regulation of ACC and at the transcriptional level by activation of the sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP) (Sanders and Griffin, 2016). In the fed period, insulin stimulates lipogenesis by PI3K-PKB-mediated activation of SRBP1c, which, once converted to a mature form, translocates to the nucleus to promote the transcription of lipogenic genes such as FASN and ACC (Peterson *et al.*, 2011; Ricoult and Manning, 2013). This results in increased TG storage in adipocytes and low-density lipoprotein (LDL) in hepatocytes (Kersten, 2001). At high glucose (postprandially and during hyperglycaemia) lipogenesis is also stimulated via activation of ChREBP by metabolites of glycolysis (in an insulin-independent manner) (Uyeda and Repa, 2006; Dentin *et al.*, 2012). Lipogenesis is suppressed in the postabsorptive period, primarily due to inhibition of ACC by activated AMPK and enhanced

cAMP-PKA signalling (Kawaguchi *et al.*, 2001; Munday, 2002; Lu and Shyy, 2006).

Dysregulated *de novo* lipogenesis can result in metabolic disease, with the most prevalent condition associated with enhanced lipogenesis being non-alcoholic fatty liver disease (NAFLD) (Kawano and Cohen, 2013). NAFLD is associated with hepatic insulin resistance and increased risk of T2D (Tilg *et al.,* 2017).

### 1.2.8 Skeletal muscle

SM is not only vital for physical movement and posture but also has critical metabolic and endocrine roles contributing to glucose disposal, lipid oxidation and protein metabolism. Muscle mass is maintained by a balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) and dysregulation of this balance leads to muscle atrophy seen in ageing (sarcopenia) (Rennie *et al.*, 2010), which is aggravated in disuse and T2D (Park *et al.*, 2006). The ability to maintain MPS in response to anabolic stimuli such as protein and AA ingestion is lost in these states, a phenomenon referred to as anabolic resistance (Hodson *et al.*, 2019) and this is associated with lipid overflow and IR (Stephens *et al.*, 2015). As muscle accounts for 80 % of whole-body insulin-stimulated glucose disposal, and is highly metabolically flexible, as proposed by Randle's glucose-FA cycle hypothesis (Randle, 1998), it is an important mediator of IR in T2D.

In the postprandial period, glucose metabolism is favoured over fat metabolism as insulin stimulates GLUT4-mediated glucose uptake. Glucose is metabolised in glycolysis, glycogen synthesis and pyruvate oxidation, while FA oxidation (FAO) is suppressed (Randle, 1998). During fasting, cellular energy depletion activates AMPK, which releases the inhibition on FAO while stimulating glucose uptake (Hardie and Pan, 2002). AMPK is also activated by an increase in the AMP/ATP ratio during exercise which increases the transcription, translation and activity of the transcriptional coactivator PPAR gamma coactivator 1-alpha (PGC1-α), which in turn co-activates several factors promoting mitochondrial biogenesis and OXPHOS (Herzig and Shaw, 2018). Increased mitochondrial biogenesis, OXPHOS and FAO improve insulin sensitivity (Pagel-Langenickel *et al.*, 2010).

In addition to insulin-mediated glucose uptake, SM partly regulates glucose homeostasis by acting as a reservoir for AAs, released during fasting to promote gluconeogenesis and sustain glycaemia. As an endocrine tissue, it releases myokines such as myostatin (Han *et al.*, 2013) and irisin (Vaughan *et al.*, 2015) as well as IL-6 (Steensberg *et al.*, 2000; Pedersen and Febbraio, 2008) and macrophage migration inhibitory factor (MIF) (Reimann *et al.*, 2010) which act in an autocrine and paracrine manner to regulate energy homeostasis.

Skeletal muscle metabolism is a major focus of this thesis so the pathways that are critical for its role in the pathophysiology of diabetes and energy homeostasis are introduced in more detail below, including insulin signalling, FAO and AA sensing and signalling.

#### 1.2.8.1 Fatty acid oxidation

Mitochondrial FAO (β-oxidation) in liver and SM provides energy from FAs when circulating levels of glucose and lipids are lower (during fasting). Figure 1.4 summarises the main steps in FA transport across plasma and mitochondrial membranes for β-oxidation. FFAs or TGs (first hydrolysed by lipoprotein lipase) are first taken up by cells, primarily by FA transporter proteins (FATP) (Bonen *et al.,* 1999). SM expresses FATP1 and FATP4, which enhance uptake of long-chain and very-long chain FAs across the membrane (Black *et al.*, 2009). Uptake is also regulated by plasma membrane FA binding proteins (FATBPpm) and FA translocase (CD36) (Cameron-Smith *et al.*, 2003) and these, along with cytoplasmic FABPs regulate the rate of FAO (Binas *et al.*, 2003). FATPs and a second family of membrane proteins, the long-chain acyl-CoA synthetases (ACSL) ensure that FAs are rapidly converted to acyl-CoAs in preparation for mitochondrial import (Soupene and Kuypers, 2008).

As mitochondrial membranes are impermeable to acyl-CoAs, these are converted to acyl-carnitines by carnitine palmitoyltransferase 1 A and B (CPT1) at the outer mitochondrial membrane. Acyl-carnitines are transported across the mitochondrial membrane by carnitine acyl-carnitine translocase (CACT) and, once inside, are converted back into acyl-CoAs by carnitine palmitoyltransferase 2 (CPT2) in the inner mitochondrial membrane, to enter the β-oxidation pathway (Van der Leij *et al.*, 2000; Ramsay *et al.,* 2001; Bonnefont *et al.*, 2004).

This pathway is a series of four enzymatic reactions in which acyl-CoAs of different lengths are oxidised to generate a two-carbon shortened acyl-CoA and acetyl-CoA as well as $FADH_2$ and NADH after each cycle. The resulting acyl-CoA enters another cycle and acetyl-CoA is used in the Krebs cycle, ultimately generating more electron carriers, which along with NADH and $FADH_2$ from this process, enhance OXPHOS.

CPT1 catalyses one of the rate-limiting steps of FAO (Bonnefont *et al.*, 2004) and CPT1 activity is directly regulated by inhibition from malonyl-CoA. One of the triggers for enhanced FAO during energy depletion is the activation of AMPK, which phosphorylates acetyl-CoA carboxylase (ACC), inhibiting its effect on the generation of malonyl-CoA from acetyl-CoA, and therefore suppressing inhibition at the level of CPT1. Another important regulator of FAO is PDH. Its upstream regulators such as pyruvate dehydrogenase kinase (PDK) are allosterically inhibited by elevated acetyl-CoA and NADH generated during FAO, which leads to phosphorylation and inactivation of PDH. This spares pyruvate for gluconeogenesis to prevent hypoglycaemia. Increased FA availability also enhances citrate levels, which inhibit PFK1 and PFK2, phosphoinositide-dependent kinase 1 (PDK1) and GLUT, suppressing glucose uptake (Garland *et al.,* 1963; Williams and O'Neill, 2018).

Appropriate lipid handling and mitochondrial function are important regulators of metabolic flexibility and both impaired FAO and mitochondrial dysfunction are important aetiological factors in the development of IR (introduced below).

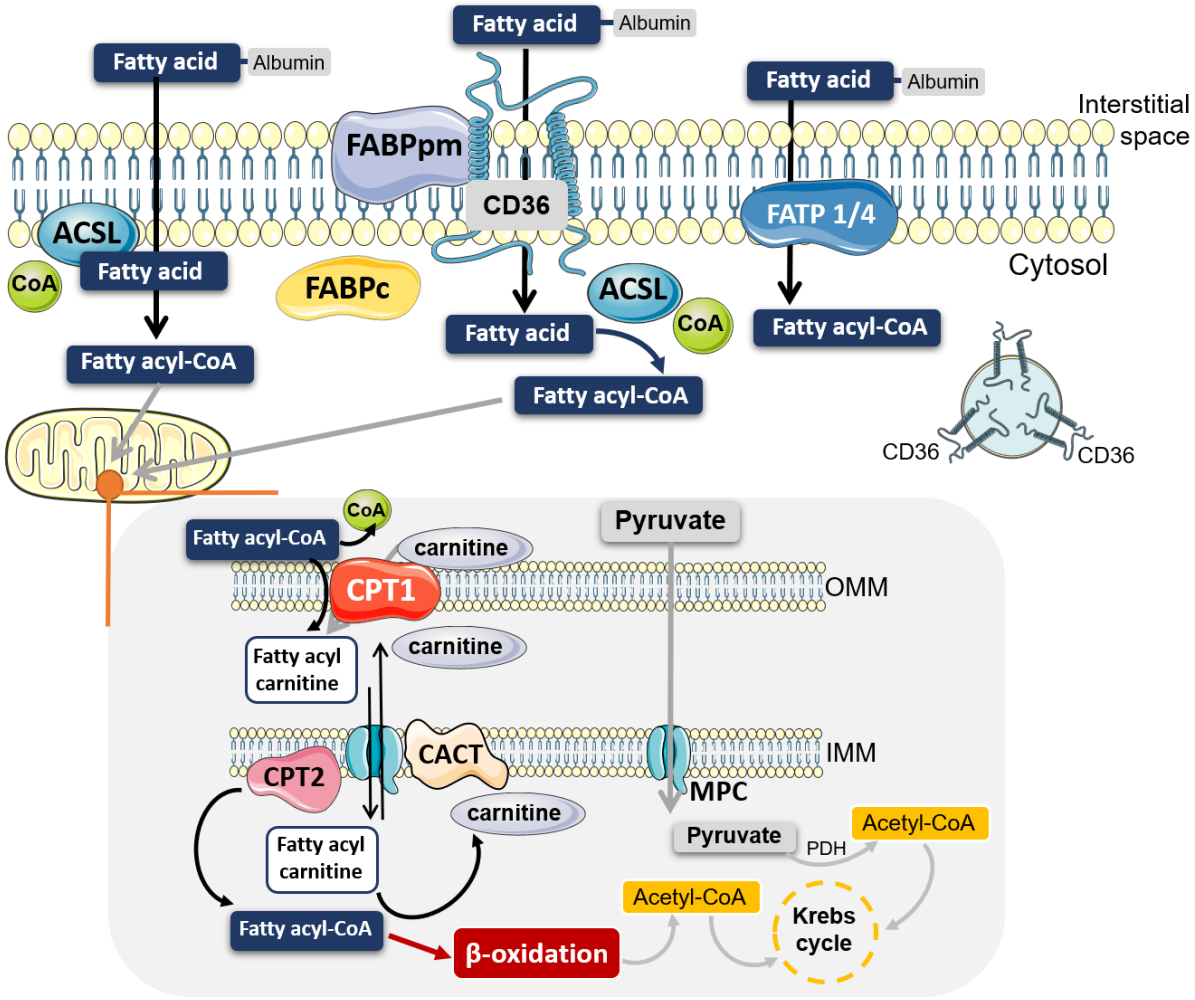



**Figure 1.4. Fatty acid transport across plasma membrane and mitochondria for β-oxidation**

Fatty acids must be taken up into tissues for utilisation. This occurs via diffusion or protein-regulated facilitated transport across membranes. Facilitator proteins include CD36 (whose extracellular site interacts with transporter protein FABPpm), which accept fatty acids and facilitates membrane translocation. In the cytosol, cytosolic binding proteins (FABPc) assist in binding and transport to utilisation sites. Before entering the mitochondria, plasma-derived or intramyocellular triglyceride-derived fatty acids are converted to acyl-CoAs by acyl-CoA synthetases (ACSL). To enter the mitochondria, a carnitine group is added to each species by carnitine palmitoyltransferase 1 (CPT1) and the resulting fatty acyl-carnitines are shuttled to the mitochondrial matrix via carnitine-acyl carnitine translocase (CACT). In the matrix, CPT2 converts carnitinized species back to fatty acyl-CoAs, which are oxidised in β-oxidation and generate acetyl-CoA. MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase complex.

### 1.2.9 Brain

The brain accounts for ~ 2 % of total body mass but consumes ~20 % of its daily energy (Rolfe and Brown, 1997), preferentially metabolising glucose (Allaman and Magistretti, 2013). The high glucose threshold of GLUT1 and GLUT3 ensures insulin-independent uptake into the brain via the blood brain barrier (BBB) and subsequent uptake into neurones (GLUT3, 4) and astrocytes (GLUT1) (Shah *et al.,* 2012). The primary regions contributing to central control of glucose homeostasis are the hypothalamus, particularly four of the eleven nuclei, the ventromedial (VMH), arcuate (ARC) paraventricular (PVN) and lateral (LHN) hypothalamic nuclei; and brainstem, namely the nucleus of the tractus solaris (NTS) (Williams *et al.*, 2001). These regions integrate stimuli of neuronal (e.g. vagal nerve), nutritional, including glucose (Routh *et al.*, 2014), FA (Lam *et al.*, 2005) and AA (Su *et al.*, 2012) levels; and hormonal (insulin, leptin, ghrelin) (Baskin *et al.*, 1999; Bellinger and Bernardis, 2002) origin to regulate energy expenditure, appetite, fat deposition and the counterregulatory response to hypoglycaemia (Morton *et al.*, 2006).

The ability to sense glucose is attributed to populations of “glucose-excited” (GE) and “glucose-inhibited” (GI) neurones, identified in the 1960s, whose electrical activity is augmented in response to increases and decreases in glucose, respectively (Anand *et al.*, 1964). GE neuronal activation by glucose is akin that of the β-cells, involving ATP-dependent closure of $K_{ATP}$ channels, causing depolarisation and increased $[Ca^{2+}]_i$ and initiation of action potential firing (Beall *et al.*, 2012). Conversely, GI neurones are depolarised in low glucose and both populations rely on AMPK, the intracellular “fuel-gauge” (McCrimmon *et al.*, 2008), to instigate these responses (Beall *et al.*, 2012).

These glucose-sensing brain regions also contribute to the regulation of appetite. Orexigenic (ghrelin, adiponectin) stimuli promote food intake whereas anorexigenic (leptin, insulin) hormones suppress it (Nakazato *et al.*, 2001; Sahu, 2003). These hormonal, along with neuronal, signals are sensed by two populations of functionally opposing neurones, the pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) and agouti-related peptide (AgRP)-containing neurones (in the ARC and NTS) which suppress and promote food intake, respectively (Betley *et al.*, 2013).

### 1.3 Therapeutic approaches to combat hyperglycaemia

Currently available therapeutic strategies primarily aim at combating hyperglycaemia in diabetes (anti-hyperglycaemic agents in T2D), with insulin replacement therapy remaining the cornerstone treatment for T1D and advanced T2D. Although drug development has improved exponentially over the past few decades, it is important to remember that lifestyle interventions, particularly dietary manipulation and exercise, play a critical role in the prevention and therapy of diabetes (primarily T2D).

#### 1.3.1 Exercise

Increased physical activity and regular exercise improve physical and mental well-being and help prevent and treat T2D. Aerobic exercise augments mitochondrial density, insulin sensitivity, compliance, lung function, immune function and cardiovascular output, improving glycaemic control in T2D (Garber *et al.*, 2011).

This is partly mediated by enhanced insulin-stimulated glucose uptake and increased expression of GLUT4 and insulin receptors (Zanuso *et al.*, 2010; Mann *et al.*, 2014). Insulin-independent mechanisms also increase muscle glucose uptake after acute bouts of aerobic exercise (Magkos *et al.*, 2008; Roberts *et al.,* 2013). In T1D aerobic training promotes cardiovascular fitness, muscle strength, insulin sensitivity and improves lipid levels and endothelial function (Colberg *et al.*, 2016). High-intensity interval training shows added benefit to muscle oxidative capacity, insulin sensitivity and glycaemic control in T2D (Fiocco *et al.*, 2013) and does not deteriorate glycaemic control in T1D. Fear of severe hypoglycaemia and lack of knowledge of strategies to avoid hypoglycaemia remain the overriding barriers to maintaining or enhancing physical activity in T1D (Brazeau *et al.*, 2008; Gomez *et al.*, 2015). Resistance training promotes muscle hypertrophy and improves glycaemic control, insulin sensitivity, fat mass and strength in T2D (Gordon *et al.*, 2009) and can minimise the risk of post-exercise hypoglycaemia in T1D (Yardley *et al.*, 2013). Regular exercise also increases muscle capillary density, an important feature given the reduced capillary density and blood flow seen in T1D (Rivard *et al.*, 1999) and impaired micro and macrovascular function associated with loss of muscle mass in T2D (Groen *et al.*, 2014).

A better understanding of the insulin-dependent and independent mechanisms by which exercise regulates whole-body glucose homeostasis and muscle mass and strength is still required to elucidate therapeutically targetable exercise-mimetics.

### 1.3.2 AMPK activators

AMPK emerged as an important therapeutic target given its role in the regulation of glucose/lipid homoeostasis (Hardie, 2004; Kahn *et al.*, 2005), food intake (Xue and Kahn, 2006), insulin signalling (Towler and Hardie, 2007) and mitochondrial function in response to metabolic stress (Fogarty and Hardie, 2010).

AMPK is a serine/threonine protein kinase that is structurally comprised of a catalytic α-subunit and two regulatory β and γ subunits. There are two isoforms of the alpha subunit α1 and α2, two β isoforms, β1 and β2 and three γ isoforms γ1,γ2,γ3 allowing for 12 combinations of heterotrimeric complexes (Carling *et al.*, 1994; Davies *et al.*, 1994). AMPKα1β1γ1 heterotrimers are ubiquitously expressed in many tissues so initial pharmacological AMPK activators were developed to target this complex.

This has proved somewhat limiting given the specific tissues distribution of distinct complexes, strengthened by evidence showing that inactivating mutations and genetic deletion of specific isoforms generates tissue-specific effects (Salt *et al.*, 1998). AMPK is endogenously activated by stimuli that deplete intracellular energy levels, such as nutritional status (decreased glucose) and toxins that inhibit respiration and exercise (Shaw *et al.*, 2004; Hawley *et al.*, 2005). Elevated AMP (and ADP) levels relative to ATP in energy-deficient cells allosterically activates AMPK (Carling *et al.*, 1989), and pharmacological AMP mimetics form part of the class of indirect AMPK activators. Table 1.1 summarises the mechanisms of action and complex specificity of AMPK activators.

Metformin remains the first line of treatment in T2D. A complex picture has developed over the past decade elucidating multiple AMPK-dependent and independent mechanisms of action of metformin (Rena *et al.,* 2017). Some evidence suggests that metformin accumulates in mitochondria and inhibits mitochondrial complex I of the ETC, which indirectly activates AMPK by increasing AMP levels (Bridges *et al.*, 2014). AMPK-dependent actions of

metformin, however, have been questioned by hepatic AMPK knockout experiments in mice (Foretz *et al.*, 2010). More recently discovered, and now most well-accepted mechanism of action of the drug, was the ability of metformin to suppress hepatic gluconeogenesis via inhibition of fructose-1-6-bisphosphatase (Hunter *et al.*, 2018). The group, concomitantly with other's observations (Shaw *et al.*, 2005; Madiraju *et al.*, 2014; Patel *et al.*, 2014), concluded that AMPK activation is neither sufficient nor necessary to acutely suppress HGP but plays an important role in the chronic and indirect attenuation of hepatic insulin resistance (Cool *et al.*, 2006; Fullerton *et al.*, 2013).

Other indirect AMPK activators include thiazolidinediones, which inhibit mitochondrial complex I and activate AMPK in liver and muscle to promote glucose uptake and FAO (Fryer *et al.,* 2002; Brunmair *et al.*, 2004); polyphenols, such as resveratrol which inhibit ATP synthase (Zheng and Ramirez, 2000; Gledhill *et al.*, 2007); ginsenosides that decrease hepatic TG and cholesterol levels and suppress HGP and lipogenesis (Jeong *et al.,* 2014); and α-lipoic acid which suppresses AMPK activity in the hypothalamus to reduce food intake (Kim *et al.*, 2004).

Direct AMPK activators were first explored with 5-aminoimisazole-4-carboxamide riboside (AICAR) which acts as an AMP mimetic (ZMP) and directly binds AMPKγ subunits (Sullivan *et al.*, 1994; Corton *et al.*, 1995). Others include A-769662 which directly binds the catalytic subunit to inhibit AMPK dephosphorylation (Moreno *et al.*, 2008; Scott *et al.*, 2014) and aspirin. Compound 911, more recently developed, specifically targets β1 (but not β2)-expressing complexes with 5-10 fold higher potency compared to A-769662 (Xiao *et al.*, 2013). Small molecule activators are also being developed and characterised. PT-1 directly activates AMPK (γ1 complexes in SM) and downstream substrate ACC independently of changes to AMP/ATP ratio (Pang *et al.*, 2008; Jensen *et al.*, 2015) and indirectly by mitochondrial respiratory chain inhibition (Jensen *et al.*, 2015). Small molecule activator MK-8722 (Van Heek *et al.*, 2017) is highly selective for β1-containing complexes and, like PF-793 (Cokorinos *et al.*, 2017), promotes SM glucose uptake *ex-vivo* in an insulin-independent manner. More recently developed small molecule PAN-AMPK activator O304 showed anti-hyperglycaemic and hyperinsulinaemic effects and attenuated IR in diet-induced

obese (DIO) mice; reduced FGC and markers of IR in humans with T2D treated for 28 days (Steneberg *et al.*, 2018).

Data suggest that AMPK activation in distinct brain regions may be glucose-raising (McCrimmon *et al.*, 2004), directly contrasting its peripheral glucose-lowering effects (summarised in Figure 1.5). Further work is necessary to assess the utility of central and peripheral AMPK activation in the control of whole-body glucose homeostasis. This is explored further in Chapter 5.

**Table 1.1. Direct and indirect AMPK activators and inhibitors**

| Compound | Mode | Mechanism of action | Molecular target | Reference |
|---|---|---|---|---|
| Troglitazone | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Brunmair *et al.*, 2004; Saha *et al.*, 2004; LeBrasseur *et al.*, 2006) |
| Pioglitazone | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Brunmair *et al.*, 2004; Saha *et al.*, 2004; LeBrasseur *et al.*, 2006) |
| Rosiglitazone | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Brunmair *et al.*, 2004; Saha *et al.*, 2004; LeBrasseur *et al.*, 2006) |
| Resveratrol | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex V | (Baur *et al.*, 2006; Gledhill *et al.*, 2007) |
| Quercetin | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex V | (Zheng and Ramirez, 2000; Gledhill *et al.*, 2007; Ahn *et al.*, 2008) |
| Genistein | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex V | (Hwang *et al.*, 2005; Gledhill *et al.*, 2007) |
| Epigallocatechin gallate | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex V | (Zheng and Ramirez, 2000; Hwang *et al.*, 2005) |
| Berberine | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Y. S. Lee *et al.*, 2006; Turner *et al.*, 2008) |
| Curcumin | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex V | (Gledhill *et al.*, 2007; Kim *et al.*, 2009) |
| Ginsenoside Rb1 | Indirect | Increase in AMP:ATP ratio | Unknown | (Shen *et al.*, 2013; Jeong *et al.,* 2014) |

| | | | | |
|---|---|---|---|---|
| Cryptotanshinone | Indirect | Increase in ROS | Unknown | (Kim *et al.*, 2007; Park *et al.*, 2014) |
| α-lipoic | Indirect | Increase in $[Ca^{2+}]_i$ | Unknown | (Lee *et al.*, 2005; Lee *et al.*, 2006; Golbidi, Badran and Laher, 2011) |
| Cordycepin | Indirect | AMP analogue | AMPKγ | (Hawley *et al.*, 2020) |
| R419 | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Marcinko *et al.*, 2015) |
| R118 (Mechanistic R419 analogue) | Indirect | Increase in AMP:ATP ratio | Mitochondrial complex I | (Jenkins *et al.*, 2014) |
| R481 | Indirect | Unknown/ mitohormesis | Unknown/ Complex I | N/A (discussed in Chapter 5) |
| AICAR | Direct | AMP analogue | AMPKγ | (Sullivan *et al.*, 1994; Corton *et al.*, 1995) |
| Thienopyridone (A-769662) | Direct | AMPKβ | β1-containing complexes | (Cool *et al.*, 2006; Scott *et al.*, 2008; Vlachaki Walker *et al.*, 2017) |
| Salicylate (Aspirin pro-drug) | Direct | AMPKβ | β1-containing complexes | (Hawley *et al.*, 2012) |
| Compound-13 (C-2 pro drug) | Direct | AMPKα | α1-containing complexes | (Gómez-Galeno *et al.*, 2010) |
| PT-1 | Direct | AMPKβ | γ1-containing complexes | (Pang *et al.*, 2008; Jensen *et al.*, 2015) |
| MT 63-78 | Direct | AMPKβ | β1-containing complexes | (Zadra *et al.*, 2014) |
| O304 | Direct | Protects from PPC2-mediated dephosphorylation | PAN-activator | (Steneberg *et al.*, 2018) |
| MK 8722 | Direct | PAN-activator | β1 and β2-containing complexes | (Myers *et al.*, 2017; Feng *et al.*,2018) |
| PF-06409577 | Direct | AMPKβ | β1-containing complexes | (Esquejo *et al.*, 2018) |
| PF-06755739 | Direct | PAN-activator | β1 and β2-containing complexes | (Cokorinos *et al.*, 2017) |
| PF-06685249 | Direct | AMPKβ | β1-containing complexes | (Salatto *et al.*, 2017; Willows *et al.*,2017) |

| | | | | |
|---|---|---|---|---|
| Compound 991 | Direct | AMPKβγ | β1/γ2-containing complexes | (Xiao *et al.*, 2013; Willows *et al.*, 2017) |
| Compound C (dorsomorphin) | Direct inhibitor | Type I ATP competitive inhibitor | ULK1 and α1β1γ1 | (Zhou *et al.*, 2001; Handa *et al.*, 2011) |
| SBI-0206965 | Direct inhibitor | Type II inhibitor AMPKα | AMPKα1β1γ1 and α2β1γ1 and ULK-1 | (Dite *et al.*, 2018) |

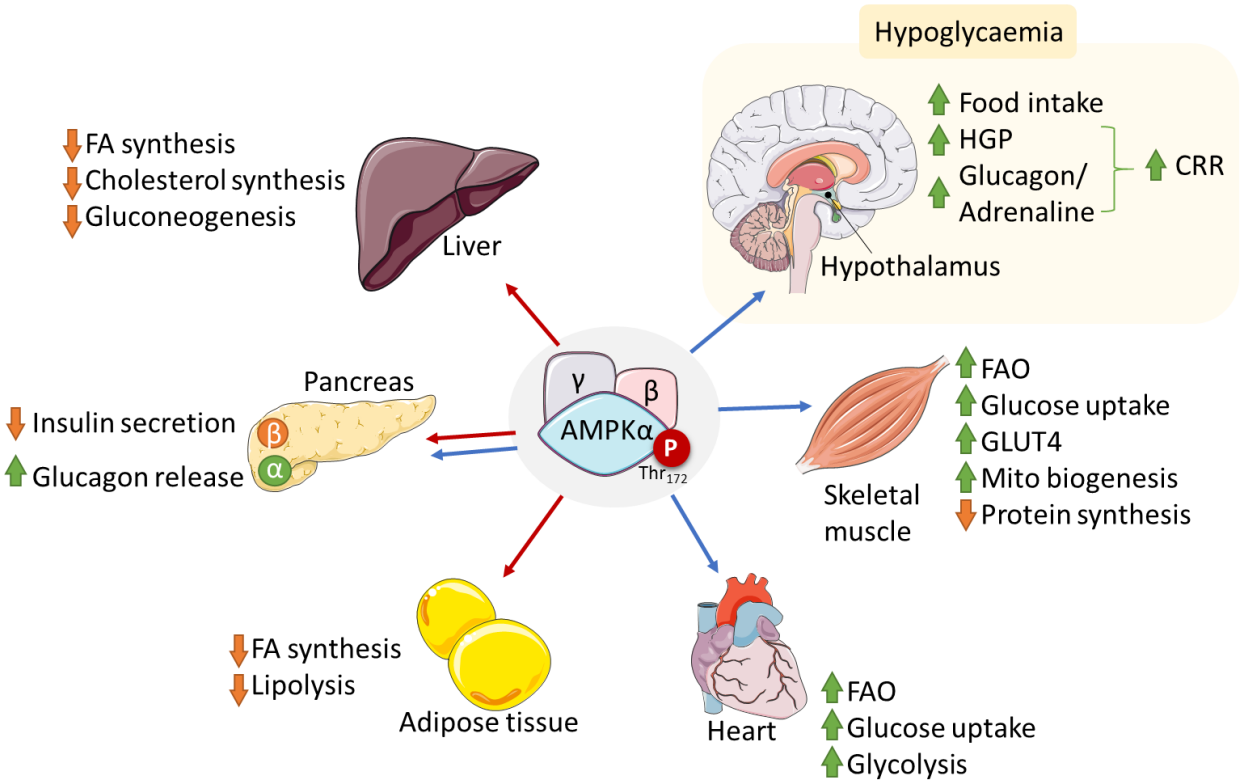



**Figure 1.5. Roles of AMPK activation in the control of whole-body glucose and lipid homeostasis.**

Activation of AMPK in peripheral tissues primarily acts to suppress anabolic processes such as protein synthesis, gluconeogenesis and fatty acid (FA) synthesis; and promote catabolic processes such as glucose uptake, glycolysis and fatty acid oxidation (FAO). These function to decrease blood glucose. Conversely, as depicted in the yellow shaded box, under conditions of hypoglycaemia, AMPK activation in energy sensing brain regions (such as the hypothalamus) has glucose raising effects. Activation of hypothalamic AMPK enhanced the counterregulatory response (CRR) to hypoglycaemia, partly via enhanced glucagon and adrenaline release and stimulates food intake and hepatic glucose production (HGP).

### 1.3.3 Other pharmacological agents

Insulin secretagogues such as sulphonylureas and meglitinides or glinides stimulate insulin release by blocking pancreatic $K_{ATP}$ channels (Madison *et al.,* 1959; Ashcroft, 1996) and their differential pharmacokinetic profiles lead to decreased FPG in the case of sulphonylureas and decreased postprandial glucose by meglitinides (Del Prato and Pulizzi, 2006). Meglitinides are associated with less hypoglycaemic side-effects and weight gain compared to sulphonylureas (Madsbad *et al.*, 2001; Plosker and Figgitt, 2004) and although the latter remain the most cost-effective option (Holman *et al.*, 2008) loss of effectiveness over time is seen in some cases (Takahashi *et al.*, 2007).

More recently developed compounds include dipeptidyl peptidase-4 inhibitors (DPP4). These compounds suppress the inhibition of glucagon-like peptide (GLP-1) and gastric inhibitory peptide (GIP) secretion by DPP4, ultimately improving islet function and glycaemic control in T2D (Deacon, 2011).

Sodium glucose co-transporter-2 inhibitors (iSGLT2) have a unique mechanism of action by inhibiting renal reabsorption of glucose and reducing hyperglycaemia by consequential increased excretion of glucose in urine (Abdul-Ghani *et al.,* 2011; Defronzo *et al.*, 2013). The independence from insulin posts a lower risk of hypoglycaemia and no risk of overstimulation or fatigue of islets (Nauck, 2014). Injectable agents have also been developed, namely GLP-1 receptor agonists (RA-GLP1), which unlike GLP-1 that is rapidly broken down by DPP4, have extended duration. RA-GLP1 treatment effectively lowers HbA1c, FGC, body weight and blood pressure with low risk of causing hypoglycaemia (Willms *et al.*, 1996; Inzucchi *et al.*, 2015).

### 1.3.4 Insulin replacement therapy

Insulin replacement therapy has been the cornerstone of treatment for T1D and advanced T2D for over 8 decades (Owens *et al.,* 2014). In that time, more and better insulin analogues have been developed, including short-acting pre-meal compounds as well as longer-acting once-daily options (Horvath *et al.*, 2007), which can be combined in basal bolus therapy. The inherent pharmacokinetic properties of these agents however, hinder the maintenance of glycaemia in range, partly due to individual variability in absorption (Morello, 2011).

Insulin injection or intranasal administration before a meal can still lead to an elevated postprandial glucose excursion with delayed hypoglycaemia, but these responses are ameliorated by the use of subcutaneously administered insulin via pumps.

The unfortunate consequence of the use of insulin or insulin secretagogues is the increased risk of hypoglycaemia (iatrogenic hypoglycaemia). Hypoglycaemia in T1D and insulin-treated T2D negatively influences quality of life (Alvarez-Guisasola *et al.*, 2010) and promotes fear of future episodes of hypoglycaemia (Anderbro *et al.*, 2010), being associated with less self-care and poorer glycaemic control as well as reluctance towards intensifying therapy (Barnard *et al.*, 2010; Haugstvedt *et al.*, 2010).

The development of strategies to prevent, detect and minimise hypoglycaemic risk is of utmost importance to reduce the social and emotional impact of this phenomenon and help individuals safely achieve glycaemic targets.

### 1.4 Hypoglycaemia

Hypoglycaemia is defined clinically by low plasma glucose levels (< 4.0 mmol/L for insulin-treated individuals), symptomatic response to administration of carbohydrates (or raised plasma glucose) and the development of autonomic or neuroglucopenic symptoms (American Diabetes Association, 2019c). The degree of severity of hypoglycaemia is determined by clinical manifestations from mild to moderate and severe, with severe episodes being characterised by the requirement of assistance from another person, potential loss of consciousness and plasma glucose level < 2.8 mmol/L (Yale, Paty and Senior, 2018), Symptoms can occur after a single hypoglycaemic episode and include autonomic responses such as sweating, anxiety, hunger and nausea; as well as neuroglucopenic symptoms such as confusion, dizziness, difficulty speaking and vision changes (Yale *et al.,* 2018). The frequency of mild hypoglycaemic events is approximately twice-weekly (Ovalle *et al.*, 1998), while severe episodes occur 1-3 times per year (Heller *et al.*, 2007), and the latter are associated with increased morbidity and mortality in T1D (Skyler *et al.*, 2009) and increased risk of cardiovascular complications in T2D (Hayward *et al.*, 2015).

Repeated exposure to hypoglycaemia leads to a reduction in the body's first line of defence against hypoglycaemia, termed the counterregulatory response (CRR) (Ma *et al.*, 2018), as well as reduced psychological and behavioural responses, referred to as impaired awareness of hypoglycaemia (IAH) (Geddes *et al.*, 2008). Some of the strategies available to manage hypoglycaemia include better education and psycho-behavioural programs to improve detection (Kinsley *et al.*, 1999), continuous subcutaneous insulin infusion (CSII) and continuous glucose monitoring (CGM), or both (sensor-augmented pump) to reduce risk of hypoglycaemia (Ly *et al.*, 2013; Little *et al.*, 2014); and islet cell transplantation, which has been shown to restore counterregulation (Rickels, 2012) and reduce hypoglycaemia in T1D (Hering *et al.*, 2016).

### 1.4.1 Counterregulatory response

The CRR to hypoglycaemia refers to the myriad of physiological and behavioural responses (neural-endocrine axis) that converge to raise blood glucose following a progressive decline in plasma glucose concentration (Schwartz *et al.*, 1987).

As plasma glucose falls within the physiological range (4.4-4.7 mmol/L), the body first responds by suppressing insulin secretion to reduce glucose utilisation (Fanelli *et al.*, 1994). If levels continue to fall below 3.6-3.9 mmol/L the release of counterregulatory hormones glucagon, adrenaline and noradrenaline is triggered, with the latter two being released from the adrenal medulla. Adrenaline acts on the liver via β-2 adrenergic receptors to increase glycogenolysis and enhances lipolysis, mobilising gluconeogenic precursors from peripheral tissues to stimulate HGP (Exton, 1987; Dufour *et al.*, 2009). SM contributes to CRR by decreasing glycogenesis (decreases demand for glucose uptake) (Cohen *et al.*, 1995) and by enhancing lactate release (Meyer *et al.*, 2005). Symptom onset occurs in healthy individuals when plasma glucose falls further, below 2.8-3.0 mmol/L, and these are mediated by parasympathetic and sympathetic responses rather than adreno-medullary activation (Exton, 1987; DeRosa and Cryer, 2004).

Glucose sensing by hypothalamic GE and GI neurones plays an important role in the CRR (McCrimmon, 2009). CNS involvement in CRR was supported by observations that intracarotid glucose infusion in dogs suppressed the CRR to hypoglycaemia (Biggers *et al.*, 1989) and intraventricular injection of 2-deoxy-d-

glucose (2-DG), which competes with glucose for cellular uptake but is not metabolisable, enhanced glucagon response to hypoglycaemia (Borg *et al.*, 1995). Hypothalamic (and NTS) glucose sensing machinery also regulates orexigenic and anorexigenic peptide release, with a drop in glucose resulting in increased feeding response. AMPK activation in these regions is believed to be important for appetite regulation (Kola, 2008) and represent a stimulus to amplify CRR (McCrimmon *et al.*, 2006). This is explored in Chapter 5.

In diabetes, disease progression and frequent exposure to hypoglycaemic events lead to impaired CRR (Kleinbaum and Shamoon, 1983; George *et al.*, 1995). This is characterised by a shift to lower plasma concentrations to trigger CRR thresholds (Mitrakou *et al.*, 1993); lack of endogenous suppression of insulin secretion (administered exogenously) and attenuated glucagon and sympathoadrenal responses (Kleinbaum and Shamoon, 1983). Impaired CRR is associated with a 25 fold (or higher) increased risk of severe iatrogenic hypoglycaemia (White *et al.*, 1983) and IAH with a 6 fold higher risk (Geddes *et al.*, 2008). The mechanisms leading to impaired CRR are incompletely understood so a better understanding is required of the central and peripheral neuroendocrine processes mediating these responses.

### 1.5 Mechanisms of insulin action and insulin resistance

The ensuing hyperglycaemia seen in T2D is largely a consequence of inadequate insulin action and understanding the mechanisms of both insulin action and IR is essential for the continued development of novel therapeutic strategies to combat glycaemia dysregulation. Insulin acts on the liver to promote glycogenesis, increase expression of lipogenic genes and suppress gluconeogenesis; while in WAT, it suppresses lipolysis and enhances lipogenesis and glucose transport (Petersen and Shulman, 2018). Insulin also has indirect actions in the CNS (Porte *et al.,* 2005) and suppresses glucagon secretion from α-cells (Cooperberg and Cryer, 2010). Although these are critical insulin target tissues, 85-90 % of the impairment in total body glucose utilisation in T2D is attributed to SM IR (Pendergrass *et al.*, 2007), so emphasis will be placed on SM here.

### 1.5.1 Insulin signalling pathway

A simplified schematic of the insulin signalling pathway in SM is summarised in Figure 1.6. Upon insulin binding, the insulin receptor (INSR) autophosphorylates and recruits insulin receptor substrates (IRS), of which IRS1 and 2 are believed to mediate most of the metabolic effects of INSR activation in SM (White *et al.*, 1988; Taniguchi *et al.,* 2006). Once bound to the INSR, IRSs become phosphorylated at tyrosine residues to propagate either mitogenic or metabolic signalling cascades. IRSs also regulate negative feedback loops, primarily by S6 kinase (S6K) when phosphorylated at serine/threonine sites. Tyrosine-phosphorylated IRS proteins recruit phosphoinositide-3-kinase (PI3K) heterodimers, primarily class I, which in turn catalyse the production of phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$) from phosphatidylinositol-4,5-bisphosphate ($PIP_2$). $PIP_3$ localises downstream effector proteins to the plasma membrane, including PDK1 and protein kinase B1 and 2 in SM (PKB, also known as AKT).

Activation of PKB is initiated by PDK1 phosphorylation at threonine 308 (Alessi *et al.*, 1997), and kinase is fully activated via phosphorylation of serine 473 by mammalian mechanistic target of rapamycin (mTOR) complex 2 (mTORC2). Once activated, PKB primarily regulates translocation of GLUT4-containing storage vesicles to the plasma membrane to promote glucose uptake (Wang *et al.*, 1999). PKB phosphorylates substrates such as GTPase-activating protein (GAP), AKT substrate of 160 kDa (AS160, or TBC1D4) and TBC1D1 (Mîinea *et al.*, 2005). These, in turn, disinhibit Rab GTPase proteins that control vesicle translocation. A second signal for PI3K-mediated GLUT4 vesicle translocation is mediated by the Rho GTPase RAC1 (Khayat *et al.*, 2000). The resulting increase in intracellular glucose availability drives glycolytic flux and promotes glycogen synthesis. Insulin stimulates these by upregulating transcription of HKII and regulates glycogen synthase by PKB-mediated inhibition of glycogen synthase kinase 3 (GSK3) (O'Brien and Granner, 1996).

Insulin signalling is tightly regulated by positive and negative feedback mechanisms. Negative feedback, resulting in attenuated proximal insulin signalling, includes INSR internalisation and dephosphorylation. Receptor internalisation is regulated by substrates such as carcinoembryonic antigen-

related cell adhesion molecule 1 (CEACAM1) (Najjar, 2002; Poy *et al.*, 2002); and dephosphorylation is mediated by protein tyrosine phosphatases (PTPases), primarily PTP1B (Galic *et al.*, 2005). Once activated, INSR contributes to feedforward amplification via activation of NAD(P)H oxidase 4 (NOX4) and NOX4-derived $H_20_2$ inhibition of PTP1B activity (Mahadev *et al.*, 2004; Wu and Williams, 2012).

Besides glycogen and lipid synthesis (in liver), insulin regulates protein synthesis by modulating mTOR signalling. Insulin activates mTORC1 by inactivating mTORC1 inhibitors tuberous sclerosis complex 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) (Inoki *et al.*, 2002; Haar *et al.*, 2007). This, in turn, phosphorylates components of the protein translational machinery such as S6K, and eukaryotic translation initiation factor binding proteins 1 and 2 (4EBP1/2). mTOR mediators are also involved in feedback mechanisms to tune insulin signalling. These include inhibition of IRS activation by S6K1, PKB phosphorylation by mTORC2 (Sarbassov *et al.*, 2005; Hsu *et al.*, 2011; Yu *et al.*, 2011) and mTORC1-mediated phosphorylation of growth factor receptor bound protein 10 (GRB10) which attenuates INSR activity (Hsu *et al.*, 2011). Activation of mTOR signalling by insulin enables the integration of other anabolic pathways, such as AA sensing, with insulin signalling.

### 1.5.2 Pathogenesis of SM insulin resistance

The pathogenesis of SM IR appears to involve an interplay between increased nutrient derived toxic metabolites (lipotoxicity), nutrient stress-mediated toxicity (inflammation) and overwhelmed substrate utilisation machinery (endoplasmic reticulum stress, ROS) (summarised in Figure 1.7). Impairments in insulin signalling pathways are primarily proximal, involving INSR, IRS1, PI3K and PKB. This was evidenced by early work showing impaired INSR receptor expression and function in human and rat obesity and diabetes (Goldfine *et al.*, 1973; Archer *et al.,* 1975). Others demonstrated impaired stimulation of IRS1-PI3K in response to insulin in acute and chronically-induced IR (Dresner *et al.*, 1999; Cusi *et al.*, 2000; Griffin *et al.*, 2000). Impairments in these early responses may mediate most of the pathophysiology of IR, but multiple signalling effectors are known to be dysregulated to bring these about.

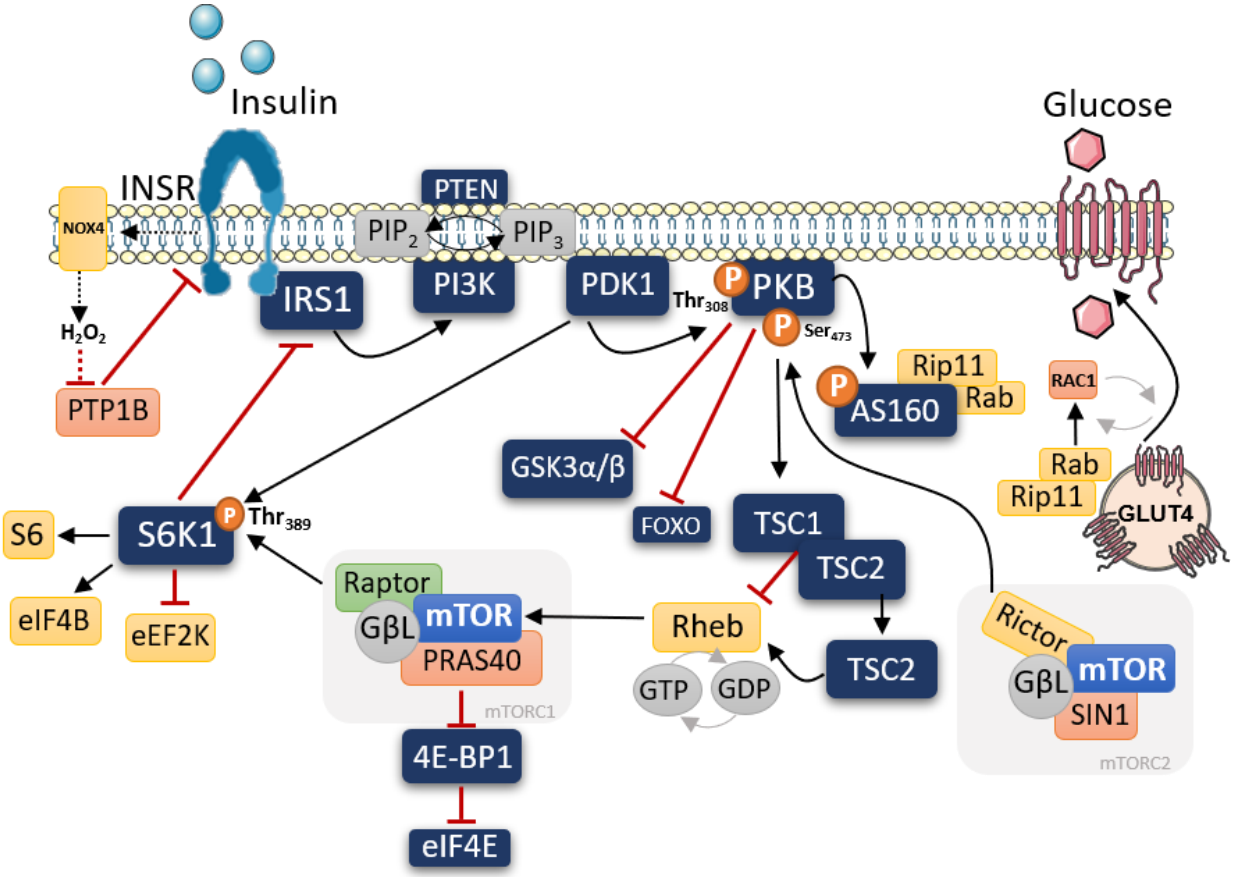



**Figure 1.6 Insulin signal transduction pathway in skeletal muscle**

Biochemical pathway by which insulin promotes glucose uptake into SM to maintain glycaemia. Inhibitory steps indicated by red arrows and stimulatory transduction in black. PTEN, phosphatase and tension homologue. Negative regulator of insulin/PI3K. SIN, mammalian stress-activated protein kinase interacting protein 1. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Of particular interest to this thesis is the contribution of excessive lipid availability, impaired mitochondrial function and inflammation to the development of IR.

### 1.5.2.1 Lipid-induced IR

Lipid-induced IR was first observed in rabbits who were resistant to insulin-induced hypoglycaemia following intravenous lipid infusion (Young., 1962), and later supported by observations of an association between elevated NEFAs and IR (Chen *et al.*, 1987; Golay *et al.*, 1987). Randle and colleagues first suggested that the FA-glucose cycle regulated the impairments in glucose metabolism brought about by FAs (Randle *et al.*, 1963; Randle *et al.,* 1964).

In that hypothesis, increased FA availability would enhance FAO, resulting in elevated acetyl-CoA and citrate which would, in turn, inhibit PDH and PFK1 and suppress glucose oxidation (Randle, 1998). Although extensive *in vitro* and *in vivo* work originally suggested that this might be the main mechanism behind glucose intolerance in T2D, the hypothesis has since been challenged and current evidence suggests that the glucose-FA cycle does not account for the impairment in insulin-stimulated glucose uptake seen following several hours of lipid infusion or in obesity/T2D (Boden *et al.*, 1991; Rahimi *et al.*, 2014).

Currently, the best described mechanisms implicate increased FFA levels (Paolisso *et al.*, 1995), IMCL accumulation (Goodpaster *et al.*, 2000) and enhanced generation of lipotoxic metabolites such as long-chain fatty acyl-CoA (LCFA-CoA) (Cooney *et al.*, 2006), diacylglycerol (DAG) (Itani *et al.*, 2002), acyl-carnitines (Aguer *et al.*, 2015) and ceramides (Summers, 2006) in the pathophysiology of muscle IR (Boden, 2011). The proposed mechanisms are summarised in Figure 1.7. DAG activates protein kinase C-θ (PKCθ), which is believed to target IRS1 and guanine exchange factor GIV/Girdin, the latter being required for insulin-stimulated glucose uptake (Laybutt *et al.*, 1999; Griffin *et al.*, 2000). Ceramides action in IR is two-fold: activation of PKCζ which phosphorylates PKB at a site that lowers affinity for phosphoinositide (Powell *et al.*, 2003) and prevents translocation (Stratford *et al.,* 2001); and by protein phosphatase 2A (PP2A)-mediated dephosphorylation of activated PKB (Fox *et al.*, 2007; Blouin *et al.*, 2010). Both DAG and ceramide accumulation suggest inappropriate anabolic shunting of excess lipid to bioactive species in these conditions.

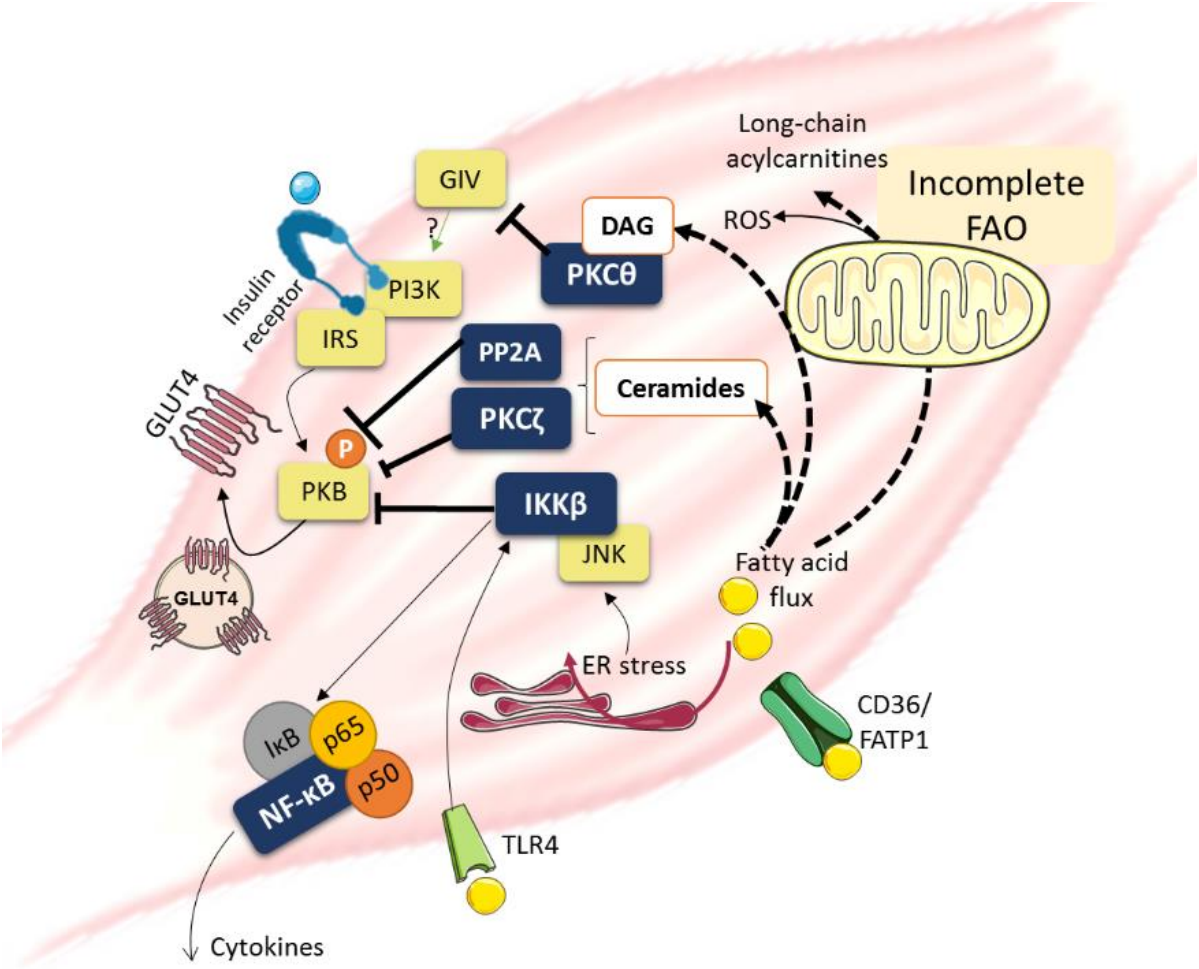



**Figure 1.7 Proposed mechanisms of lipid-induced skeletal muscle insulin resistance.** Diacylglycerol (DAG) is proposed to act via activation of protein kinase θ (PKCθ). Targets of PKCθ, although not clearly defined, may include insulin receptor substrate 1 (IRS1) and guanine nucleotide-binding protein α subunit-interacting vesicle-associated protein (GIV). PI3K, phosphoinositide-3-kinase. Ceramides are proposed to decrease PKB activity via protein phosphatase 2A (PP2A) or PKCζ. Incomplete fatty acid oxidation (FAO) may lead to acylcarnitine species accumulation which can directly contribute to insulin resistance or lead to increased reactive oxygen species levels (ROS). Signalling of species such as palmitate via toll-like receptors (TLR4) may contribute to insulin resistance by activation of inflammatory signalling pathways, which can also be activated by the effect of increased intracellular fatty acid flux on endoplasmic reticulum (ER) stress. JNK, c-Jun N-terminal kinase. IKK, IκB kinase.

### 1.5.2.2 Mitochondrial dysfunction

The two main fates of lipids entering SM are FAO and storage as TGs. Impaired ATP synthesis in T2D is associated with IMCL accumulation and initial hypotheses linked the accumulation of lipid species in muscle with the development of IR (Pan *et al.*, 1997). This was supported by evidence showing decreased mitochondrial activity (Kelley *et al.*, 2002; Fabbri *et al.*, 2017) and SM mitochondrial content (Mootha *et al.*, 2003) and increased mitochondrial damage (Chomentowski *et al.*, 2011) in insulin resistant humans.

An increasing body of evidence now suggests that, contrary to the original hypotheses, a relative increase in β-oxidation flux itself drives IR. Rodents on a high-fat diet (HFD) develop SM IR coupled with an increase in FAO and blocking β-oxidation leads to improved insulin sensitivity, despite IMCL accumulation (Guerre-Millo *et al.*, 2001; Finck *et al.*, 2005; Koves *et al.*, 2008). FAO is also upregulated in response to increased FA availability in diabetic models. However, this relative increase in FAO is not accompanied by markers of complete β-oxidation, such as increased $CO_2$ production, suggesting incomplete FAO (Koves *et al.*, 2008). This results in accumulation of the lipotoxic moieties described above as well as acyl-carnitine and generation of ROS, which impair insulin signalling at the levels of PKB and via activation of inflammatory pathways and mitochondrial damage (Samocha-Bonet *et al.*, 2012).

### 1.5.2.3 Inflammation

Impaired insulin signalling primarily affects the metabolic pathway (PI3K/PKB) while mitogenic pathways such as the mitogen-activated protein kinase (MAPK) cascades remain activated (Cusi *et al.*, 2000). Signalling via MAPK results in upregulated inflammatory pathways associated with IR such as the inhibitor κB (IκB)/nuclear factor κB (NF-κB) and c-Jun $NH_2$-terminal kinase (JNK) cascades (Lee *et al.*, 2003). Activation of IκB/NF-κB signalling pathway is also implicated in SFA-induced IR via toll-like receptor 4 (TLR4) (Shi *et al.*, 2006). These signalling cascades mediate the expression and release of cytokines/myokines, the best described of which in the context of IR being TNF-α, interleukin 10 (IL-10) and IL-6. TNF-α primarily suppresses insulin-stimulated glucose uptake (Bouzakri and Zierath, 2007), whereas IL-10 prevents SFA-induced IR in muscle cells (Kim *et al.*, 2004).

IL-6 has demonstrated both pro and anti-inflammatory roles depending on concentration and length of exposure (Scheller *et al.*, 2011). Figure 1.8 summarises the contribution of inflammatory markers to SM IR (adapted from Wu and Ballantyne, 2017).

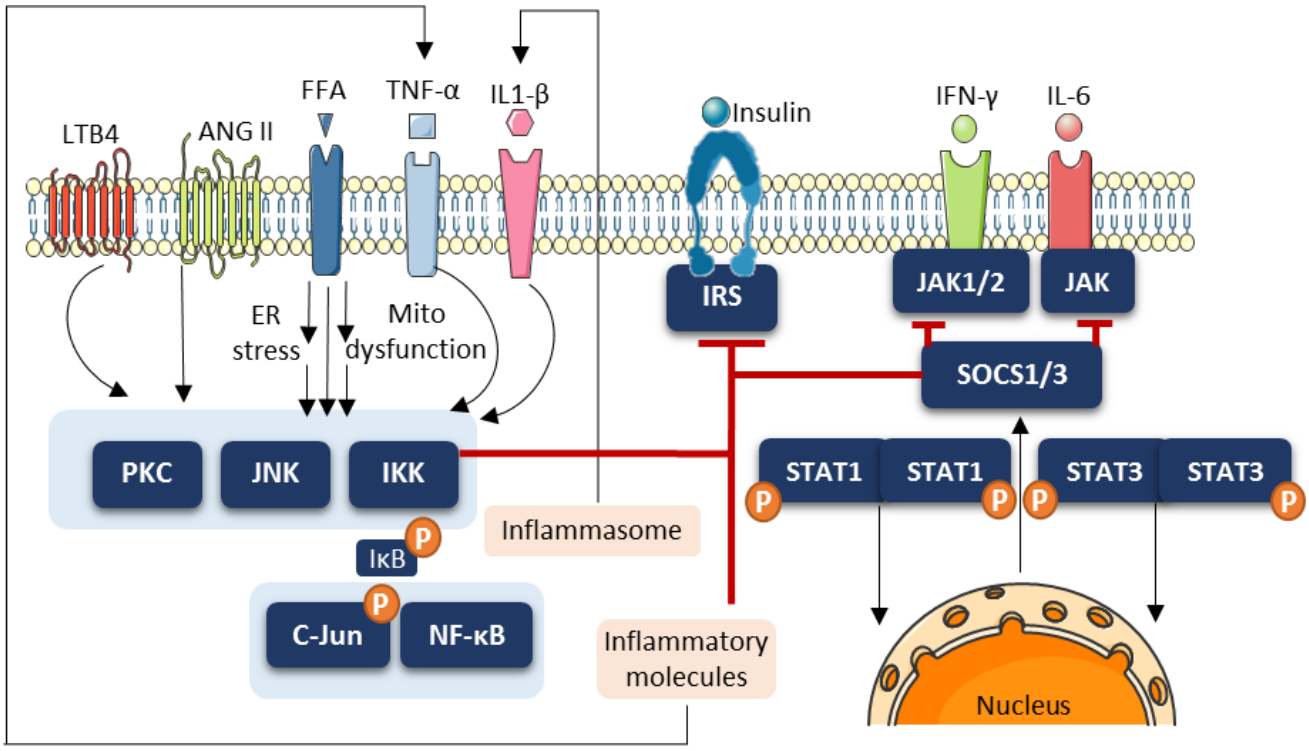



**Figure 1.8 Inflammatory signalling-mediated skeletal muscle insulin resistance**

Extracellular cytokines and inflammatory molecules such as arachidonic acid-derived leukotriene B4 (LTB4), angiotensin II (ANG II), FFAs, TNF-α and interleukin 1β (IL1-β) act on their respective receptors (TLRs in the case of FFAs) to activate PKC/JNK/NF-κB signalling pathways in muscle cells. These can interfere with INSR signalling by disrupting tyrosine phosphorylation and enhancing serine/threonine phosphorylation of INSR or IRSs. IFN-γ and IL-6 activate JAK/STAT signalling pathways that impair insulin signalling, partly via suppressor of cytokine signalling (SOCS) proteins-mediated suppression of INSR/IRS activation. Adapted from Wu and Ballantyne (2017).

### 1.6 Amino acid metabolism

Anabolic resistance to feeding underlies the accelerated loss of muscle mass and function seen in ageing and T2D so a better understanding of impaired AA metabolism is required to better understand its aetiology.

The effect of mixed meal consumption on postprandial MPS was established over 3 decades ago (Rennie *et al.*, 1982), and it was quickly realised that the bioactive components necessary and sufficient to maintain MPS were the essential AAs (EAA): leucine, isoleucine, valine, lysine, tryptophan, phenylalanine, methionine and histidine (Madden *et al.*, 1943). These cannot be synthesised *de novo*, originating from the diet, and, as such, act as important signals for nutritional status in the body. Conversely, non-essential AAs (NEAA) such as glycine, arginine and serine, which are endogenously synthesised, cannot stimulate MPS (Smith *et al.*, 1998). NEAAs are, however, important in the CNS acting as neurotransmitters or neurotransmitter precursors.

Of the EAA, the branched-chain AAs (BCAA - leucine, valine, and isoleucine) are primarily metabolised in SM, so that their metabolites also have anabolic properties. Leucine alone robustly stimulates MPS, even in the absence of other EAAs (Buse and Reid, 1975) and its metabolites, such as β-hydroxyl-β-methylbutarate (HMB), have emerged as potent stimulators of MPS and inhibitors of MPB (Van Koevering and Nissen, 1992; Wilson *et al.*, 2009; Wilkinson *et al.*, 2013). A catabolite of valine, 3-hydroxyisobutyrate (3-HIB) has recently been shown to be released from muscle and act in a paracrine manner to regulate myocellular lipid uptake (Jang *et al.*, 2016). Interestingly, this bioactive moiety has also been implicated in the development of lipid-induced IR, as BCAAs start to emerge as potential mediators of IR (Mardinoglu *et al.*, 2018). This is explored further in Chapter 4.

In the postprandial period, the levels of BCAAs (namely leucine) become elevated in the brain and are sensed by hypothalamic and NTS nuclei to modulate food intake (Heeley and Blouet, 2016). Direct leucine administration into the third ventricle of rodents suppresses fast-induced refeeding (Blouet *et al.*, 2009) and POMC ARC neurones become activated in response to leucine (Blouet *et al.*, 2009; Smith *et al.*, 2015).

Leucine administration into the medial basal hypothalamus (MBH) during pancreatic glucose clamps in rats also decreases plasma glucose due to inhibition of HGP, without affecting glucose disposal into SM (Arrieta-Cruz *et al.,* 2016). Anabolic resistance in central AA sensing is also evidence following high-SFA feeding in rodents (Arrieta-Cruz *et al.,* 2016). Impaired CNS nutrient-sensing dysregulates glucose homeostasis and appetite regulation so a better understanding of the influence of AA sensing and availability in these processes is required to develop better therapeutic strategies for glycaemic control.

### 1.6.1 Amino acid sensing and signalling

The uptake, release and exchange of AAs across cells and organelles are facilitated by the presence of AA transporters (AATs) located in cell and organelle membranes. AATs belong to the superfamily of solute carriers (SLC) and there are 66 known AATs within 11 of the subfamilies of the SLCs (Kandasamy *et al.*, 2018). AATs are also classified into systems such as system A, N, ASC, B, L, T, $X^-_C$ and $Y^+$ according to their mode of transport and specificity. For instance, stimulation with EAA upregulates the mRNA expression of AATs in SM, such as L-type AA transporter 1 (LAT1), sodium-coupled neutral AA transporter 2 (SNAT2) and CD98 at the plasma membrane and proton assisted AA transporter (PAT1) in lysosomes; and this coincides with enhanced MPS (Drummond *et al.*, 2010, 2011).

An increase in MPS in response to increased AA availability is accompanied by activation and increased expression of mTORC1 related signalling markers (Bond, 2016), which enhance anabolic and suppress catabolic processes such as initiation of protein translation and autophagy, respectively (Kimball *et al.*, 1999; Wang *et al.*, 2001; Raught *et al.*, 2004). The upstream regulators of mTORC1 activation were largely unknown in AAs sensing and, although not fully characterised, are now believed to incorporate AA sensors SLC38A9 (Jung, Genau and Behrends, 2015), Sestrin1/2 (Budanov and Karin, 2008; Chantranupong *et al.*, 2014) and cellular arginine sensor for mTORC1 (CASTOR1/2) (Saxton *et al.*, 2016); and signalling pathways implicating MAPK kinase kinase 4 (MAP3K4) (Findlay *et al.*, 2007), Rag GTPases 1-4 (Sancak *et al.*, 2008) and vacuolar protein sorting 34 (Vps34) (Nobukuni *et al.*, 2005).

Although insulin also activates mTORC1, this is not required for EAA-induced MPS, given that blocking PI3K/PKB does not block AA-stimulated MPS (Bolster *et al.,* 2004).

Once signals converge at mTORC1, the complex phosphorylates and inhibits 4E-BP1 which is removed from its association with eukaryotic initiation factor 4F (eIF4F) to enable translation initiation (Sonenberg and Hinnebusch, 2009). mTORC1 also hyperphosphorylates S6K1 at threonine residue 389, which in turn phosphorylates S6 and regulates MPS initiation via eIF4B and elongation via eukaryotic elongation factor 2 (eEF2) (Raught *et al.*, 2004) (Figure 1.5). Phosphorylated mTORC1 levels are poorly detectable in human SM in response to anabolic stimuli (Mitchell *et al.*, 2015), which further supports the existence of more than one input relaying AA sensory information.

Anabolic resistance in older individuals is reflected in impaired S6K1 phosphorylation in response to AA/protein intake compared to younger individuals (Guillet, 2004). However, it is unlikely that this impairment results from a reduction in the available AA pool for MPS, considering intracellular EAA concentrations are higher in older compared to younger individuals (Cuthbertson *et al.*, 2005). A better understanding is still required of the mechanisms impairing anabolic sensitivity with age, which is exacerbated in conditions such as T2D and following disuse (Wall and van Loon, 2013). Assessments of AA metabolism and MPS were made easier by technological advances in detection and sensitivity of mass spectrometry, along with the development of stable AA isotope tracers (Kim *et al.*, 2016), making it possible to assess dynamic changes in uptake, synthesis and breakdown and better understand the development of anabolic resistance. This is explored in Chapter 4.

### 1.7 Purinergic signalling system

Pioneering work by Geoffrey Burnstock in the 1970s elucidated a role for nucleotides and nucleosides in cell-to-cell communication, coining the term purinergic signalling (Burnstock, 1972; Burnstock and Verkhratsky, 2009). This work questioned the role of ATP and other purine and pyrimidine nucleotides such as ADP, uridine triphosphate (UTP), uridine diphosphate (UDP), UDP-

glucose; and nucleosides (adenosine) as purely universal intracellular energy sources, identifying novel extracellular signalling properties (Burnstock *et al.*, 1970). The idea was received with scepticism and only became accepted when the first ATP receptor was cloned (Webb *et al.*, 1993). The purinergic signalling field has since grown exponentially and these nucleotides/sides have been shown to be implicated in a plethora of physiological and pathophysiological processes.

### 1.7.1 Mechanisms of nucleotide release

Intracellular nucleotide release is regulated by processes including vesicular exocytosis, macrovesicles and by a variety of channels and transporters (Burnstock, 2007; Abbracchio *et al.*, 2009). Large amounts of nucleotides can also be released in a non-regulated manner, as a consequence of shear stress induced by mechanical strain, hypoxia, membrane damage or stress induced by cytotoxic agents (Forrester and Williams, 1977; Kroemer *et al.*, 2013). This also occurs during inflammation from disrupted cell membranes where these nucleotides/sides act as “danger signals” leading to immune cell recruitment and activation (Burnstock, 2007, 2008).

In the case of ATP, the nucleotide is produced during cellular respiration and stored intracellularly in cytosolic granules by the action of the vesicular nucleotide transporter (VNUT); and vesicular exocytosis occurs in a SNARE-mediated manner (Sudhof and Rothman, 2009; Imura *et al.*, 2013; Moriyama *et al.*, 2017). Transporters for ATP release include primarily pannexins hemichannels (Pnx) and connexin hemichannels/gap junctions (Cnx); as well as ATP binding cassette transporters (ABC), calcium homeostasis modulators (CALHM) and purinergic receptor X7 (P2X7) (Taruno, 2018). Levels of extracellular ATP can also increase by backward ATP re-synthesis by extracellular activity of adenylate kinase (Dzeja and Terzic, 2009; Tsai *et al.*, 2012), by nucleoside diphosphate kinase (NDPK) (Yegutkin *et al.,* 2001; Donaldson *et al.,* 2002) and ATP synthases, converting AMP to ADP and ATP (Yegutkin, 2008, 2014). These pathways are summarised in Figure 1.9.1.

Once outside cells, the levels of extracellular nucleotides are tightly regulated by the actions of cell surface enzymes (Zimmermann, Zebisch and Sträter, 2012).

There are four main families of nucleotide hydrolysing enzymes, or ecto-nucleotidases, including the ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases), ecto-5'-nucleotidases (5'-NT), ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs) and alkaline phosphatases (AP) (Zimmermann, 2006). The ENTPDases (such as CD39) are the main nucleotide hydrolysing enzymes as they break down nucleoside triphosphate and diphosphates into nucleoside monophosphates (such as ATP>ADP>AMP) (Kukulski *et al.,* 2011). 5'NT (CD73) then acts to convert AMP and adenosine. The CD39/CD73 axis is important in attenuating the inflammatory effects of extracellular nucleotides, particularly ATP (Eckle *et al.*, 2007; Friedman *et al.*, 2009). Adenosine can be taken up by adenosine transporter proteins, such as equilibrative nucleoside transporters 1-4 (ENT) and concentrative nucleoside transporters (CNTs) (Young *et al.*, 2013; Young, 2016). A summary of extracellular nucleotide metabolism is represented in Figure 1.9.2.

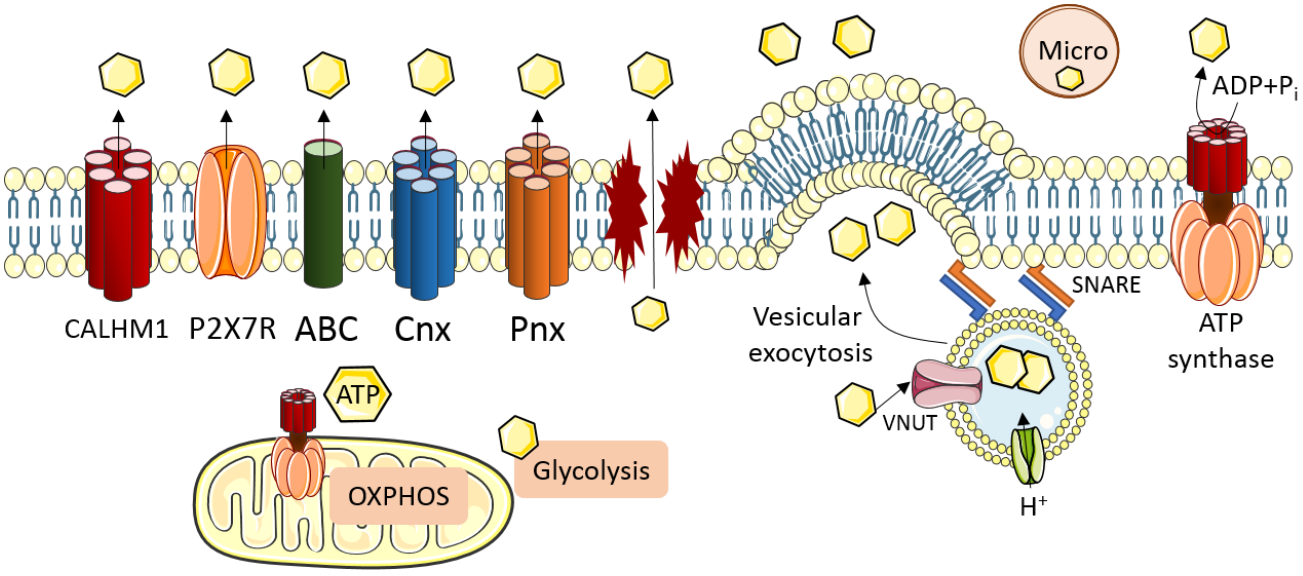



**Figure 1.9.1 Mechanisms of ATP release**

ATP produced via glycolysis and OXPHOS can be released in a non-regulated manner as a consequence of mechanical or sheer stress, or via regulated mechanisms. Regulated ATP release can occur via vesicular exocytosis, membrane-derived microvesicles, connexin (Cnx) and pannexin (Pnx) hemichannels, ATP binding cassette (ABC) transporters, calcium homeostasis modulators (CALHM) channels or purinergic receptors (eg. P2X7R).

### 1.7.2 Purinergic (P2) receptors

Extracellular nucleotides and nucleosides are recognised by specific and widely distributed purinergic (P2) receptors (P2R). There are two classes of P2Rs, the metabotropic P2Y and ionotropic P2X (Burnstock and Kennedy, 1985; Ralevic and Burnstock, 1998; Abbracchio *et al.*, 2006). The affinity of P2Rs for extracellular nucleotides ranges from nanomolar/low micromolar (100 nM-20 μM) to high micromolar/millimolar (300 μM-1 mM) (Valera *et al.*, 1994; Coddou *et al.*, 2011).

There are 7 identified ligand-gated P2XRs (P2X1-7) which allow influx of $Na^+$ and $Ca^{2+}$ ions and efflux of $K^+$ (Valera *et al.*, 1994; Abbracchio *et al.*, 2009). P2XRs are assembled as hetero or homo-trimers with three ATP-binding sites that all need to be engaged for channel/receptor opening. P2X7R forms unique macropores and has the lowest affinity for ATP, being implicated in primarily pro-inflammatory processes such as inflammasome activation, and inflammatory cytokine signalling (Leo Bours *et al.*, 2011; Franceschini *et al.*, 2015).

The G-protein coupled P2Y receptors identified to date, of which there are 8, are P2Y1, 2, 4, 6, 11, 12, 13 and 14. These display differential specificity for nucleotides as well as tissue and cellular distribution and their downstream signal transduction is dependent on the G-protein to which they are coupled (Abbracchio *et al.*, 2006; Jacobson and Müller, 2016). P2Y1, 2, 4 and 6 activate $G_q$ and phospholipase C-β (PLC-β), which in turn generates inositol-1,4,5-triphosphate (IP3) triggering the release of $Ca^{2+}$ from intracellular stores; and DAG, activating PKC (Zimmermann, 2016). Activation of $G_i$ by P2Y12, 13 and 14 causes inhibition of adenylyl cyclase (AC) and decrease in cyclic AMP (cAMP) levels (Torres *et al.,* 2002; Zhang, Zhang and Ding, 2017). Signalling via P2Y11, which does not appear to be expressed in rodents, only humans, activates $G_q$ and $G_s$ enhancing cAMP and $[Ca^{2+}]_i$ (Kennedy, 2017). The preferred agonists for P2YRs are ATP (P2Y11), UTP (P2Y2, 4), ADP (P2Y1, 12, 13), UDP (P2Y6) and UDP-glucose (P2Y14). Other pathways are also modulated by activation of P2YRs, such as activation of PI3Kγ, MAPKs and PLC-β2/β3 (von Kugelgen and Harden, 2011; Erb and Weisman, 2012).

P2Rs can also be activated by ATP analogues such as 2-methylthio-ATP, 2-cloro-ATP, adenosine-5'-*o*-(3-thiotriphosphate) (ATPγS), and 2' (3')-*O*-4-benzoylbenzoyl-ATP (BzATP) (Evans *et al.*, 1995). The thio substitution at phosphate ends of ATP makes compounds such as ATPγS more resistance to rapid hydrolysis by ecto-nucleotidases. P2R activity can be antagonised by nucleotide derivatives such as trinitrophenyl-ATP (TNP-ATP) (Virginio *et al.*, 1998) as well as pharmacological inhibitors such as suramin (Lambrecht *et al.*, 2005), truncated forms of suramin (Damer *et al.*, 1998) and pyridoxal 5-phosphate 6-azolphenul-2',4'-disulfonic acid (PPADS) (Jacobson *et al.,* 2002).

Extracellular adenosine can be further inactivated by cell-surface associated adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) (Franco *et al.*, 2007; Yegutkin, 2008), re-enter cells to replenish intracellular stores via ENT/CNT (Boison, 2013; Virtanen *et al.*, 2014) or elicit signalling roles by activation of the P1 adenosine receptor family ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$) (Ralevic and Burnstock, 1998). Activation of these G-protein coupled receptors activates ($A_1$ and $A_3$) and inhibits ($A_{2A}$ and $A_{2B}$) AC and consequently increases or decreases cAMP levels and $[Ca^{2+}]_i$ (Fredholm *et al.*, 2001; Abbracchio *et al.*, 2009). Signal transduction then involves the MAPK pathways involving extracellular signal regulated kinase 1/2 (ERK1/2), JNK and p38-MAPK (May *et al.*, 2006).

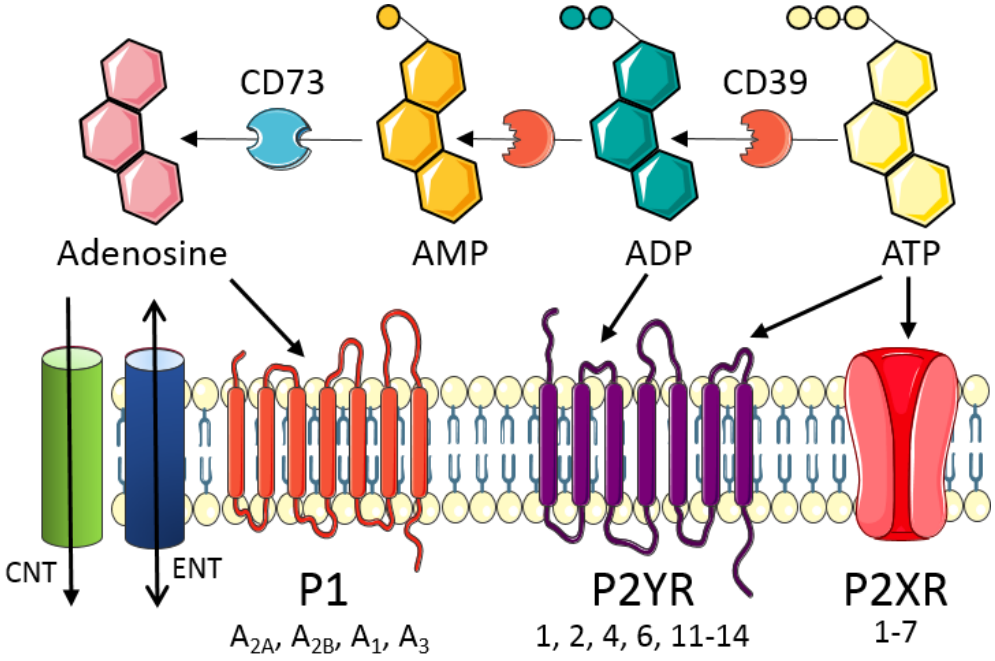



**Figure 1.9.2. Nucleotide and nucleoside receptor mediated signalling**

Extracellular nucleotides (eg. ATP) activate different ionotropic (P2X) and metabotropic (P2Y) receptors, resulting in altered intracellular ion concentrations, particularly calcium and altered cAMP levels. Ecto-nucleotidases (eg. ENTPDase 1/CD39 and 5'NT/CD73) break down ATP to generate ADP, AMP and adenosine. Adenosine can be released (or taken up) via the equilibrative transporter (ENT) and taken up via the concentrative nucleoside transporter (CNT). Extracellular adenosine exerts signalling functions via P1 receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$).

### 1.7.3 Pathophysiology of purinergic signalling

Extracellular purines and pyrimidines participate in the regulation of highly heterogeneous cellular functions across most cell and tissue types, given the widespread P2R distribution (Verkhratsky and Burnstock, 2014; Zimmermann, 2016).

ATP was first recognised as a neurotransmitter, participating in non-adrenergic, non-cholinergic neurotransmission but several roles in physiological and pathophysiological processes have since been established for ATP and its derivatives. The full range of known functions is beyond the scope of this thesis and the reader is referred to comprehensive reviews describing these in more detail. Briefly, physiological roles include cellular proliferation and differentiation (Kaebisch *et al.*, 2015), neuromodulation and transmission (Burnstock, 2013), wound healing (van der Vliet and Bove, 2011), hormone secretion (Burnstock, 2014) and tissue specific roles in liver (Vaughn *et al.,* 2014), muscle/bone

(Burnstock *et al.,* 2013) platelets (Gachet, 2008) and kidneys (Menzies *et al.*, 2017). Purinergic signalling also regulates the pathophysiology of conditions such cancer (Di Virgilio, 2012), CNS/neurodegenerative conditions (Burnstock, 2008), cardiovascular disease (Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015) and processes such as immune response (Cekic and Linden, 2016), infection (Di Virgilio *et al.*, 2017), and inflammation (Leo Bours *et al.*, 2011; Franceschini *et al.*, 2015). With relevance to this thesis, extracellular nucleotides act as important mediators of myocyte differentiation and proliferation and have been shown to be implicated in obesity and diabetes.

Extracellular nucleotides are most commonly associated with propagating immune responses, where they act as “danger signals”; promoting systemic inflammatory responses; inflammasome activation; participating in host-tumour interactions and modulating vascular function (vasodilation). In the musculoskeletal system, ATP was first seen to participate in the neuromuscular junction, being released as a co-transmitter with acetylcholine (Ach) to promote contraction (Buchthal and Folkow, 1948). Work in the 1980s then suggested that myoblasts and myotubes in culture possibly responded to extracellular ATP via ATP-sensitive cation channels (Kolb and Wakelam, 1983). Subsequent studies elucidated differential patterns of P2Rs across development and concluded that P2XRs are critical for early development of SM but expression reduces significantly after birth (Meyer *et al.*, 1999). P2YRs were then found to be expressed in developing murine myotubes (C2C12) and embryonic SM (Henning *et al.*, 1993).

Treatment of SM with extracellular nucleotides, primarily ATP, stimulates muscle contraction in a P2R-PKC-dependent manner (Teplov *et al.*, 2006) and increases $[Ca^{2+}]_i$ (Cseri *et al.*, 2002). P2XR-mediated influx of $Ca^{2+}$ into C2C12 cells contributes to phosphorylation of ERK1/2 (Banachewicz *et al.*, 2005; May *et al.*, 2006), and this was later seen to occur downstream of ATP-mediated signalling via P2YRs in human myotubes (May *et al.*, 2006). ATP is released by SM upon mechanical (Taguchi *et al.*, 2008) and electrical stimulation (Osorio-Fuentealba *et al.*, 2013) and acts in an autocrine manner to stimulate both fast (P2XR) and slow (P2YR) calcium transients; with the latter acting to maintain calcium flux (Szigeti *et al.*, 2007) and promote gene expression of molecules such as IL-6

(Jaimovich *et al.*, 2011). Purinergic signalling modulators also participate in SM pathologies, such as Duchenne Muscular Dystrophy (DMD), where loss of α-sarcoglycan (membrane protein with ecto-ATPase activity) leads to excessive release of ATP and enhanced expression of P2XRs, leading to $Ca^{2+}$ overload and muscle fibre death (Hack *et al.*, 1998; Rybalka *et al.*, 2014).

### 1.7.4 Purinergic signalling in obesity and diabetes

Evidence from recent years suggests that purinergic signalling modulators are implicated in obesity and diabetes, both at central and peripheral levels (reviewed by Burnstock, 2017; Burnstock and Gentile, 2018).

Main findings suggest that P2Y6Rs are implicated in hypothalamic regulation of food intake in DIO mice (Steculorum *et al.*, 2017); ATP enhances lipid accumulation in adipocytes (Kita and Arakaki, 2015) and regulates leptin secretion via P2Y1R (Laplante *et al.*, 2010); and P2Y11R activation stimulates lipolysis (H. Lee *et al.*, 2005). Furthermore, P2YR activation in β-cells promotes insulin secretion (Balasubramanian *et al.*, 2014; Wang *et al.*, 2014). Early work also suggested that ATP inhibits insulin-stimulated glucose uptake into adipocytes and inhibited glucose oxidation (Chang and Cuatrecasas, 1974; Halperin, Mak and Taylor, 1978). More recently, studies in rat adipocytes demonstrated that ATP treatment caused inflammation and AT IR (Yu and Jin, 2010). Adenosine signalling via $A_{2B}$ receptors prevents HFD-induced AT inflammation and IR (Johnston-Cox *et al.*, 2012); via $A_1$ receptors protects against obesity-related IR (Dhalla *et al.*, 2009) and promotes leptin release from adipocytes (Cheng *et al.*, 2000; Rice, Fain and Rivkees, 2000); and via $A_{2A}$ activation in adipocytes counteracts DIO (De Oliveira Moreira *et al.,* 2017) and enhances β-cell proliferation (Matsumoto *et al.*, 2015). Adenosine also promotes glucagon release (Loubatières-Mariani *et al.,* 1982) and inhibits GSIS (Ismail *et al.,* 1977).

Extracellular nucleotides and nucleosides appear to play paradoxical roles in glucose homeostasis and in the pathophysiology of metabolic conditions. A better understanding of the possible involvement of the purinergic signalling system in these pathways is therefore required, particularly in the context of SM IR, as this is largely unexplored.

### 1.8 Project aims

Although the understanding of diabetes as a metabolic disease has significantly evolved since insulin was discovered in the 1920s, individuals with T1D and T2D are still challenged daily with unpredictable fluctuations in blood glucose. As such, the main aim of this thesis was to better understand the complex integrated physiology of glucose homeostasis and identify therapeutically targetable markers involved in the pathophysiology of impaired glucose control. The work presented in this thesis aimed to:

- Elucidate the *in vitro* (muscle cells) and *in vivo* (human) mechanisms regulating lipid-induced insulin and anabolic resistance in skeletal muscle.
    - Does lipid overflow diminish muscle anabolic sensitivity and promote inflammatory cytokine release and mitochondrial dysfunction via alterations to the purinergic signalling system (Chapter 3).
        - Hypothesis: Palmitate-induced increase in ATP release promotes insulin and anabolic resistance in skeletal muscle cells

    - Does lipid overflow (by high-fat overfeeding) exacerbate insulin and anabolic resistance brought about by inactivity in humans (Chapter 4)
        - Hypothesis: Increased intake of dietary saturated fat (2.5 fold increase) exacerbates the development of insulin and anabolic resistance in immobilised forearm muscle tissue.

- Pharmacologically target AMPK (as an important whole-body and cellular energy sensor) to better understand the peripheral and central regulation of blood glucose (Chapter 5).
        - Hypothesis: Peripheral administration of brain permeable AMPK activator R481 amplifies the counterregulatory response to hypoglycaemia in healthy Sprague Dawley rats.

# Chapter 2

# Materials and methods

## 2.1 Materials

### 2.1.1 Chemicals

**Table 2.1 General chemicals and consumables**

| Chemical | Supplier/ Manufacturer | Catalogue number |
|---|---|---|
| 0.05 % (w/v) Trypsin EDTA | Thermo Fisher | 25300-062 |
| 2-Deoxy-d-glucose | Agilent | 103020-100 |
| 2-mercaptoethanol | Sigma-Aldrich | M3148 |
| 5-BDBD | Biotechne | 3579 |
| A438079 | Biotechne | 2972 |
| Accu Check Performa Test strips | (Roche) Williams Medical | D213 |
| Actrapid Insulin 100 IU/mL | Centaur Services | 20411387 |
| Adenosine tri-phosphate (ATP) | Sigma-Aldrich | A9187 |
| Adenosine-5'-(γ-thio)-triphosphate tetralithium salt (ATPγS) | Biotechne | 4080 |
| Amino acid solution (50x MEM) | Thermo Fisher | 11130051 |
| Ammonium persulfate (APS) | Sigma-Aldrich | A3678 |
| Apyrase | Sigma-Aldrich | A6410 |
| ATPLite luminescence assay system | PerkinElmer | 6016947 |
| BD Vacutainer® fluoride/oxalate tubes | Thermo Fisher | 12947676 |
| BD Vacutainer® PST Lithium Heparin tubes | Thermo Fisher | 12947676 |
| BD Vacutainer® SST II tubes | Thermo Fisher | 12927696 |
| Benzamidine | Sigma-Aldrich | 12072 |
| Bio-Rad protein assay dye reagent | Bio-Rad | 500-0006 |
| Biotrace nitrocellulose membranes (0.2µm pore) | VWR | 732-3031 |
| Bromophenol blue sodium salt | Sigma-Aldrich | B5525 |
| BSA Factor V, fatty acid free | Sigma-Aldrich | 10775835001 |
| BSA lyophilised powder | Sigma-Aldrich | A2153, |
| BSA standard (2 mg/mL) | Thermo Fisher | 11811345 |
| Cotton buds | Williams Medical | D500 |
| D-glucose | Sigma-Aldrich | G7021 |
| Dimethyl sulphoxide (DMSO) | Sigma-Aldrich | D2650 |
| DMEM glucose free | Fisher | 11966 |
| DMEM high glucose | Sigma-Aldrich | D5671 |
| DMEM no glucose or phenol red | Fisher | 12307263 |
| Drawing up needle 18G | Williams Medical | D5789 |
| EDTA pH 8 | Sigma-Aldrich | E1644 |
| EGTA pH 8 | Melford | E1102 |
| EMLA 5 g crème | Williams Medical | XD-102059 |
| Ensure Plus | Abbott Nutrition | N/A |
| FCCP | Agilent | 103015-100 |
| Fluo-4 Direct calcium assay reagent | Life Technologies | F10471 |

| | | |
|---|---|---|
| Foetal bovine serum | Gibco | 11573397 |
| Glucose 50 % w/v solution (IV infusion, POM, 25 g in 50 mL) | Centaur Services | 30098718 |
| Glucose uptake-Glo assay | Promega | J1341 |
| Glycerol | Sigma-Aldrich | 49767 |
| Glycine | Melford | G0709 |
| Glycolytic stress kit | Agilent | 103020-100 |
| Grade 3 MM Chr blotting paper | Fisher | 11330744 |
| Heparin sodium | Centaur service | 30172843 |
| HEPES | Sigma-Aldrich | H0877 |
| Hexamethonium bromide | Sigma-Aldrich | H0879 |
| Human insulin ELISA kit | Oxford Biosystems Ltd | DX-EI1-2935 |
| Hypo needle 25G | Williams Medical | K2126 |
| Hypromellose | Sigma-Aldrich | H3785 |
| L-glutamine | Fisher | 25030-024 |
| L-leucine (1-13C, 99 %) | Cambridge Isotope Laboratories Inc | CLM-468-PK |
| L-Phenylalanine (RING-D5, 98 %) | Cambridge Isotope Laboratories Inc | DLM-1258-PL |
| Methanol | Sigma-Aldrich | 34860 |
| Microlance needle 21G x 1.5 | Williams Medical | D5887 |
| Mito stress test kit | Agilent | 103015-100 |
| Mouse Interleukin-6 (IL-6) Duoset ELISA | R&D Systems | DY406 |
| Mouse Macrophage migration inhibitory factor (MIF) Duoset ELISA | R&D Systems | DY1978 |
| Oligomycin | Agilent | 103015-100 |
| Palmitic acid | Sigma-Aldrich | P0500 |
| Penicillin/streptomycin | Fisher | 15070-063 |
| Phenylmethylsulphonyl fluoride (PMSF) | Sigma-Aldrich | P7626 |
| Phosphate buffered solution (PBS) | Thermo Fisher | 10209252 |
| Poly-L-lysine hydrobromide | Sigma-Aldrich | P1274 |
| Ponceau S solution red | Sigma-Aldrich | P7170 |
| PPADS | Tocris | 0625/10 |
| Protogel Acrylamide 30% | Scientific-laboratory supplies | NAT1260 |
| R419 | Rigel Pharmaceuticals | N/A |
| R481 | Rigel Pharmaceuticals | N/A |
| Rat Adrenaline ELISA | Demeditec | DEE5100 |
| Rat C-peptide ELISA | Mercodia | 10-1150-01 |
| Rat Glucagon ELISA | Mercodia | 10-1281-01 |
| Rotenone/Antimycin | Agilent | 103015-100 |
| SBI-0206965 | Cayman | 18477 |
| Seeblue plus 2 pre-stained protein standard | Life Technologies | LC5925 |
| Skimmed milk powder | Oxoid | LP0031 |

| | | |
|---|---|---|
| Sodium bicarbonate | Sigma-Aldrich | S5761 |
| Sodium chloride (NaCl) | Sigma-Aldrich | S7653 |
| Sodium Dodecyl Sulphate (SDS) | Melford | B2008 |
| Sodium fluoride NaF | Sigma-Aldrich | S7920 |
| Sodium hydroxide NaOH | Sigma-Aldrich | |
| Sodium orthovanadate ($Na_3VO_4$) | Sigma-Aldrich | S6508 |
| Sodium Pyrophosphate Tetrabasic Decahydrate - NaPPi | Sigma-Aldrich | S6422 |
| Sodium pyruvate | Sigma-Aldrich | P5280 |
| Standardised chow diet – EU rodent diet 14% | Lab Diet | 5LF2 |
| Sterile water for injections | Medisave | WIA210 |
| Sucrose | Melford | S0809 |
| Suramin hexasodium salt | Tocris | 1472/50 |
| TEMED | Sigma-Aldrich | T7024 |
| Terumo Luer Slip tip Syringe 5 mL | Medisave UK Ltd | BS-05S |
| Terumo Tuberculin Syringe 1mL | Medisave UK Ltd | BS-01T |
| Tris (hydroxymethyl) aminomethane (TRIS) | Melford | B2005 |
| Triton 100x | VWR | M143 |
| Tween 80® | Sigma-Aldrich | P6474 |
| Tween® 20 | Sigma-Aldrich | P2287 |
| Vetivex saline solution (0.9%) | Centaur services | 30227005 |

### 2.1.2 Equipment

**Table 2.2 General equipment**

| **Equipment** | **Supplier** |
|---|---|
| Accu-Check glucose meter | Roche |
| BODPOD Body Composition System | COSMED |
| Cobas 8000 modular analyser | Roche Diagnostics |
| Doppler ultrasound (1000 Apogee) 10MHz transducer probe (L10LC). | Shantou Institute of Ultrasonic Instruments |
| Dynamometer, hand-held | T.K.K 5001, Takei Scientific |
| Odyssey CLx | LI-COR Biosciences |
| PHERAstar FS microplate reader | BMG Labtech |
| Seahorse XFe96 Bioanalyser | Agilent Technologies |
| TC20 Automated Cell counter | Bio-Rad |
| DCi8 S Widefield inverted microscope | Leica Microsystems |
| YSI 2300 Stat Plus, Glucose Analyzer | YSI Life Sciences |
| BeneCast fibreglass casting tape | BeneCare Medical |
| Heated hand warmer – Hotbox 55 °C | Nottingham University |

**Table 2.3 Software**

| Software | Supplier |
|---|---|
| Brachial Analyser for Research software, Version 6 | Medical Imaging Applications |
| GraphPad Prism 8 | GraphPad Software |
| Image Studio Lite Ver 5.2 | LI-COR Biosciences |
| Nutritics Education v4.315 | Nutritics |
| Wave Desktop | Agilent Technologies |
| LAS X Life Science | Leica Microsystems |

**Table 2.4 Cell culture materials**

| Consumable | Supplier | Catalogue number |
|---|---|---|
| 60 mm tissue culture treated dishes | Starstedt | 83.3901 |
| 96 well plate - Clear flat bottom | Starlab | E2996 |
| DMEM high-glucose | Sigma-Aldrich | D5671 |
| DMEM low glucose | Thermo Fisher | 11966, |
| DMEM low glucose, no phenol red | Thermo Fisher | 12307263 |
| DMEM Seahorse XF base medium | Agilent | 103193 |
| Earl's balanced salt solution 10x | Sigma-Aldrich | E7510 |
| Foetal bovine serum (FBS) | Gibco | 11573397 |
| Horse serum | Thermo Fisher | 10368902 |
| L-glutamine | Thermo Fisher | 25030 |
| Penicillin/streptomycin solution 5,000 U/mL | Thermo Fisher | 15070 |
| Seahorse XFe96 culture microplate | Agilent | 101085-004 |
| T75 $cm^2$ flasks | Starstedt | 83.3911.002 |
| Cryovials | Corning | 430659 |
| FluoroDish cell culture dish 35 mm | World Precision Instruments | FD35-100 |

**Table 2.5 Glucose clamping equipment**

| Consumable | Supplier | Catalogue number |
|---|---|---|
| 25G dual channel stainless steel swivel | Instech Labs | 375/D/25 |
| 3-way Y connector, 22ga, sterile | Instech Labs | SCY22 |
| Catheter for rat carotid artery, PU 1.9-3Fr 16.5 cb, collar @ 3.5 cm, 8 cm sleeve. Fits 22G. | Instech Labs | C19PU-RCA1936 |
| Catheter for rat jugular vein, PU 3 Fr 30 cm, collar @ 3.0 cm, sleeve, square tip. Fits 22G. | Instech Labs | C30PU-RJV1303 |
| Clear animal enclosure, 15i n H, holes for MWATER and MFEEDER | Instech Labs | MTANK/WF |
| Counter-balanced lever arm for rats (15 cm) | Instech Labs | MCLA |

| | | |
|---|---|---|
| Luer stub, 22G (blue) x 0.5 in (12 mm), non-sterile | Instech Labs | LS22 |
| Pin-port injectors | Instech Labs | PNP3M |
| PinPorts, 22G red | Instech Labs | PNP3F22R |
| Protective aluminium cap for magnetic rat vascular access button | Instech Labs | VABRC |
| Co-extruded PE/PVC tubing for 22G, .024x.064in, spool, nonsterile | Instech Labs | BTCOEX-22 |
| Pump 11 Elite infuse only programmable syringe pump | Instech Labs | HA1100 |
| Tether for magnetic 2 channel rat VAB, 12 in | Instech Labs | VABR2T/25 |
| Vascular access button for rat, magnetic, 2 channel, 22G, injector | Instech Labs | VABR2B/22 |
| Water bottle for MTANK | Instech Labs | MWATER |

### 2.1.3 Antibodies

**Table 2.6 Primary antibodies**

| **Antibody** | **Supplier** | **Catalogue number** |
|---|---|---|
| GAPDH | Sigma Aldrich | G9545 |
| pS473 PKB | Cell signalling | 9271 |
| pS536 NF-κB p65 | Cell signalling | 13346 |
| pS79 ACC | Cell signalling | 3661 |
| pT172 AMPK | Cell signalling | 2535 |
| pT389 p70S6K1 | Cell signalling | 9205 |
| Total ACC | Merck Millipore | 05-1098 |
| Total AMPK | Cell signalling | 2793S |
| Total p70S6K1 | Cell signalling | 9202 |
| Total PKB | Cell signalling | 9272 |
| Total β-Actin | Biotechne | NB600-501 |
| p44/42 MAPK (ERK1/2) | Cell Signalling | 4370 |

**Table 2.7 Secondary antibodies**

| **Antibody** | **Supplier** | **Catalogue number** |
|---|---|---|
| Alexa Fluor 680 goat anti-mouse IgG | Thermo Fisher | A21057 |
| Anti-IgG (Rabbit) Goat polyclonal Ab | VWR | ROCK611-132-122 |

## 2.2 *In vitro* methods

### 2.2.1 Cell culture

All cell lines used were maintained in culture under sterile conditions, handled only in category-2 biological safety-rate hoods and maintained at 37°C in humidified 5% $CO_2$ incubators.

The C2C12 (ATCC CRL-1772, *Mus musculus*) murine myoblast cell line was kindly gifted by colleagues at the University of Dundee. The cells' ability to retain differentiation potential during prolonged cultivation (Yaffe, 1968) make them a useful tool in muscle biology. C2C12 myoblasts were cultured in differing media according to the stage of differentiation. When relevant, cells were also deprived of AAs for treatments. Media characteristics are described in Table 2.8.

GT1-7 murine hypothalamic neurones were kindly gifted by Dr. Pamela Mellon of the University of San Diego. Media characteristics for GT1-7 culture are described in Table 2.9

**Table 2.8 C2C12 culture media**

| Medium | Components |
|---|---|
| Growth medium | High glucose (25 mM) DMEM supplemented with 10 % FBS 4 % (v/v) l-glutamine, 2 % penicillin/streptomycin (pen/strep) |
| Plating medium | Glucose-free DMEM with 4 % l-glutamine supplemented with 5.5 mM glucose, 10 % FBS and 2 % pen/strep |
| Differentiation medium | Glucose-free DMEM with 4 % l-glutamine supplemented with 5.5 mM glucose, 2 % horse serum and 2 % pen/strep |
| AA deprived medium (EBSS) | Earl's balanced salt solution 1 x, supplemented with sodium bicarbonate (2.2 g/L) and 0.34 mM AA solution. |
| Freezing medium | Growth medium supplemented with 10 % DMSO. |
| Seahorse XF DMEM | Glucose-free XF DMEM with 5.5 mM glucose, 2.5 mM sodium pyruvate, 2 mM l-glutamine, pH 7.4 |

**Table 2.9. GT1-7 culture media**

| Medium | Components |
|---|---|
| Stock medium | High glucose (25 mM) DMEM supplemented with 10 % FBS 4 % (v/v) l-glutamine, 2 % penicillin/streptomycin (pen/strep) |
| Plating medium | Glucose-free DMEM with 4 % l-glutamine supplemented with 7.5 mM glucose, 10 % FBS and 2 % pen/strep |
| Experimental medium | Glucose-free DMEM with 4 % l-glutamine supplemented with 2.5 mM glucose, without serum. |

### 2.2.1.1 Culturing and passaging of cell lines

C2C12 myoblasts were maintained in culture in 75 $cm^2$ flasks (T75) containing 10 ml of growth medium and incubated in humidified 5 % $CO_2$ incubators, at 37$^o$C. Cells were passaged every 48 hours (60-70 % confluence) and maintained for no more than 10 subsequent passages (below passage 30). When passaging cells to maintain and expand culture, these were plated at $1x10^6$ per flask in T75 flasks. 48 hours later, media was aspirated, flasks washed with pre-warmed sterile phosphate buffer solution (PBS) and subsequently incubated with 2 mL of trypsin/EDTA (0.05 %) for 3-5 minutes at 37$^o$C. Cells were mechanically displaced from the bottom of the flask and observed under the microscope to ensure detachment before the effects of trypsin were neutralised by the addition of 5 mL of growth medium. The cell suspension was then centrifuged at 1000 rpm for 5 minutes to separate trypsin in the media from cell pellet. Media was then aspirated and cell pellet resuspended in 1 mL of growth media by gently pipetting up and down with a p1000 pipette with a filter tip. Cells ($1 x10^6$) were resuspended in fresh growth media and transferred to a new T75 flask. For experiments, cells were counted using an automated cell counter (TC10, Bio-Rad) and the volume corresponding to the desired seeding density was resuspended in plating medium. Table 2.10 summarises the seeding density for each culturing platform.

**Table 2.10 C2C12 myoblast seeding density**

| Culture platform | Density per well/dish |
|---|---|
| 60 mm dish | 2 x $10^5$ |
| Clear F-bottom 96 well plate | 5 x $10^3$ |
| Black F-bottom 96 well plate | 5 x $10^3$ |
| Seahorse XFe96 microplate | 1.6 x $10^3$ |

GT1-7 cells were maintained in 75 $cm^2$ flasks (T75) stock medium (Table 2.9) at 50-80 % confluence and every 3 to 4 days, cells were passaged according to the method described above, with the difference that this cell line is non-adherent, requiring a pre-coat in culture flasks with poly-L-lysine (PLL) (10 ng/mL). Flasks were coated with PLL for 30 minutes before solution was collected and flasks washed with PBS and allowed to dry before cells were added. GT1-7 cells were plated 24 hours before experimentation in 60 mm dishes at 450,000 cells/dish in plating medium (Table 2.9). A 2 hour incubation period in experimental medium was conducted before each experiment.

#### 2.2.1.2 Differentiating C2C12 myoblasts

C2C12 myoblasts were maintained in culture at sub confluence before differentiation. To initiate differentiation, cells were plated in plating media at densities indicated in Table 2.10 and were incubated for 48 hours. After 48 hours (100 % confluence), growth media was replaced with differentiation media. Media was changed every day for 6 consecutive days to replenish glucose and remove waste. Cells were differentiated for a total of 7 days, when mature myotubes were used for experimentation. Cells were also plated at 2 x$10^5$ cell/dish in glass-bottom FluoroDish culture dishes and differentiated as above for live cell imagining. Images were taken using a Leica DMi8 inverted widefield system, fitted with a Hammamatsu digital camera C11440 ORCA-flash 4.0, LED external light source EL600, HC Plan-Apochromat x40 objective, motorised stage, and temperature-controlled environmental chamber (Pecon) and controlled by Leica Application Suite X on a PC. Images were taken at days 1, 3, 5 and 7 of differentiation and processed using LAS X Life Science software.

### 2.2.1.3 Freezing cells

C2C12 myoblast and GT1-7 cell stocks were maintained in liquid nitrogen for long term storage. To freeze C2C12 myoblasts, cells were passaged (as above) until reaching the pellet stage, when they were resuspended in freezing medium (Table 2.8). Cells were frozen at 1 x $10^6$/mL in cryovials and transferred to polystyrene insulators before being slow frozen at -80 $^oC$ overnight and subsequently transferred to liquid nitrogen. GT1-7 cells were passaged as above until the pellet stage, when the cells were resuspended in DMEM supplemented with 25 mM glucose and 10 % DMSO and frozen down at 1 x $10^6$/mL in cryovials and frozen as described for C2C12 cell line.

### 2.2.2 Preparation of cell lysates and media collections

All C2C12 myotube experiments were conducted after 7 days of differentiation. Depending on treatments, cells were either incubated in differentiation medium (day 6 to 7) or deprived of serum (maximum of 16 hours) or in the morning of day 7 (maximum 2 hours). Treatments were carried out in serum free media (serum free DMEM or EBSS) supplemented with the desired treatments. Experiments in dishes were terminated by media collection (placed immediately on ice) and one warmed PBS wash. PBS was then replaced with ice cold lysis buffer, following which cells were scraped, on ice, and cell lysates were collected into eppendorf tubes and frozen at -20 $^0C$. Lysis buffer contained magnesium and calcium chelators to remove cofactors for protein kinases and was supplemented with serine/threonine protein kinase inhibitors, protein tyrosine phosphatase inhibitors, and protease inhibitors (Table 2.11). For analysis, lysates were thawed on ice and insoluble cellular debris removed by centrifugation at 14,800 rpm, at 4 $^oC$, for 20 minutes; before supernatants were transferred to separate tubes. Media was thawed on ice and spun at 1,000 rpm for 5 minutes, at 4 $^oC$.

**Table 2.11 Lysis buffer preparation**

**a. Lysis buffer stock preparation**

| Reagent | Stock concentration | 1x Lysis buffer |
|---|---|---|
| Tris HCL pH7.4 | 1 M | 25 mM |
| NaF | 750 mM | 50 mM |
| NaCl | 4 M | 0.1M |
| EDTA pH 8 | 0.5 M | 1 mM |
| EGTA pH 8 | 0.2 M | 5 mM |
| Triton 100x | 100 % | 1 % |
| NaPPi | 50 mM | 10 mM |
| Total volume | | 500 mL |

**b. Lysis buffer + inhibitors for lysate preparation**

| Reagent | Final lysis buffer |
|---|---|
| 1 x lysis buffer | 1 mL |
| Sucrose | 92 mg / mL |
| 2-Mercaptoethanol (0.1 % v/v) | 1 µL/mL |
| $Na_3VO_4$ (1 mM) | 2 µL/mL |
| Benzamidine (1 Mm) | 2 µL/mL |
| PMSF (1 mM) | 2 µL/mL |

### 2.2.3 Cell treatments

#### 2.2.3.1 Essential amino acid treatment

The composition of the mixed essential amino acid solution used to treat C2C12 myotubes is summarised in Table 2.12

**Table 2.12 Mixed essential amino acid solution composition**

| Amino acid | Molecular weight | Concentration (mg/mL) 50 X | Concentration (mM) 50 X | Final concentration (mM) 1 X |
|---|---|---|---|---|
| L-Arginine hydrochloride | 211.0 | 6320 | 29.95 | 0.60 |
| L-Cysteine | 240 | 1200 | 5.0 | 0.1 |
| L-Histidine hydrochloride-$H_20$ | 210.0 | 2100 | 10.0 | 0.2 |
| L-Isoleucine | 131.0 | 2620 | 20.0 | 0.4 |
| L-Leucine | 131.0 | 2620 | 20.0 | 0.4 |
| L-Lysine Hydrochloride | 183.0 | 3625 | 19.81 | 0.39 |
| L-Methionine | 149.0 | 755 | 5.07 | 0.10 |

| | | | | |
|---|---|---|---|---|
| L-Phenylalanine | 165.0 | 1650 | 10 | 0.2 |
| L-Threonine | 119.0 | 2380 | 20 | 0.4 |
| L-Tryptophan | 204.0 | 510 | 2.5 | 0.05 |
| L-Tyrosine | 181.0 | 1800 | 9.95 | 0.02 |
| L-Valine | 117.0 | 2340 | 20.0 | 0.4 |

#### 2.2.3.2 Palmitate preparation

Palmitic acid was dissolved in 90 % ethanol and heated to 70 ºC for 10 minutes at a stock concentration of 100 mM. The stock was then conjugated to a 10 % (w/v) solution of fatty acid free bovine serum albumin (BSA) made up in the solvent of interest (different media), and incubated at 37ºC for 1 hour. Conjugated stocks were 10-100 times more concentrated than target concentrations to maintain ethanol content in cell treatment below 0.5 % and BSA below 1 %. Control cells were treated with BSA with relevant ethanol concentration, in the absence of palmitate.

#### 2.2.3.3 R481/R419 preparation

R481/R419 were kindly provided by Rigel Pharmaceuticals (San Francisco, California). Both drugs were suspended in a solution of hypromellose (HPMC) (0.5 % w/v + 0.1 % Tween-80 v/v), which was also used as the vehicle control for experiments using those compounds. HPMC solution was prepared by heating ¾ of the total volume of distilled water necessary to make a 0.5 % w/v solution to 70 °C under sterile conditions. The appropriate mass of hypromellose was subsequently added to the solvent and the mixture stirred with a magnetic stirrer for 2 minutes. The remaining volume of water was then added (cold), solution removed from heat and stirred until it reached 37 °C. At this stage, the appropriate volume of Tween-80 was added (final 0.1 % v/v) and solution stirred until combined. Solution was cooled to room temperature before R481/R419 was added. To make R481/R419 the relevant mass was weighed onto a sterile container able to fit a magnetic stirrer, HPMC (0.5 % + 0.1 % Tween-80) then added to the powder and solution stirred at maximum rpm for approximately 2 hours. Both vehicle and compound preparations were aliquoted into small volumes and frozen at -20 °C before use.

### 2.2.4 Protein expression analysis

#### 2.2.4.1 Total protein content determination

The total protein of each lysate was assessed using the Bradford assay method (Bradford, 1976). An initial assessment was conducted to determine whether cell lysate dilution was necessary and, when required, dilutions were conducted in lysis buffer (Table 2.11) before performing the assay.

The assay was adapted for use in a clear flat-bottomed 96 well plate, where 1 µl of sample was incubated with 200 µl of Bradford reagent, before absorbance was read at 595 nm in a PHERAstar microplate reader (BMG LABTECH). Absorbance values were compared to a standard curve plotted with known concentrations of BSA.

#### 2.2.4.2 Preparation of homemade gels for SDS-PAGE

Gels were prepared according to Table 2.13 in mini-PROTEAN® Tetra handcast equipment from Bio-Rad. Gels with different acrylamide percentages (10-15 %) were used accordingly, to accommodate for the different molecular weights of the proteins of interest.

**Table 2.13. Acrylamide gel composition**

| Reagent (2x gels) | Upper stacking 4 % | 10 % acrylamide | 15 % acrylamide |
|---|---|---|---|
| dd$H_2O$ | 2.8 mL | 4.15 mL | 2.3 mL |
| 0.5 M Tris pH 6.8 | 1.25 mL | | |
| 1.5 M Tris pH 8.8 | - | 3.15 mL | 3.15 mL |
| 30 % acrylamide | 850 µL | 3.75 mL | 5.6 mL |
| 10 % SDS | 50 µL | 110 µL | 110 µL |
| 20 % APS | 50 µL | 55.4 µL | 55.4 µL |
| TEMED | 5.35 µL | 11 µL | 11 µL |

#### 2.2.4.3 SDS-PAGE

Samples were prepared for SDS-PAGE by combining a calculated volume of sample supernatant with sample buffer (Table 2.14) and lysis buffer (Table 2.11). Samples were prepared at 1 µg/µL. Samples were then heated to 37 $^{o}$C for 10 minutes before 20 µg were loaded onto each well of the relevant handmade gel. Running conditions were set at 90 volts (V) for 15 minutes followed by 150 V for 60-95 minutes, in running buffer (Table 2.14).

**Table 2.14. SDS-PAGE buffers**

| Buffer | Components |
|---|---|
| Running buffer | 25 mM Tris, 192 mM glycine, 0.1 % SDS |
| Transfer buffer | 20 % (v/v) methanol, 48 mM Tris-base, 39 mM Glycine, pH 8.3 |
| TBS-T | 150 mM NaCl, 20 mM Tris/HCL pH 7.4, 0.05 % (v/v) Tween-20 |
| Sample buffer | 125 mM Tris-HCL pH 6.8, 20 % (v/v) glycerol, 4 (w/v) % SDS and bromophenol blue with 1/1000 dilution of 2-mercaptoethanol |

### 2.2.4.3 Protein transfer into nitrocellulose membranes

Wet electrotransfer was conducted following SDS-PAGE to transfer the separated proteins onto nitrocellulose membranes. The sandwich method was used by placing a nitrocellulose membrane against the gel and stacking filter paper and sponges on both sides before mounting them onto a cassette and tank where a current could be applied. Nitrocellulose membranes non-specifically bind proteins and enable for protein quantification with immunodetection methods. A current of 100 V was applied for 60-75 minutes. Rapid visualisation of transfer efficiency was conducted using a reversible non-specific protein stain, Ponceau-S Red, which was subsequently washed off with transfer buffer (Table 2.14).

### 2.2.4.4 Immunodetection

Immediately after the protein transfer, nitrocellulose membranes were blocked with a solution of 5% (w/v) dried semi-skimmed milk in TBS-T, for 1 hour, at room temperature. Blocking the membrane prevents non-specific binding of the antibodies to proteins in the membrane with open recognition sites. The block was washed off with TBS-T and membranes incubated with primary antibodies, either overnight at 4 $^{o}$C or for 1 hour at room temperature. Suppliers and catalogue numbers summarised in Table 2.6 and dilutions and solvents are summarised in Table 2.14. The primary antibodies were then removed and membranes washed 6 times (5 minutes each) with TBS-T. Membranes were then incubated with secondary antibody preparations for 1 hour, at room temperature, before this was removed and membranes washed 6 additional times (10 minutes each) in TBS-T. All secondary antibodies were conjugated to fluorescent dyes, which enabled the simultaneous imaging of multiple proteins on each membrane with high sensitivity.

Suppliers in Table 2.7, dilutions and solvents in Table 2.15. Blots were subsequently imaged for fluorescence at 680 nm and 790 nm using the LI-COR Odyssey Imaging System.

**Table 2.15. Antibody preparations**

| Protein | Dilution | Solution | Species | Incubation conditions |
|---|---|---|---|---|
| Alexa Fluor 680 anti-mouse IgG | 1/10000 | TBST | Goat | 1 hour RT |
| Anti-IgG anti-rabbit polyclonal Ab | 1/10000 | TBST | Goat | 1 hour RT |
| GAPDH | 1/10000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 1 hour RT |
| p44/42 MAPK (ERK1/2) | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| pS473 PKB | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| pS536 NF-κB P65 | 1/1000 | 2.5% (w/v) BSA/TBS-T | Mouse | 4º C overnight |
| pS79 ACC | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| pT172 AMPK | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| pT389 p70-S6K1 | 1/1000 | 5% (w/v) milk/TBS-T | Mouse | 4º C overnight |
| Total ACC | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| Total AMPK | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| Total p70-S6K1 | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| Total PKB | 1/1000 | 2.5% (w/v) BSA/TBS-T | Rabbit | 4º C overnight |
| Total β-Actin | 1/10000 | 2.5% (w/v) BSA/TBS-T | Mouse | 1 hour RT |

### 2.2.4.5 Western blot analysis

Densitometric analysis of bands of interest was conducted using the Image Studio Lite (Ver 5.2) software. Regions of interest were drawn around the detected bands of interest, including areas for background detection and signal measured using pixel intensity. Levels of phosphorylated proteins were normalised to levels of total protein of interest or to loading controls β-actin or GAPDH. When relevant, changes in relative protein expression were normalised to control groups (designated a value of 1) within experiments.

### 2.2.5 Quantification of ATP

The extracellular concentration of ATP was measured using a commercially available assay kit and used according to manufacturer's instructions (ATPLite, PerkinElmer). This bioluminescence plate-based assay uses firefly luciferase-mediated reaction between ATP and d-luciferin to generate light. Emitted light is directly proportional to ATP concentration, making this assay highly sensitive. The following illustrates the reaction:

$$ATP + D\text{-}Luciferin + O_2 \xrightarrow{magnesium,\ luciferase} Oxyluciferin + AMP + PPi + CO_2 + Light$$

ATP assays were conducted on media collected from cells in culture. Briefly, 100 µl of media supernatant was used per sample, in black-walled 96 well plates. Mammalian cell lysis solution (50 µL) was added to each sample and mixed on an orbital shaker for 5 minutes before substrate solution (50 µL) was added and plate mixed in the same manner. Following this step, the plate was dark-adapted for 10 minutes before luminescence was read using the PHERAstar microplate reader. ATP concentrations were calculated in pmol/L or normalised to total protein assessed from lysates from the same experiment using the Bradford method to generate ATP concentrations in pmol/mg.

The same methodology was used to measure the levels of intracellular ATP from intact cells. Briefly, GT1-7 cells were plated in black-walled 96 well plates at $5 \times 10^4$ per well in plating medium (Table 2.9) and incubated overnight at 37 °C. Cells were subsequently incubated for 2 hours in serum free experimental medium (2.5 mM glucose), followed by 30 minute incubation in fresh serum free experimental medium supplemented with R481/vehicle. As above, cell lysis buffer and substrate solution were then added and luminescence read.

### 2.2.6 Intracellular calcium

Changes in intracellular calcium were assessed using the fluorescent calcium indicator Fluo-4 Direct. C2C12 myotubes were plated in clear, flat bottomed 96 well plates at a seeding density of $5x10^3$ cells/well in plating medium and differentiated (as before) for 7 days. Cells requiring overnight serum deprivation were incubated for 16 hours with EBSS prior to the start of the experiment, whereas others were maintained in differentiation media for the 7 days. On the day of the experiment, cells were serum starved for up to 2 hours before being incubated with Fluo-4

Direct-containing phenol red free media (supplemented with 25 mM HEPES and 5.5 mM glucose) for 60 minutes at 37$^{o}$C. Following incubation, fluorescence was captured using a PHERAstar FS plate reader with 485 nm and 520 nm excitation and emission wavelengths, respectively. Measurements were recorded every 0.2 seconds with compounds used to stimulate calcium responses being injected after 30 seconds. Changes in calcium were expressed as relative fluorescent units normalised to a baseline value of 1.

### 2.2.7 Assessment of cellular metabolism

Cellular metabolism was assessed by measuring changes to oxygen consumption rate (OCR) as a measure of mitochondrial function and extracellular acidification rate (ECAR) as a proxy for glycolytic rate using the Seahorse Bioscience XFe96 extracellular flux analyser (Agilent).

#### 2.2.7.1 Mitochondrial stress test

Mitochondrial stress tests were performed according to manufacturer's instruction with refinements described below (Agilent, UK). C2C12 myoblasts were seeded at 1.6x10$^{3}$ cells/well in Seahorse XFe96 cell culture microplates in 200 µL of plating media and subsequently differentiated, as described above, for 7 days. On the day prior to the experiment, Seahorse XFe96 sensor cartridges were hydrated with Seahorse XF calibrant solution and maintained at 37 $^{o}$C in a non-$CO_2$ incubator overnight. For experiments assessing the chronic effect of palmitate on mitochondrial function, treatments were conducted for 16 hours prior to the experiment, in EBSS (supplemented as described in Table 2.8). Cells were treated with increasing concentrations of palmitate (250-750 µM) conjugated as described above, to 2 % FA-free BSA. On the day of the experiment, media was aspirated and cells washed with Seahorse XF DMEM before being incubated for 1 hour with XF DMEM, at 37$^{o}$C in a non-$CO_2$ incubator. Overnight treatments were re-introduced at this stage. During this time, cartridges were loaded with inhibitors or compounds of interest to be injected during the assay. For mitochondrial stress tests, cartridges were loaded with Oligomycin (final concentration 2 µM), FCCP (final concentration 1 µM) and Rotenone/Antimycin A (1:1 ratio, final concentration 1 µM). Oligomycin inhibits mitochondrial complex V, or ATP-synthase, and the resulting decrease in OCR is reflective of oxygen consumption associated with ATP synthesis. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)

transports protons ($H^+$) through the inner mitochondrial membrane, interfering with the proton gradient and therefore uncoupling oxidative phosphorylation and allowing for maximum oxygen consumption. Rotenone and antimycin A inhibit mitochondrial complexes I and III, respectively blocking the remainder of the respiratory chain. The cartridges were then calibrated in the Seahorse bioanalyser. Following 1 hour de-gas, cell plate was loaded onto the machine with pre-loaded cartridges and protocol (Table 2.16) initiated.

**Table 2.16 Mitochondrial stress test protocol steps**

| | Baseline | Oligomycin | FCCP | Rotenone + Antimycin A |
|---|---|---|---|---|
| Cycles | 4 | 4 | 4 | 4 |
| Mix (min) | 03:00 | 03:00 | 03:00 | 03:00 |
| Injection | - | Port A | Port B | Port C |
| Measure (min) | 03:00 | 03:00 | 03:00 | 03:00 |

Upon completion of the assay, cell plates were removed from the analyser and remaining media aspirated. Cells were lysed using sodium hydroxide (50 mM, 100 µL/well) and protein quantified in the lysates using the protein quantification methods described above, with minor alterations. Briefly, sodium hydroxide was used instead of lysis buffer in standard curve wells. Changes in OCR and ECAR were normalised to total protein concentrations. Calculations of cellular metabolic parameters were done according to Table 2.17.

**Table 2.17 Mitochondrial stress test parameter calculation**

| **Parameter** | **Equation** |
|---|---|
| Basal respiration | (last rate measurement before oligomycin injection) – (non-mitochondrial respiration) |
| Oligomycin-sensitive OCR | (last rate measurement before oligomycin injection) – (minimum rate measurement after oligomycin injection) |
| Maximal respiration | (maximum rate measurement after FCCP) – (non-mitochondrial respiration rate) |
| Spare respiratory capacity | (maximal respiration) – (basal respiration) |
| Proton leak | (minimum rate measurement after oligomycin injection) – (non-mitochondrial respiration) |

### 2.2.7.2 Glycolysis stress test

Glycolytic stress tests were conducted in the same manner as the mitochondrial stress tests with the following differences: during the de-gas incubation period, cells were deprived of glucose to assess their non-glycolytic ECAR (baseline) before testing the cells' ability to respond to different stimuli and inhibitors. The glycolysis stress test measures glycolysis as the ECAR reached by a given cell after the addition of a saturating amount of glucose (10 mM). Glycolytic capacity is then assessed by shutting down oxidative phosphorylation with oligomycin (2 µM) and driving the cell to maximise glycolytic rate. The cells glycolytic reserve is assessed as the difference between the response to glucose and the maximum rate achieved following oligomycin injection as an indication of the cellular capacity to respond to energetic demand. Finally, 2-deoxyglucose (50 mM) was used to suppress glycolysis and achieve a measure of non-glycolytic acidification. Effect of acute treatments on glycolytic rate was assessed by injecting compounds prior to glucose, followed by the steps above. Protocol in Table 2.18 and calculation of different glycolytic parameters described in Table 2.19.

**Table 2.18 Glycolysis stress test protocol steps**

| | Baseline | Glucose | Oligomycin | 2-DG |
|---|---|---|---|---|
| Cycles | 4 | 4 | 4 | 4 |
| Mix (min) | 03:00 | 03:00 | 03:00 | 03:00 |
| Injection | - | Port A | Port B | Port C |
| Measure (min) | 03:00 | 03:00 | 03:00 | 03:00 |

**Table 2.19 Glycolysis stress test parameter calculation**

| **Parameter** | **Equation** |
|---|---|
| Glycolysis | (Maximum rate measurement before oligomycin injection) – (last rate measurement before glucose injection) |
| Glycolytic capacity | (Maximum rate measurement after oligomycin injection) – (last rate measurement before glucose injection) |
| Glycolytic reserve | (Glycolytic capacity) – (Glycolysis) |
| Non-glycolytic acidification | Mean ECAR prior to glucose injection |

### 2.2.8 Extracellular cytokine quantification

The levels of extracellular cytokines present in conditioned media from treated cells were quantified using enzyme linked immunosorbent assays (ELISAs). MIF and IL-6 were measured using mouse Duo-set ELISA kits (Biotechne), performed according to manufacturer's instructions. Briefly, assays were conducted on 96 well microplates incubated overnight with the capture antibody of interest. Following incubation, antibody was washed off (0.05 % Tween® 20 in PBS) and replaced with blocking solution (1 % BSA in PBS) and incubated for 1 hour. Following a second wash step, standards and samples were loaded onto the plate and allowed to bind to capture antibodies for 2 hours, after which the plate was incubated with detection antibody for an additional 2 hours. Once washed off, this was replaced with streptavidin-horseradish peroxidase (HRP)-conjugated antibody and incubated for 20 minutes at room temperature, protected from light. Finally, a wash was performed and substrate solution (50 % v/v $H_2O_2$, 50 % v/v tetramethylbenzidine) added to each well and incubated for precisely 20 minutes before a stop solution (2 N $H_2SO_4$) was added to stop the colour changing reaction. At this point, absorbance was read in a PHERAstar plate reader at 450 nm. Wavelength correction was performed by subtracting substrate reading at 570 nm from those at 450 nm. After wavelength correction, data were analysed using a 4-parameter logistic curve fitting/regression (S-shaped). MIF Duoset specificity: 25.0 ng/mL of recombinant human MIF reads as 551 pg/mL (2.2 % cross-reactivity). Detection range 125 – 8000 pg/mL. IL-6 Duoset sensitivity: 50 ng/mL human/rat/porcine recombinant IL-6 assayed demonstrates no cross-reactivity or interference. Detection range 15.6 – 1000 pg/mL.

### 2.2.9 Glucose uptake assay

Glucose uptake into C2C12 myotubes was assessed using the Glucose Uptake-Glo assay (Promega), according to manufacturer's instructions. This non-radioactive plate-based assay uses a bioluminescence method to detect 2-deoxyglucose-6-phosphate (2DG6P), which is formed upon entry of 2-DG into cells and cannot be metabolised further by glycolytic enzymes. Briefly, C2C12 myotubes were differentiated in 96-well plates for 7 days (as above). On day 7, wells were washed with PBS before 2 hour incubation in DMEM (11966; 5.5 mM glucose) starved of serum, at 37 °C.

Media was replaced with serum free, glucose free DMEM and incubated for 1 hour at 37 °C. Cells were then pre-treated with relevant treatment stated in each experiment and subsequently incubated for 15 minutes with 100 µM 2-DG (dissolved in PBS). To terminate the reaction a stop buffer was added to lyse the cells (acid detergent solution) and this was neutralised by the addition of a high-pH buffer solution (neutralising solution). A detection reagent containing glucose-6-phosphate dehydrogenase (G6PDH), NADP+, reductase, luciferase and proluciferin substrate were then added. 2DG6P is oxidised by G6PDH to 6-phosphodeoxygluconate and this reduces NADP+ to NADPH, which is used by the reductase enzyme to convert proluciferin to luciferin. Luciferin is used by the luciferase to produce a luminescent signal that is proportional to the concentration of 2DG6P. Luminescence was detected with 0.3-1 second integration using the PHERAstar FS microplate reader. Glucose uptake assays were performed as follows:

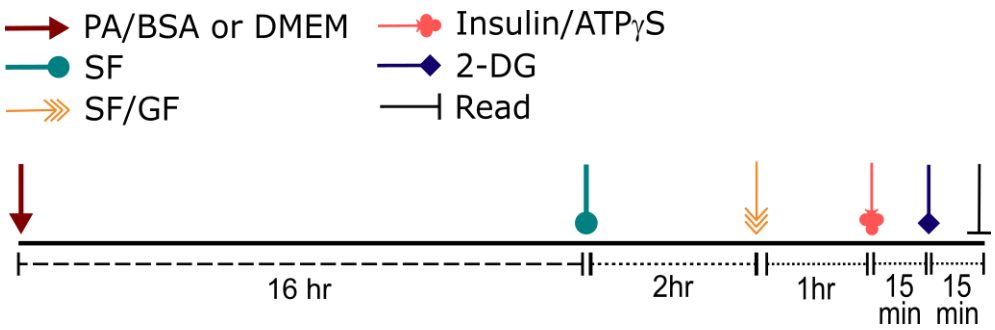



**Figure 2.1 Glucose uptake assay**

Schematic representation of glucose uptake experiments. PA, palmitate; SF, serum-free DMEM; GF, glucose-free, 2-DG, 2-deoxy-d-glucose

## 2.3 *In vivo* methods

### 2.3.1 Experimental animals

All experimental procedures were conducted under a Home Office Project License and in accordance with the University of Exeter and Home Office regulations (UK) as well as Animals (Scientific Procedures) Act (1986). Efforts were maximised to employ the 3Rs, reduction, replacement and refinement, when possible, and animal welfare carried out routinely with help from staff at the Biomedical Services Unit, Living Systems Institute (University of Exeter).

Unless otherwise stated, procedures were performed in male Sprague-Dawley rats (75-400 g), obtained from Charles River Laboratories (Margate, UK), some of which were pre-implanted with carotid artery and jugular vein catheters exteriorised via vascular access buttons and protected with button caps. Animals were group housed (groups of 2 or 3) when not undergoing procedures and had *ad libitum* access to food (standardised chow diet, LabDiet, comprised of 27% protein, 13 % fat and 59 % carbohydrate in palatable pellet form) and water while being maintained in temperature (22-23 °C) and humidity (55 %) controlled facilities with a dark and light cycle of 12 hours respectively.

### 2.3.2 Compounds

Compounds were obtained from commercial sources and adequately prepared for experimental use in a sterile manner. Suppliers and catalogue numbers listed in Table 2.1. Compounds used in animals were administered intravenously (i.v.), orally or intraperitoneally (i.p.) in adequate buffers and volumes concordant with The Laboratory Animal Science Association (LASA) guidelines.

### 2.3.4 Feeding studies

The effect of different compounds on feeding behaviour was assessed in healthy, male, Sprague-Dawley rats throughout the studies presented in Chapter 5. Unless otherwise stated, animals were singly housed during feeding studies and had *ad libitum* access food (standardised chow) and water. Food assessments in response to R481 were conducted as follows: *Ad libitum* food measurements were performed by injecting rats with R481/vehicle (i.p.) shortly before the start of their dark-phase, where they are most active and consume the largest amount

of calories in the day, and food measurements taken after 1 and 4 hours. For assessment of hyperinsulinaemia-induced feeding, animals were fasted for 16 hours, following which they were administered R481/vehicle (orally) and injected with insulin (0.75 U/Kg) one hour later. Food was introduced after 60 minutes and intake measured 1 and 4 hours later. The effect of fasting on feeding behaviour was also assessed by fasting rats for 16 hours, administering R481/vehicle (i.p.), immediately re-introducing food and measuring intake after 1, 2 and 4 hours. Weights were monitored before and after all feeding studies.

### 2.3.5 Intraperitoneal glucose tolerance test (IPGTT)

Healthy male Sprague-Dawley rats were fasted for 16 hours, and fasting blood glucose measured from a tail vein sample (made using a 21 gauge needle) and measured using a hand-held glucometer (AccuCheck, Roche). Approximately 15 minutes prior to first tail vein prick rat tails were coated with local anaesthetic (lidocaine and prilocaine, EMLA 5 %). For studies using SBI-0206965 and hexamethonium, compounds were administered (i.p.) 30 minutes prior to administration of R481/R419/vehicle (i.p.) and glucose (2 g/Kg; i.p.). Blood glucose was measured 15, 30, 60 and 120 minutes after glucose administration.

### 2.3.6 Insulin-induced hypoglycaemia

Rats were fasted (16 hours) and basal blood glucose measured, as above. Animals were then administered R481/vehicle by oral gavage and 60 minutes later with insulin 0.75 U/Kg (i.p.). Blood glucose was measured 30 minutes after insulin administration, as above.

### 2.3.7 Glucose clamping

#### 2.3.7.1 Hyperinsulinaemic-euglycaemic clamping

The hyperinsulinaemic-euglycaemic clamp was first developed as a method to assess insulin sensitivity in humans (DeFronzo, Tobin and Andres, 1979), but has since been adapted for use in rodents. Gold-standard glucose clamping in rats is performed in conscious, freely moving animals, which have undergone surgical implantation of carotid artery and jugular vein catheters. Catheters are externalised via connection to a vascular access button (VAB) which can be directly accessed using a syringe (combined with a pinport adaptor) or a tether, allowing for continuous venous infusion and arterial sampling.

A magnetic vascular access button cap is placed over the buttons to allow rats to be group-housed when not undergoing procedures. Catheters are maintained patent over time with the use of a heparinised glucose lock solution which prevents blood from entering the catheters and blocking them. During a hyperinsulinaemic-euglycaemic clamp, insulin is infused at a constant dose, aimed to induce a steady state of hyperinsulinaemia, concomitantly with a variable dose of dextrose to maintain euglycaemia; while allowing for sampling via the carotid artery for measures of glucose and hormones. Here, healthy, male, Sprague-Dawley rats underwent catheter implantation surgery at Charles River (Margate, UK) seven days before being transported to the Biomedical Services Unit (Living Systems Institute, Exeter). Daily health checks and weight monitoring were conducted during a 7 day acclimatisation period following arrival. During acclimatisation, catheter patency was checked (every 3-4 days) by drawing catheter content, flushing with sterile saline (0.9 % NaCl) and re-locking the catheters with a heparinised glucose lock solution (100-500 U/mL heparin in 50 % w/v glucose solution). The day before the clamp study, rats were weighed and food removed for 16 hours. After the 16 hour fast, rats were re-weighed and basal glucose samples taken (from the arterial catheter). R481/vehicle was then administered (i.p.) and 60 minutes later another blood sample was taken, venous catheter unlocked and animals connected to magnetic tethers. Insulin and dextrose infusions were started simultaneously, marking the start of the clamp. Insulin infusion was maintained constant, while glucose infusion rate was changed in response to blood glucose levels measured from blood samples collected every 10 minutes throughout the clamp. Small (<50 µL) samples were taken every 10 minutes and larger samples (~150 µL) were collected every 30 minutes. To collect a blood sample, the content of the carotid catheter was removed using a syringe attached to the pinport at the end of the tether with a pinport adaptor. A small amount of blood (<50 µL) was taken to ensure no lock solution was present in the sample. Blood was then collected. Catheters were flushed with saline and re-locked with heparinised glycerol solution. Note that lock solution on the day of clamping was not comprised of glucose and heparin, rather glycerol and heparin (300 U/mL heparin in 80-100 % glycerol) to ensure that residual lock solution in the catheter did not alter blood glucose reading from samples.

Large samples were centrifuged at 5,000 rpm for 10 minutes at 4 °C and plasma fraction transferred to separate storable eppendorf tubes and immediately frozen at -20 °C.

Target blood glucose during euglycaemic clamp was 5.5 mmol/L glucose. Clamp duration was 120 minutes, after which animals were disconnected from the clamping apparatus and sacrificed by intravenous injection of pentobarbitone sodium (20% w/v, 200 mg/mL administered at 60-100 mg/Kg). Animals were exsanguinated via carotid artery catheter using a syringe with pinport adaptor and blood processed for plasma preparations by centrifugation at 3,500 rpm for 15 minutes, at 4 °C. Plasma was collected and stored at -20 °C.

#### 2.3.7.2 Hyperinsulinaemic-hypoglycaemic clamping

Hyperinsulinaemic-hypoglycaemic clamps are useful to assess the counterregulatory response to hypoglycaemia under controlled conditions. Clamps were performed as above with small adjustments. A bolus dose of insulin (80 mU/Kg/min) was administered for 10 minutes as rats were connected to the tethers, followed by continuous infusion as before. A variable 20 % dextrose solution was infused based on glucose infusion rates aiming to maintain blood glucose at 3 mmol/L. Clamps were terminated after 90 minutes and animals sacrificed as above.

#### 2.3.7.3 Calculating glucose infusion rates

Once blood glucose concentration is clamped (at steady state), maintained by a variable glucose infusion, the glucose infusion rate equates to glucose uptake by tissues. The following equation was used to calculate glucose infusion rate (GIR, in mg/Kg/min) during glucose clamps:

$$GIR = \frac{Volume\ glucose\ infusion\ (\mu L/\min) \times \text{mass infused glucose (mg)}}{weight\ (Kg)}$$

(where volume of glucose infusion was the variable rate infused during clamp, mass of glucose corresponds to glucose solution (eg. 20 % or 50 % w/v glucose = 0.2 or 0.5 mg) and weight refers to rat weight).

### 2.3.8 Plasma hormone and metabolite analysis

Plasma glucagon analysis was performed using Mercodia Glucagon ELISA (Uppsala, Sweden) according to manufacturer's instruction. Briefly, pre-coated plates were loaded with 10 µL per well of calibrant or plasma along with 50 µL of enzyme conjugate and incubated at 4°C for 20 hours, shaking at 800 rpm.

The following day, six wash steps were performed using wash buffer and well subsequently incubated for 3 minutes with TMB substrate. The reaction was stopped with stop solution and absorbance read at 450 nm. Assay specificity: highly specific. Does not detect GLP-1, GLP-2 or mini-glucagon. Detects mouse (7 %) human (1 %) and rat (1 %) glicentin. Detection range 7-627 pg/mL).

Plasma adrenaline was analysed using Demeditec Adrenaline ELISA (Kiel, Germany) according to manufacturer's instruction. Standards and samples were pipetted onto two 48 well plates (10 µL) and diluted in 90 µL of distilled water. Subsequently, 25 µL of TE buffer were added per well and the plate incubated for 1 hour at room temperature, shaking at 600 rpm. Two wash steps were then performed with wash buffer and 150 µL of acylation buffer added to each well. Acylation reagent (25 µL) was then pipetted onto each well and the plate incubated for 20 minutes at room temperature. Two more wash steps were performed before plates were incubated for 10 minutes at room temperature in the shaker with 100 µL hydrochloric acid in each well. The steps above describe the preparation of the extraction plates. The samples from this plate were then transferred to a microtiter plate (90 µL/sample), to which 25 µL of enzyme solution were added. Plate was incubated for 1 hour at RT, followed by 2 hrs at 37°C. The samples from this plate were then transferred into adrenaline microtiter plates (100 µL). Adrenaline antiserum (50 µL) was added to each well and plate incubated for 1 hour at RT, in the shaker, followed by an overnight incubation at 2-8°C. Four wash steps were then performed and plates incubated with 100 µL enzyme conjugate, per well, for 30 minutes, at RT. Four more wash steps were undertaken. Finally, 100 µL substrate was added per well and plate incubated for 30 minutes at RT, protected from light. Reaction was stopped with stop solution (100 µL) and absorbance read at 450 nm within 10 minutes. Assay analytical sensitivity: 3.3 pg/mL; detection range 0.5 – 80 ng/mL.

Levels of C-peptide in plasma were measured using Mercodia rat C-peptide ELISA (Uppsala, Sweden) according to manufacturer's instructions. Briefly, samples and calibrators (10 µL) were incubated with assay buffer for 1 hour at room temperature in a shaker (800 rpm). This was followed by six wash steps using wash buffer. Subsequently, 100 µL of enzyme conjugate were added to each well and plate incubated for another hour under the same conditions. Washes were repeated and were followed by a 15 minute incubation with 200 µL of substrate TMB.

Reaction was stopped with 50 µL stop solution, plate briefly shaken and absorbance read at 450 nm within 30 minutes. Highly specific (tested cross-reactivity with insulin (0.0006 %), proinsulin (5 %), proinsulin Des 31-32 (3 %), proinsulin Split 32-33 (2 %), proinsulin Des 64-65 (74 %), proinsulin Split 65-66 (10%). Detection range 0.015 – 0.85 ng/mL.

## 2.4 Human methods

### 2.4.1 Participant characteristics and screening

Thirteen healthy, recreationally active, young (18-40 y) males volunteered to participate in the study presented in Chapter 4. Participants were recruited from the University of Exeter student and staff bodies as well as the local community via poster and social media advertisement. Upon first contact with potential participants, these were provided with a participant information sheet (Appendix A) that they were asked to read before attending a screening visit. At the start of the screening visit, subjects were duly informed about the nature of the study, including potential risks of the experimental procedures being undertaken, before informed consent was obtained. Exclusion criteria applied to subjects that presented with a body mass index (BMI) below or above 18 and 30 $Kg{\cdot}m^{-2}$, respectively, were smokers, had a history of thrombosis, epilepsy or schizophrenia or any disorders of cardiovascular, metabolic or motor nature. Exclusions were also applied to individuals with elevated blood pressure at the time of screening as well as those chronically using nutritional supplements or pharmaceuticals; or that had taken part in blood donations or research studies in the three months leading to this study.

After clarifying details of the study with the investigator, participants were asked to complete a medical health questionnaire (Appendix B) as well a physical activity questionnaire (International physical activity questionnaire, iPAQ, long-last self-administered format, 2002) (Appendix C). Following this, heath parameters such as height, body mass, BMI, blood pressure and body composition were measured. Body composition regarding fat and lean mass was assessed using air-displacement plethysmography with the BODPOD Body Composition System (COSMED). The screening visit was concluded with a brachial ultrasound assessment to ensure that the investigator was able to locate the brachial artery. Before leaving, participants were provided with a three-day diet diary, in which they were asked to record their habitual food and drink intake, in detail.

This was used at the end of the study to assess habitual energy intake using a nutrition analysis software (Nutritics Education v4.315). In addition, participants were made aware that they were free to withdraw at any stage of the study with no disadvantages to themselves. Upon completion of the study, all participants received an inconvenience allowance. The Ethics Committee of Sport and Health Sciences of the University of Exeter approved all aspects of the studies described in this thesis. Clinical trial was registered at www.clinicaltrials.gov under registration number NCT02980952. No adverse effects were recorded during the study. Subject characteristics are presented in Table 2.20.

**Table 2.20 Participant characteristics**

| | **CON (n=8)** | **HFD (n=5)** |
|---|---|---|
| **Age (y)** | 25.4 ± 2.42 | 23.8 ± 2.82 |
| **Height (m)** | 1.76 ± 0.04 | 1.80 ± 0.03 |
| **Body mass (Kg)** | 74.27 ± 6.39 | 68.5 ± 1.32 |
| **BMI ($Kg \cdot m^{-2}$)** | 23.7 ± 1.38 | 21.2 ± 0.72 |
| **Body fat (%)** | 19.2 ± 4.41 | 16.1 ± 8.59 |

### 2.4.2 Experimental Design

This study represents a randomised controlled trial where participants were subjected to 7 days of single forearm immobilisation by means of a forearm cast. During the immobilisation period, participants were fed a controlled diet comprised of packaged meal plans including ingredients and recipes to prepare and consume at home. Subjects were randomised (www.randomization.org) into control (CON) eucaloric diet or a high-fat diet (HFD), where they consumed 50 % of their daily caloric intake in excess. Subjects visited the laboratory on 4 occasions after the screening visit. An initial baseline test day was performed to measure the parameters of interest before the start of the immobilisation period, followed by a visit in which the cast was fitted and two consecutive test visits 2 and 7 days after the start of the immobilisation period. Experimental visit design is depicted in Figure 2.1.

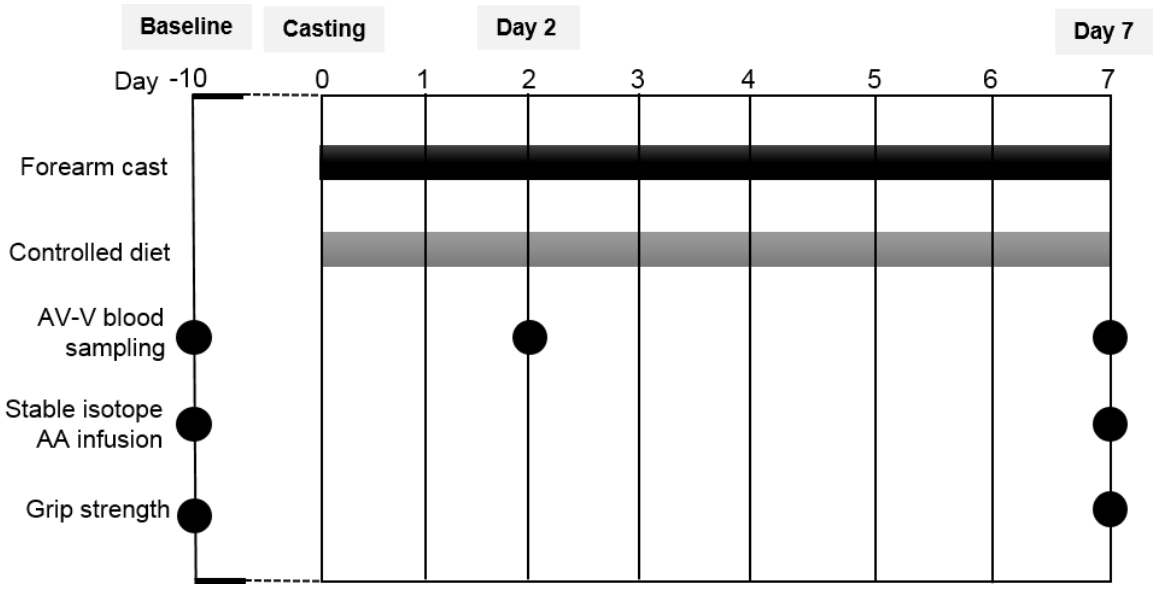



**Figure 2.2. Schematic summary of study design**

#### 2.4.2.1 Experimental visits

Participants visited the laboratory for three experimental visits. The first baseline visit occurred up to ten days before the start of the immobilisation period, with two consecutive visits taking place at days 2 and 7 of immobilisation. During the baseline visit, participants arrived in the laboratory in the fasted state (12 hour fast) and voided their bladders before being weighed in digital scales, and settling in a bed in the Nutritional Physiology labs, where they would remain for the

duration of the test day in a semi-supine position. At this point, an antegrade cannula was inserted in an antecubital vein in the dominant arm for stable isotope infusion. A second cannula was then inserted retrograde in a heated superficial dorsal hand vein in the dominant arm, after which the hand was inserted in an air-warming unit (hotbox, heated to 55$^{o}$C) for arterialised-venous blood sampling. Blood arterialised using this method generates oxygen saturation levels akin those of arterial samples (Liu *et al.*, 1992), providing an approach which is both practical and safe and has been validated for measures of glucose, insulin, glucagon, AAs and non-esterified FAs (Abumrad *et al.*, 1981). A final cannula was inserted retrograde in a deep antecubital vein in the contralateral arm, used for sampling of venous blood draining directly from the forearm muscle bed (Gallen and Macdonald, 1990). All cannulae were maintained patent using a 0.9 % saline infusion. Following the first venous baseline sample, taken as the cannula was inserted, a total of two postabsorptive blood samples and nine postprandial samples were taken, occurring at 20 minute intervals.

Preceding every blood sample, measurements were taken using a Doppler ultrasound to assess arterial diameter and blood velocity, which would later be used to calculate blood flow and factor into measures of nutrient uptake. Immediately after the baseline blood sample (at t = -120 minutes), a single intravenous dose of the isotope tracer (2 μmol·Kg$^{-1}$ L-[D5]-Phenylalanine and 7.6 μmol·Kg$^{-1}$ L-[1-$^{13}$C]-Leucine) was administered to prime the reserves of leucine and phenylalanine in the body and this was immediately followed by a stable continuous infusion (0.05 μmol·Kg$^{-1}$·min$^{-1}$ L-[D5]-Phenylalanine and 0.13 μmol·Kg$^{-1}$·min$^{-1}$ L-[1-$^{13}$C]-Leucine). The start of the infusion marked the start of a 120 minute postabsorptive period where two additional blood samples were taken (t = -20 and t = 0) before a mixed meal test drink was given. The ingested volume of the drink (Ensure Plus, Abbott Nutrition) was adjusted between participants so that 0.2 g fat, 0.3 g protein and 1.1 g carbohydrates per Kg of body weight were consumed and finalisation of the drink marked the start of the 180 minute postprandial period. Simultaneous arterialised-venous and deep venous blood samples (AV-V) were taken at 20 minute intervals during the 180 minute postprandial period. At the end of the test day, participants were provided with a meal and asked to perform a grip strength test using a hand-held dynamometer.

The two consecutive experimental visits were undertaken in the same manner as the baseline visit, except day 2 after immobilisation, which was void of a tracer infusion and grip strength assessment. Experimental visit design is summarised in Figure 2.2.

The AV-V model was applied in this study to assess dynamic changes to forearm glucose and amino acid metabolism. Two pool AV-V amino acid and glucose kinetics across the forearm muscle bed were calculated according to the principles and calculations detailed in Wolfe and Chinkes (Wolfe and Chinkes, 2005). Nutrient concentrations do not differ substantially in arterial circulation in the body and arterialised venous blood accurately represents brachial artery glucose and amino acid concentrations (Forster *et al.*, 1972; Macdonald, 1999; Wolfe and Chinkes, 2005). Implicit in using this approach is the assumption that the sampling sites represent delivery and drainage from muscle tissue with little contribution from fat and skin tissues. This is an acknowledged assumption but is supported by evidence suggesting that at least 85% of arterial delivery is to the muscle tissue (Zierler and Rabinowitz, 1963; Pozefsky *et al.*, 1976).

As a result, a systematic but slight overestimation of muscle metabolic balance may occur. Taking into account those assumptions, the metabolic measures across the forearm were referred to here as muscle metabolism.

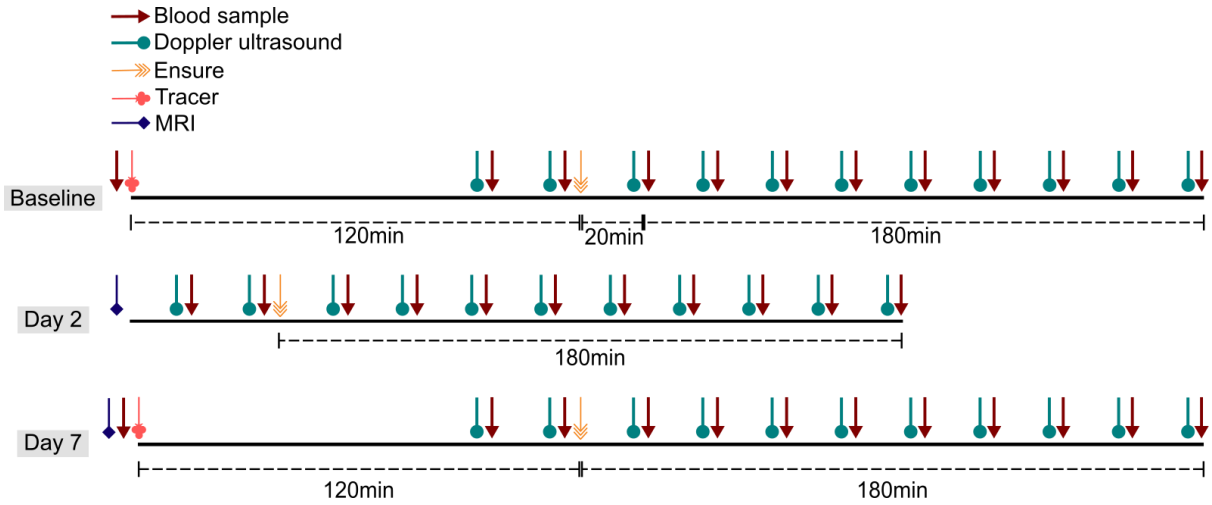



**Figure 2.3 Experimental test day schematic**

### 2.4.3 Control of energy intake

Energy intake was controlled throughout the entire immobilisation period. Participants were provided with pre-packaged meals and recipes ready to cook. Subjects were instructed to consume only the provided food in addition to water and non-caloric drinks and to refrain from vigorous physical activity, particularly 48 hours before each test visit. Food was provided for two days on the casting visit and for the remaining five days after two days of immobilisation. Energy intake was based on individual requirements taking into account participants' basal metabolic rate and physical activity. Basal metabolic rate (BMR) was calculated using the Henry equations (Henry, 2005) based on age, gender, weight and height (e.g. BMR of male (18-30y): $14.4^{*}$weight + $313^{*}$height + 113). Physical activity was not monitored throughout the study but was evaluated during the screening visit using a self-completed questionnaire (International Physical Activity Questionnaire) (Hagstromer, Oja and Sjostrom, 2006), which generated a physical activity level (PAL) based on a metabolic equivalent (MET) score. The product of the calculated PAL score of each individual and the BMR was used as the calculated total energy expenditure (TEE) for each day. Participants in the CON group were fed in energy balance with protein content of the diet being maintained at 1.2 $g \cdot Kg^{-1} \cdot d^{-1}$, carbohydrates (CHO) at 50-55 % daily energy intake and total fat and fibre comprised the remaining energy intake. Participants in the HFD group were fed an additional 50 % of their calculated TEE, mostly comprised of fat, while protein was maintained at 1.2 $g \cdot Kg^{-1} \cdot d^{-1}$ and CHO at 30-35 %.

### 2.4.4 Limb immobilisation

On the second visit to the laboratory, 7-15 days after the baseline test visit, participants reported to the Nutritional Physiology labs to have a forearm cast fitted on their non-dominant arm. Firstly, stockinette and undercast padding were applied to protect the skin covering the forearm from the elbow to the tips of the fingers. Secondly, a fibrecast (BeneCast, BeneCare Medical) cast was fitted to the arm by soaking the fibrecast in warm water and applying in from the elbow, distally, and moulding it to best fit the forearm and immobilise the wrist. Once dry and hardened, the cast extended from 5 cm distal of the antecubital fossa to 2 cm proximal of the fingertips. Participants were provided with a sling and

instructed to wear it during the daytime to keep the hand elevated above the elbow. A waterproof cover was provided to keep the cast dry while showering. Casts were removed at the end of the last test day using a cast saw. Application and removal of the casts was performed by trained personnel.

### 2.4.5 Brachial artery diameter and blood velocity measurements

Brachial artery diameter and mean blood velocity were measured using high-resolution ultrasound imaging in duplex mode (~12 MHz, Apogee, 1000. SIUI). For luminal diameter measures, the probe was placed at the distal portion of the biceps brachii, approximately 5-10 cm proximal to the antecubital fossa of the non-dominant arm, while the arm was placed away from the body with the elbow slightly flexed. Brightness mode (B or 2D-mode) ultrasound was used to locate the brachial artery and enabled the length and lumen of the artery to be insonated, at a 60$^{o}$ angle, at which point a two-second recording was taken. Mean blood velocity was assessed at the same anatomical position by integration of the pulsed-wave Doppler signal for a minimum of 8 cycles (~10-20 seconds) (Hoskins, 1990). Arterial diameter and volumetric blood flow were analysed using the Brachial Analyser for Research software (V 6.8.5, Medical Imaging Applications) (Mancini *et al.*, 2002).

### 2.4.6 Assessment of grip strength

Grip strength was assessed by maximal voluntary contraction using a hand-held dynamometer (T.K.K 5001, Takei Scientific). Assessments were performed at the end of the baseline visit and at the end of the last day of immobilisation after casts were removed and participants were provided with a meal and given time to readjust to not wearing a cast. Maximum voluntary contraction was measured 12 times on each arm and the mean of the last three readings was used as a measure of grip strength (Kg).

### 2.4.7 Processing blood samples

Simultaneous arterialised-venous and deep venous blood samples were taken and processed immediately for storage as plasma and serum. Whole blood was collected in sodium fluoride and potassium oxalate-coated vacutainers and rotated at room temperature for 2 minutes before blood glucose was measured (YSI 2300 PLUS, Yellow Springs, OH, USA).

For plasma collection, blood was collected in lithium heparin-coated vacutainers and centrifuged at 2,900 g for 10 minutes at 4$^{o}$C. For serum, blood was collected in silica particle-coated tubes and allowed to clot at room temperature for 30-60 minutes before centrifuging the samples at 2,900 g for 10 minutes at 4$^{o}$C. Plasma and serum samples were frozen in liquid nitrogen and subsequently stored at -80$^{o}$C. Stored samples were used for biochemical analysis.

#### 2.4.7.1 Plasma, serum and whole blood analysis

**Glucose**: Glucose concentration of arterialised-venous and deep venous blood was measured in whole blood in a clinical glucose analyser (YSI 2300 Stat Plus, Glucose Analyzer; YSI Life Sciences). Glucose uptake (mmol/min) was determined as the product of the arterialised-venous difference in glucose concentration (mmol/L) and arterial blood flow (mL/min) at each time point.

**Insulin**: Insulin was analysed using a commercially available immunoassay kit (DRG Instruments GmbH, Germany) according to manufacturer's instructions. Endogenous serum insulin was measured in arterialised-venous serum during the immobilisation period in fasting as well as postprandial samples. Serum samples for analysis were thawed on ice. Briefly, 25 µL of sample or standard were dispensed into wells (in duplicate) of a microtiter 96 well plate. Enzyme conjugate (mouse monoclonal anti-insulin antibody conjugated to biotin) was dispensed onto each well (25 µL) and plate briefly shaken (10 seconds in plate shaker) and incubated at room temperature for 30 minutes. Wells were subsequently washed three times with wash solution. Wells were emptied and dried (striking on absorbent paper) and subsequently incubated with 50 µL of enzyme complex (streptavidin-HRP) for 30 minutes at room temperature. Wash steps were repeated followed by a 15 minute incubation in substrate solution (tetramethylbenzidine). The enzymatic reaction was stopped by dispensing 50 µL of stop solution ($H_2SO_4$) into each well. Absorbance was read at 450 nm. Assay sensitivity range: 1.76 – 100 µIU/mL.

Insulin sensitivity indices HOMA-IR (Matthews *et al.*, 1985) and Matsuda (Defronzo, 1999) were calculated using the following equations:

HOMA-IR = fasting insulin (µU/L) x fasting glucose (nmol/L)/22.5.

Matsuda index = 10,000/√ ([fasting glucose x fasting insulin] x [mean glucose x mean insulin during 180 minute postprandial period])

**Amino acids:** Analysis of endogenous leucine and phenylalanine plasma levels was conducted by the University of Texas and performed using gas chromatography-mass spectrometry (GC-MS), as described previously (Wolfe and Chinkes, 2004). Analytical method (provided by collaborators) was as follows: Briefly, 10 µL internal standards of leucine and phenylalanine were added to the plasma samples. Plasma samples were deproteinised on ice with 500 µL of 15 % 5-sulfosalicylic acid. Free AAs were purified by acid-washed cation exchange columns (AG 50W-X8 resin: Bio-Rad, CA, USA) and AAs eluted from the column with 8 mL of 2N ammonium hydroxide. The eluate was subsequently dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA). Samples then underwent derivatization by adding 40 µL of *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1 % *tert*-Butyldimethylchlorosilane and 40 µL of acetonitrile to samples. Samples were vortexed and heated to 95°C for 40 minutes (Bornø *et al.,* 2014). Samples were subsequently analysed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies) in duplicate using electron impact ionisation and selected ion monitoring for measurement of isotope ratios (Zabielski *et al.*, 2013) by injecting 1 µL of sample splitless mode (injector temperature 280 °C). Peaks were resolved using an HP5-MS 30 m × 0.25 mm ID × 0.25 µm capillary column (Agilent). Helium was used as carrier gas at a flow rate of 1.2 mL/minute. The temperature ramp was set from 80 – 245°C at 11 °C/minute, followed by 280°C at 40°C/minutes (Zabielski *et al.*, 2013). Selected ion recording conditions were used to monitor fragments m/z 336, 341 and 346 for phenylalanine and m/z 274 and 280 for leucine. The reagents used above have not been included in the list of chemicals used in this thesis as this analysis was not performed in Exeter.

### Net amino acid balance

Leucine and phenylalanine net balance was calculated according to the Fick principle:

$$NB = BF \times (C_A - C_V)$$

(where NB is the net balance, BF is blood flow, $C_A$ is the arterialised venous concentration and $C_V$ is the deep venous concentrations of the specific amino acid adjusted for steady-state conditions (Zierler and Rabinowitz, 1963; Hoskins, 1990).

## 2.5 Statistical analysis

Significant differences between two groups were determined using unpaired t-tests. In Western Blotting experiments, significance in phosphorylated or total protein expression relative to control (normalised to 1) was determined using a One-sample t-test. Comparisons between three or more groups of treatments were compared using One-way ANOVA. Repeated measures data were analysed using a two-way or three-way ANOVA with repeated measures. In instances where data sets with repeated measures were missing values or values were excluded from analysis as they were generated in response to erroneous data collection, a mixed-effects analysis was performed. In human studies, repeated measure ANOVA analysis was conducted with day (Baseline vs Day 2 vs Day 7) and time (post-absorptive and postprandial period during experimental day) as within-subject factors, and diet intervention (CON vs HFD) as between-subjects' factor. In the case of significant interactions, donated by $P<0.05$, multiple comparisons were made using Bonferroni's posthoc tests. Statistical analysis was performed using GraphPad Prism (Prism 8, GraphPad, La Jolla, CA, USA). Unless otherwise specified, results were represented as mean ± standard error of the mean (SEM), with $P<0.05$ being considered statistically significant.

# Chapter 3

# The effect of extracellular ATP signalling on glucose metabolism and fatty acid-induced metabolic dysregulation in skeletal muscle cells

# Introduction

Skeletal muscle accounts for 40-50 % of total body mass and handles 30 % of basal and 90 % of insulin-mediated glucose disposal (DeFronzo *et al.*, 1981; Katz *et al.*, 1983). In T2D 85-90 % of the impairment in total body glucose utilisation is attributed to muscle IR (Pendergrass *et al.*, 2007). The aetiology behind loss of insulin sensitivity, which is a predominant feature in metabolic conditions such as obesity and T2D, is incompletely understood, but it is well accepted that behaviours such as inactivity and chronic overeating and consequentially enhanced lipid availability, play an important role in its development. It is also increasingly recognised that in addition to attenuated insulin sensitivity, individuals with these conditions suffer from loss of muscle mass and strength and that excess lipid may modulate muscle protein synthesis in response to AAs (anabolic resistance, explored further in Chapter 4) impairing not only function but also growth (Stephens *et al.*, 2015).

There is ample evidence suggesting that increased FFAs (Paolisso *et al.*, 1995), IMCL accumulation (Goodpaster *et al.*, 2000; Malenfant *et al.*, 2001) and enhanced generation of lipotoxic metabolite are strongly associated with the development of SM IR (Belfort *et al.*, 2005). The metabolic flexibility in utilisation of glucose and/or lipids during post-absorptive and post-prandial periods is also impaired, leading to metabolic inflexibility (Kelley and Mandarino, 2000). Intravenous lipid infusion, which elevates plasma FFAs, induces IR in individuals with and without diabetes (Boden, 1999). In contrast, acutely lowering elevated FA levels improves insulin sensitivity in obese individuals with and without diabetes (Santomauro *et al.*, 1999). Furthermore, FA transporter expression and rate of FA uptake are upregulated in obesity and T2D in an effort to lower circulating lipid levels (Bonen *et al.*, 2004). This leads to an initial increase in β-oxidation that ultimately fails to match demand and causes incomplete fat oxidation, leading to build-up of lipotoxic moieties and ROS generation (Muoio and Neufer, 2012; Aon, Bhatt and Cortassa, 2014). Mitochondrial dysfunction is also observed in obese humans, demonstrating decreased activity of CPT1, citrate synthase and ATP synthase, compared to lean individuals (Simoneau *et al.*, 1999; Kim *et al.*, 2000). The resulting increase in lipid metabolites, including ceramides (Summers, 2006), LCFA-CoA (Cooney *et al.*, 2006), DAG (Itani *et al.*,

2002) and acylcarnitines (Aguer *et al.*, 2015) impair insulin action and glucose metabolism (mechanisms summarised in Figure 1.6).

Systemic low-grade inflammation also features in metabolic disease and is associated with the pathophysiology of IR. SM inflammation is emerging as an important autocrine regulator of muscle metabolic function and paracrine/endocrine mediator of systemic IR and T2D (DeFronzo and Tripathy, 2009). Inflammatory markers contribute to SM IR through the mechanisms summarised in Figure 1.8. Importantly for this study, SFAs act as inflammatory moieties by signalling via TLRs (TLR2 and TLR4 in SM) and transducing signals via PKC, JNK and IKK/NF-κB signalling pathways (Wei *et al.*, 2008). Expression of TLRs (Wei *et al.*, 2008) and NF-κB pathway (Green *et al.*, 2011; Patsouris *et al.*, 2014) are upregulated in SM of individuals with obesity and diabetes. FA treatment of muscle cells activates IKK/NF-κB signalling pathways and upregulates the expression and release of myokines such as IL-6 and chemokines that promote immune cell migration and activation and contribute to SM IR (Wu and Ballantyne, 2017). A fraction of SM inflammation in metabolic disease is also attributed to immune cell infiltration, particularly macrophages and T lymphocytes, primarily localised in muscle adipose depots (Khan *et al.*, 2015).

SFAs are believed to be the main culprits in the development of lipid-induced IR, as evidence suggests that mono and poly-unsaturated fatty acids (PUFA) play paradoxical roles and may negate the effects of SFA (Fedor and Kelley, 2009; Jans *et al.*, 2012). However, the physiological balance between these species is disturbed in pathophysiology so that SFA/PUFA ratio increases (Imamura *et al.*, 2016). Palmitic acid (palmitate) is the most abundant dietary derived and synthesised SFA in the body, accounting for 20-30 % of all FAs (Carta *et al.*, 2017), and, as such, is extensively used *in vitro* to study mechanisms of IR. In line with this, palmitate treatment impairs insulin signalling in several models of SM (Aas *et al.*, 2005).

Although there is a clear association between elevated lipid availability and the development of insulin and anabolic resistance, the mechanisms by which these develop are not fully understood. There is, therefore, a need to elucidate the processes that regulate glucose and AA metabolism, which may help identify

viable therapeutic targets to ameliorate the pathophysiology of metabolic disease. One such candidate may be ATP.

ATP, along with other nucleotides and nucleosides acting as extracellular signalling molecules, has emerged as an important mediator of a plethora of physiological and pathophysiological processes (as introduced in Chapter 1). Early work postulated an association between purinergic signalling and diabetes (Mikhail and Awadallah, 1977; Tang *et al.*, 1996) and there is now a better understanding of the involvement of ATP and adenosine in the pathophysiology and treatment of diabetes (Atkinson and Eisenbarth, 2001; Antonioli *et al.*, 2015). Relevant to this study, ATP has been reported to induce IR and inflammation in AT (Yu and Jin, 2010); ADP blocks INSR signalling and PKB activation in hepatocytes via P2Y13 (Chatterjee and Sparks, 2012); hyperglycaemia stimulates nucleotide release from blood, smooth muscle and endothelial cells (Solini *et al.*, 2005; Nilsson *et al.*, 2006); CD39-null mice demonstrate impaired hepatic insulin sensitivity and glucose clearance (Enjyoji *et al.*, 2008); and ATP stimulates insulin release from β-cells and islets (Tozzi *et al.*, 2018). Adenosine is also an important regulator of insulin sensitivity and glucose homeostasis in liver (Figler *et al.*, 2011; Johnston-Cox *et al.*, 2012) and AT (Csóka *et al.*, 2014), often playing paradoxical roles to its nucleotide counterparts.

The effect of purinergic signalling on glucose homeostasis and inflammation in SM remains understudied. However, as SM is a major regulator of impaired insulin sensitivity and glucose metabolism in diabetes, it is important to elucidate the contribution of extracellular purinergic mediators in this pathophysiology. ATP is released from SM following electrical stimulation, contraction and exercise and acts in an autocrine and paracrine manner via a wide range of P2Rs, leading to increased $[Ca^{2+}]_i$ (Cseri *et al.*, 2002; Ito *et al.,* 2018) and transactivation and transinhibition of pathways such as MAPK/ERK and PKC (von Kugelgen and Harden, 2011; Erb and Weisman, 2012).

Given the role of extracellular ATP (eATP) in systemic inflammation and glucose homeostasis, it was hypothesised that eATP may, in part, mediate the metabolic impairments brought about by SFA treatment in muscle cells.

The aim of this study was, therefore, to establish whether elements of the purinergic signalling system are involved in FA-induced SM insulin and anabolic resistance and exacerbate inflammatory cytokine release. This was approached by utilising a SM cell model (C2C12 myotubes) of FA-induced IR, displaying loss of anabolic sensitivity, mitochondrial dysfunction and upregulated inflammatory cytokine secretion (features of metabolic disease pathophysiology) in response to palmitate treatment.

# Results

## 3.1 Differentiation of C2C12 myotubes at physiological glucose concentration

The C2C12 murine myoblasts cell line was used as these have been extensively characterised in culture as a tool to study SM metabolism. Conventional culture of C2C12 myoblasts and differentiation into myotubes is conducted under hyperglycaemic conditions (25 mM glucose). Here, a more physiologically relevant approach was taken and cells were differentiated and experimented on under euglycaemic conditions, at 5.5 mM glucose (Figure 3.1).

Prior to differentiation or experiments, cells were maintained in media supplemented with 25 mM glucose, 10 % FBS, 4 % L-glutamine and 2 % Pen/Strep. For differentiation, upon reaching 70-80 % confluence, myoblasts were plated into 60 cm dishes in DMEM supplemented with 5.5 mM glucose and 10% FBS. After 24 hours, myoblasts were displayed as individual fusiform or star-shaped mononucleated (Figure 3.1A). After 48 hours (90-100 % confluence), media was changed to DMEM supplemented with 5.5 mM glucose and lower serum content, 2 % horse serum (Day 1). At day 3 of differentiation, cells began to align (Figure 3.1B) and by day 5, cells fused and became elongated (Figure 3.1C). After 7 days of differentiation, myotubes were fully formed and displayed Y-shaped morphology (Figure 3.1D). These morphological changes are consistent with those observed when differentiating myoblasts in hyperglycaemic conditions (25 mM) (Burattini *et al.*, 2004). This differentiation protocol was used for all experiments described in this chapter.

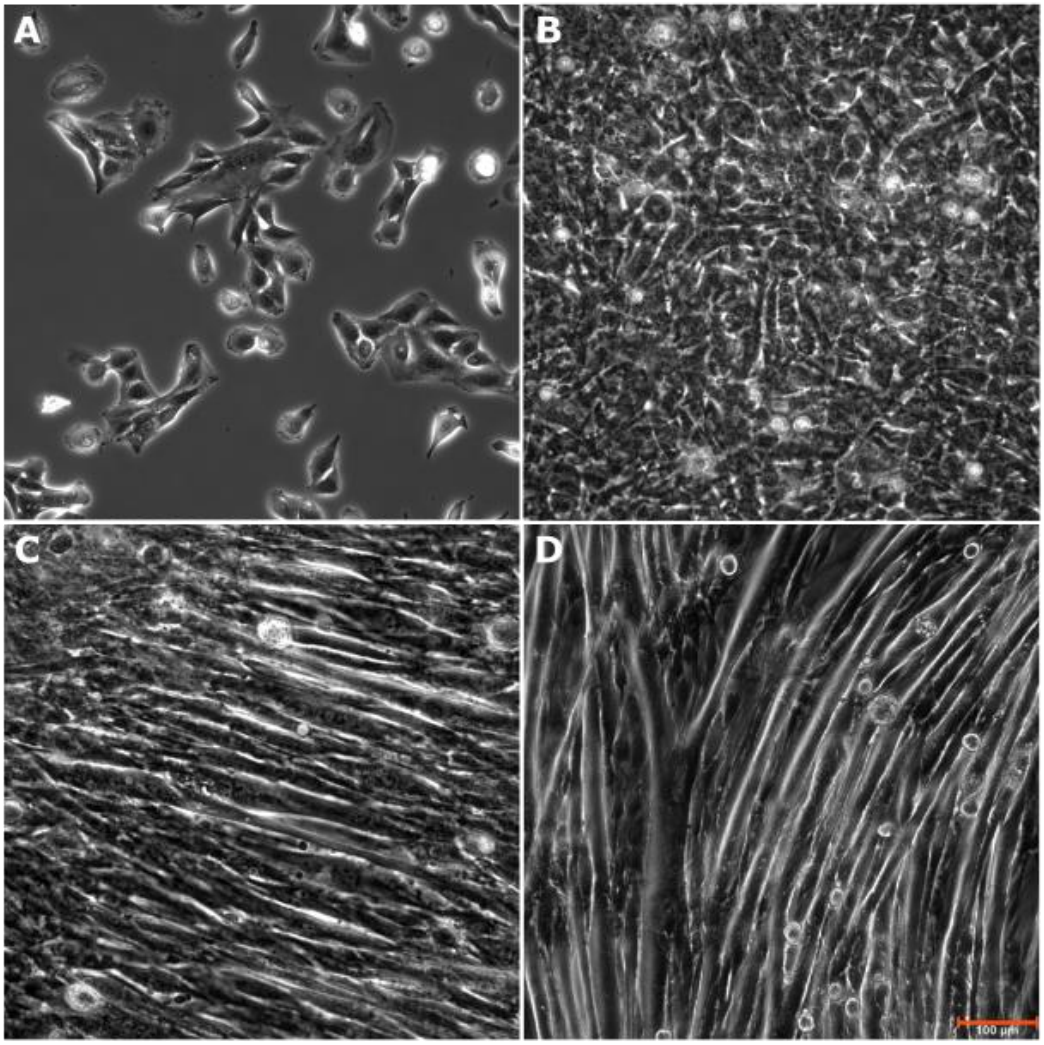



**Figure 3.1. Differentiation of C2C12 myoblasts at physiological glucose concentration**

C2C12 mouse myoblasts differentiated in culture for 7 days under physiological glucose concentration (5.5 mM). **A)** Day 1 of differentiation after 24 hour culture in 25 mM glucose. **B)** Day 3 myoblasts grown to confluence after 48 hour culture at 5.5 mM glucose. **C)** Observable myotubes at day 5. **D)** High density of myotubes at day 7 of differentiation. Scale bar = 100 µm (red line).

### 3.2 Insulin and mixed essential amino acids increase phosphorylation of anabolic signalling proteins in C2C12 myotubes

Insulin and AA sensitivity were assessed in C2C12 myotubes by treating cells with insulin (0-100 nM), mixed essential AA (3.34 mM, referred to throughout as AA) or a combination of both for 60 minutes and determining changes to protein phosphorylation within the PI3K/PKB and mTOR signalling pathways. At 10 nM, insulin caused a 2.7 ± 0.02 fold increase in PKB phosphorylation (Figure 3.2A) ($n=4$; $P<0.0001$) and 1.4 ± 0.06 fold increase in phosphorylation of p70-S6K1 (Figure 3.2B; $n=7$; $P<0.001$) which increased further in a concentration-dependent manner to 5.4 ± 0.2 and 1.6 ± 0.06 fold at 100 nM, respectively ($P<0.001$ for both). AAs (3.34 mM) significantly increased the levels of phosphorylated p70-S6K1 ($n=7$; $P<0.01$) and this response was further enhanced in the presence of insulin ($P<0.01$ for 50 nM insulin + AA versus AA control; Figure 3.2C). These responses provided a model of anabolic sensitivity to work within this study.

### 3.3. Palmitate treatment blunts insulin and amino acid-induced activation of anabolic signalling proteins in C2C12 myotubes

To assess the effect of and generate a model of palmitate-induced insulin and anabolic resistance, C2C12 myotubes were serum and AA deprived for 15 hours before anabolic sensitivity was assessed. Cells were incubated for 15 hour in serum free EBSS (supplemented with 5.5 mM glucose and 0.3 mM AAs), with increasing concentrations of palmitate (0-750 μM) conjugated to bovine serum albumin (BSA, 0.1% w/v) or BSA (0.1 % w/v) controls. Following 15 hour treatment, cells were treated with insulin (20 nM) and AAs (3.34 mM) alongside palmitate (or BSA controls) for 60 minutes. In the absence of palmitate, insulin and AAs significantly increased the levels of phosphorylated PKB ($n=6$; $P<0.0001$) but pre-treatment with 500 μM palmitate prevented this response ($P>0.99$ for 500 μM versus 500 μM + insulin and AAs; Figure 3.3A). In BSA controls, insulin and AA treatment significantly increased p70-S6K1 phosphorylation ($n=5$; $P<0.001$; Figure 3.3B) and this response was significantly blunted in cells treated with 750 μM palmitate ($P>0.99$ for 750 μM palmitate against 750 μM + insulin and AAs).

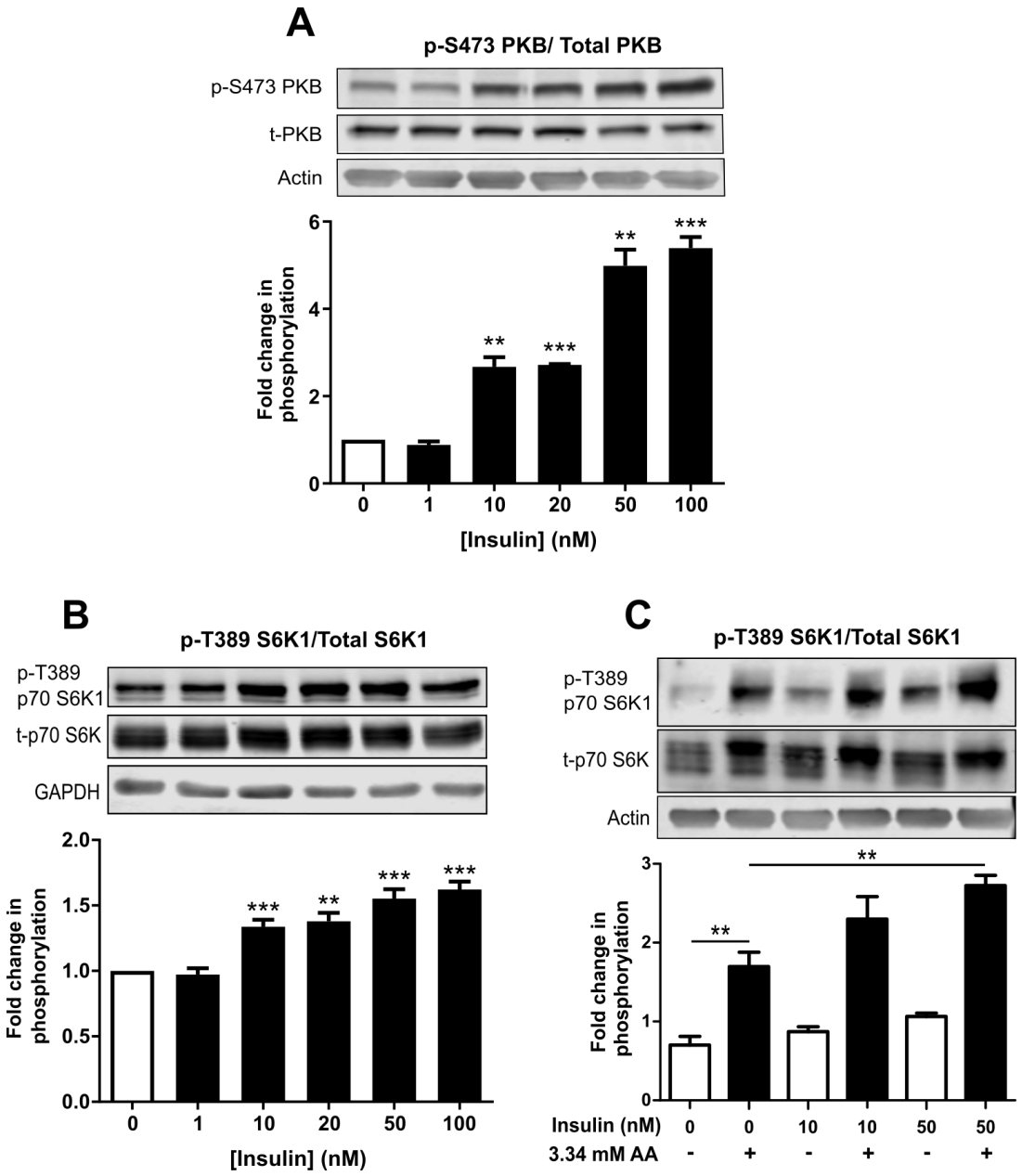



**Figure 3.2 Insulin and mixed essential amino acids increase phosphorylation of anabolic signalling proteins in C2C12 myotubes**
C2C12 myotubes treated for 60 minutes with increasing concentrations of insulin and mixed essential amino acids (AA). Representative immunoblots for PKB (pS473) normalised to PKB **(A)** and p70-S6K1 (pT389) normalised to p70-S6K1 **(B)** with densitometric analysis below for C2C12 myotubes treated with increasing concentrations of insulin (0-100 nM) for 60 minutes (One-sample t-test in comparison to control; **P<0.01; ***P<0.001; n=4-7). Actin and GAPDH were used as loading controls. **C)** Representative immunoblot for p70-S6K1 (pT389) normalised to p70-S6K1 for C2C12 myotubes treated for 60 minutes with AA (3.34 mM) or combined with insulin (0-50 nM) for 60 minutes (One-way ANOVA with multiple comparisons with Bonferroni's; **P<0.01; n=3).

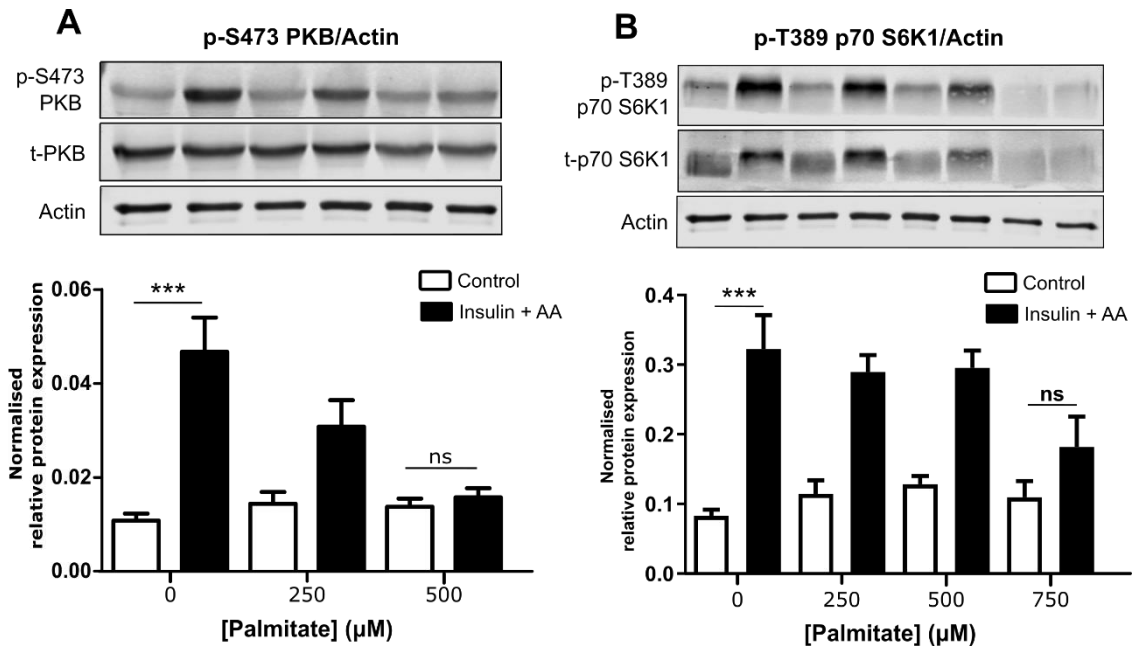



**Figure 3.3. Palmitate treatment blunts insulin and amino acid-induced activation of anabolic signalling proteins in C2C12 myotubes**

C2C12 myotubes treated for 16 hours with palmitate and treated acutely with insulin and mixed essential amino acids. Representative immunoblots for PKB (pS473) normalised to actin **(A)** and p70-S6K1 (pT389) normalised to actin **(B)** with densitometric analysis below for C2C12 myotubes treated with increasing concentrations of palmitate (0-750 μM) for 16 hours and insulin (20 nM) and amino acids (AA, 3.34 mM) for 60 minutes (One-way ANOVA with multiple comparisons; ***P<0.001; ns P>0.05; n=5-6).

### 3.4 Palmitate treatment enhances inflammatory cytokine release in C2C12 myotubes

The extracellular levels of myokines IL-6 and macrophage migration inhibitory factor (MIF) were quantified to establish the effect of palmitate treatment on inflammatory cytokine secretion in this model. Palmitate treatment (500 μM; 16 hours) significantly increased the levels of IL-6 ($n=6$; $P<0.05$; Figure 3.4A) and MIF ($n=5$; $P<0.001$; Figure 3.4B) in C2C12 myotubes.

### 3.5 Palmitate treatment impairs mitochondrial function in C2C12 myotubes

To assess the effect of palmitate treatment on C2C12 myotube mitochondrial function, extracellular flux assays were performed. Cells were incubated for 16 hours with palmitate (250-750 μM) as described above, followed by 1 hour incubation in XF basal medium (supplemented with 5.5 mM glucose, 2 mM l-glutamine and 2.5 mM sodium pyruvate) and oxygen consumption measured using the Seahorse Extracellular Flux Analyser (Agilent, UK). Basal oxygen consumption was assessed (pmoles/min/μg) before and after cells were injected with oligomycin (2 μM) to inhibit mitochondrial complex V and assess ATP-synthase activity; FCCP (1 μM), which uncouples mitochondrial membranes to allow for maximal respiration to ensue; and rotenone/antimycin in a 1:1 mix (1 μM) to inhibit the remainder of the respiratory chain. Representative extracellular flux trace during a mitochondrial stress test shown in Figure 3.5A. Palmitate treatment for 16 hours at 250 μM increased basal OCR, whereas, at 500-750 μM, it significantly decreased basal OCR compared to control ($n=26$-$28$; $P\leq0.01$, $P<0.001$ and $P<0.001$ for 250, 500 and 750 μM palmitate against control, respectively; Figure 3.5B). Oligomycin-sensitive OCR, a measure of ATP synthase activity, was maintained after 250 μM palmitate ($P>0.05$ against control), but significantly decreased with 500 μM and 750 μM palmitate treatment ($P<0.001$ for both against control; Figure 3.5C). Furthermore, following uncoupling with FCCP, cells treated with 250 μM demonstrated increased spare respiratory capacity ($P<0.001$), but this was decreased in cells treated with 500-750 μM palmitate ($P<0.001$ and $P<0.001$ for 500 and 750 μM against control, respectively; Figure 3.5D). Proton leak was significantly increased with palmitate treatment ($P<0.001$ for all concentrations against control) (Figure 3.5E).

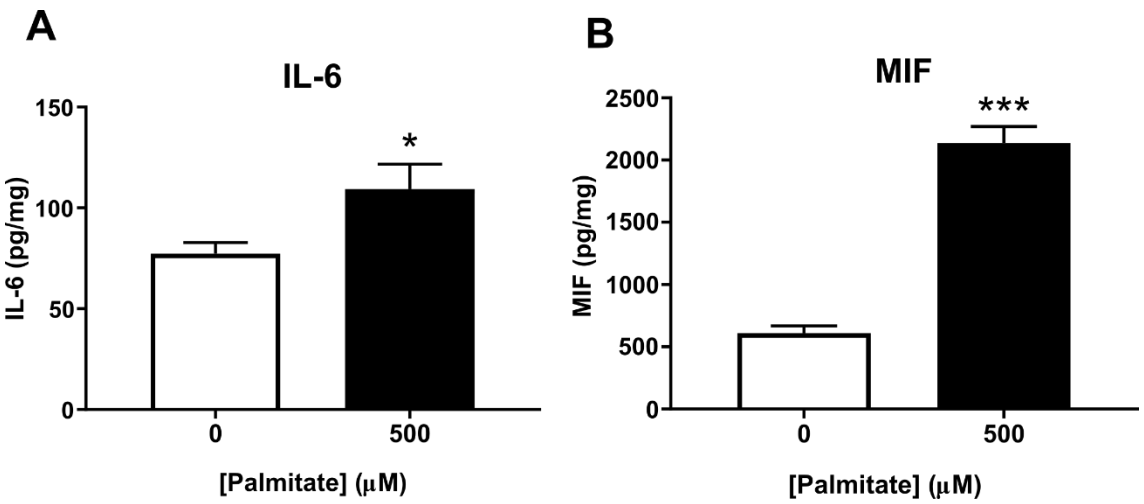
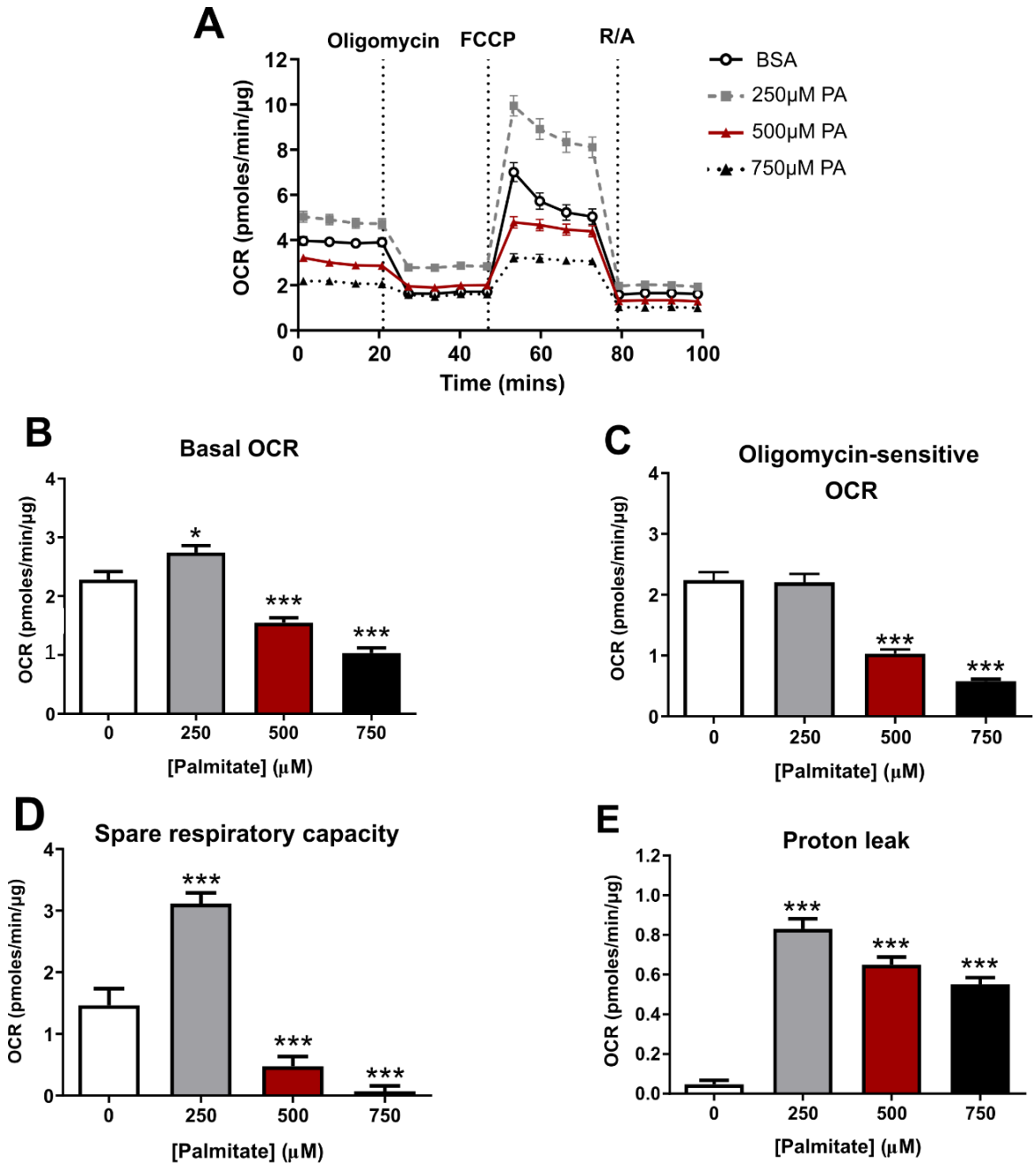



**Figure 3.4 Palmitate treatment enhances inflammatory cytokine release in C2C12 myotubes**

Extracellular cytokine levels measured by ELISA after palmitate treatment. Palmitate (500 μM) significantly enhanced the levels of IL-6 **(A)** and MIF **(B)** after 16 hour treatment in C2C12 myotubes, compared to BSA (0.1 % w/v) controls (Two-tailed unpaired t-test; *P<0.05; ***P<0.001; n=6 and n=5 respectively).




## Figure 3.5. Palmitate treatment impairs mitochondrial function in C2C12 myotubes

Extracellular flux analysis of C2C12 myotubes incubated for 16 hours with palmitate (250-750 μM) conjugated to 0.02% BSA or BSA controls. **A)** Representative extracellular flux assay demonstrating oxygen consumption rate (OCR; pmoles/min/μg) at baseline and after acute injection of oligomycin (2 μM), FCCP (1 μM) and rotenone/antimycin A (R/A; 1:1 ratio; 1 μM) at the indicated stages (n=26-28). **B)** Mean basal OCR before oligomycin (pmoles/min/μg) (*P<0.05; ***P<0.001 against control). **C)** Oligomycin-sensitive OCR measured as the difference between average baseline OCR and average OCR after oligomycin (***P<0.001 against control). **D)** Spare respiratory capacity, calculated as the difference from baseline levels to highest response after FCCP (***P<0.001 compared to control). **E)** Proton leak measured as a difference from oligomycin-insensitive OCR and non-mitochondrial respiration (OCR before FCCP minus OCR after R/A) (***P<0.001 against control). All group statistics performed using One-way ANOVA with Bonferroni's multiple comparisons analysis.

### 3.6 Apyrase treatment hydrolyses ATP released after palmitate treatment but does not alter effect on IL-6 and MIF in C2C12 myotubes

To test the hypothesis that eATP may modulate FA effects on anabolic signalling and inflammation, the effect of palmitate on eATP levels was examined (measured using luminescence based plate assay). Palmitate treatment at 500 μM, but not 250 μM, increased the levels of eATP in C2C12 myotubes after 16 hours ($n=7$; $P<0.001$; Figure 3.6A). This response was abolished by apyrase, an ATP-diphosphohydrolase which catalyses the conversion from ATP to ADP and ADP to AMP. Apyrase (0.5 units/mL) decreased the palmitate-induced increase in eATP in C2C12 myotubes treated for 16 hours with palmitate (500 μM; or BSA controls) ± apyrase and incubated for 1 hour with insulin (20 nM) and AAs (3.34 mM) (Figure 3.6B).

To test whether eATP contributed to palmitate-induced cytokine release, C2C12 myotubes were co-treated with palmitate (500 μM; or BSA controls; 16 hours) and apyrase (0.5 units/mL). Apyrase did not alter IL-6 (Figure 3.6C) or MIF (Figure 3.6D) secretion in the presence or absence of palmitate.

### 3.7 Apyrase treatment does not alter palmitate-induced insulin and anabolic resistance in C2C12 myotubes

C2C12 myotubes were treated with palmitate (500 μM) and apyrase (0.5 units/mL; 16 hours) followed by treatment with insulin (20 nM) and AAs (3.34 mM) for 1 hour. Palmitate significantly blunted insulin and AA-mediated phosphorylation of PKB ($n=6$; $P<0.001$; Figure 3.7A-B) and p70-S6K1 ($n=6$; $P<0.05$; Figure 3.7A,C) whilst apyrase treatment did not alter insulin/AA-mediated phosphorylation in BSA controls or affect palmitate-induced insulin/anabolic resistance ($P>0.05$). Phosphorylation of ERK1/2 was significantly increased by palmitate ($n=6$; $P<0.001$) and was unchanged in the presence of insulin and AA ± apyrase ($n=6$; $P<0.001$ and $P\leq0.001$ against control with same treatments; $P>0.05$ against palmitate; Figure 3.7D).

Subsequent experiments assessed the effect of direct treatment with ATP, or non-hydrolysable ATPγS, on intracellular signalling and inflammation, to better replicate the continuous release of ATP by exercising muscle *in vivo* (Crecelius *et al.*, 2013; Zarębska *et al.*, 2018).

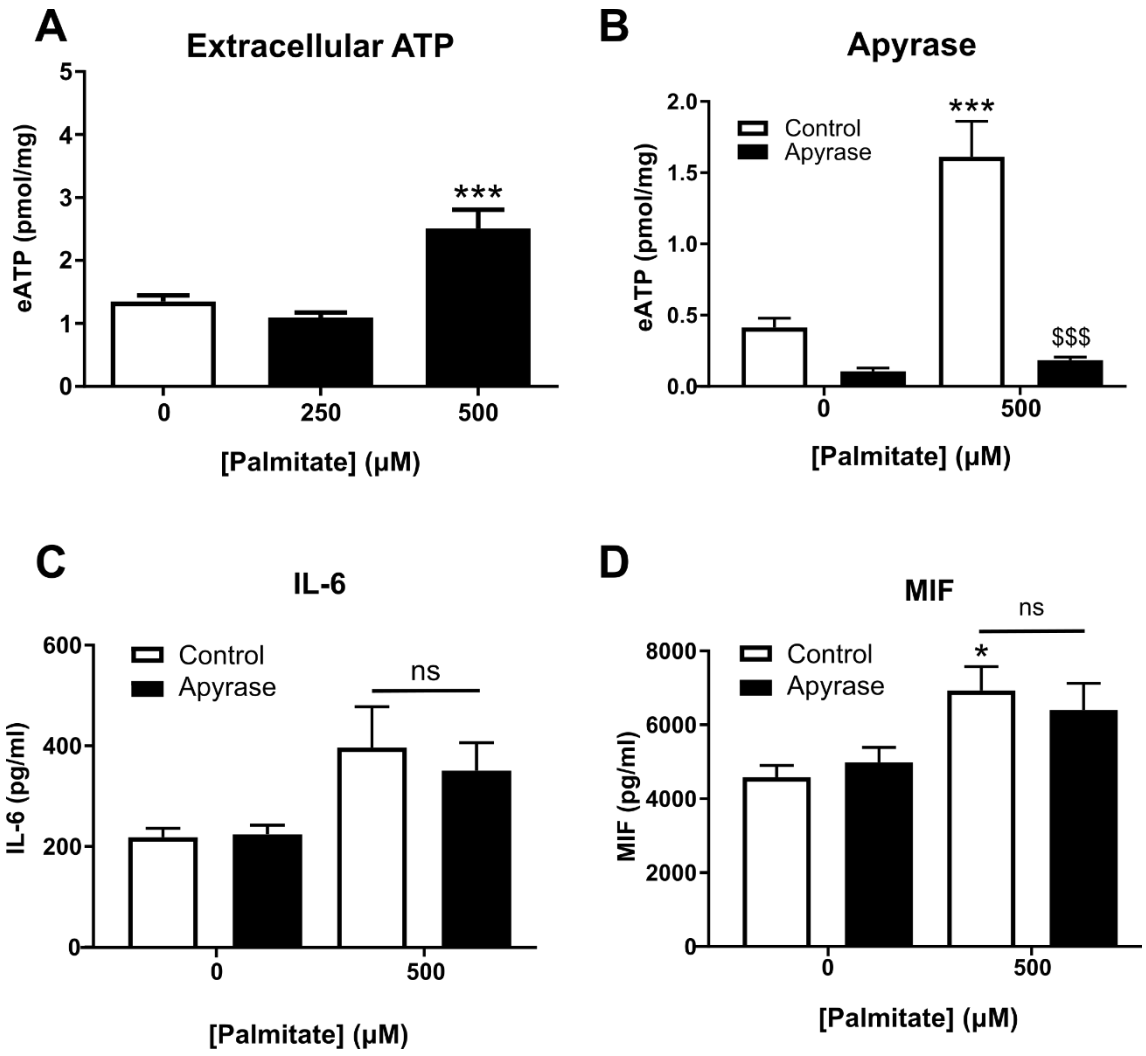



**Figure 3.6. Apyrase treatment hydrolyses ATP released after palmitate treatment but does not alter effect on IL-6 and MIF secretion in C2C12 myotubes**

**A)** Palmitate (500 μM; 16 hours) significantly increased eATP measured using luminescence based assay ATPLite (One-way ANOVA ***P<0.001; n=7) **B)** C2C12 myotubes treated with palmitate (500 μM) or BSA (0.1 % w/v; 16 hours) ± apyrase (0.5 units/mL) and spiked with insulin (20 nM) and AAs (3.34 mM) for 1 hour (One-way ANOVA with Bonferroni's multiple comparisons analysis; ***P<0.001 for palmitate against control and $$$P<0.001 for palmitate + apyrase against palmitate; n=6). **C)** Palmitate (500 μM; 16 hours) modestly enhanced levels of extracellular IL-6 (non-significant) and this was not altered with apyrase (0.5 units/mL) (n=6). **D)** Palmitate (500 μM; 16 hours) treatment enhanced extracellular MIF and this was not altered by apyrase (0.5 units/mL) (One-way ANOVA with Bonferroni's multiple comparisons analysis *P<0.05 for palmitate against control, ns=not significantly different for apyrase effect; n=5). Cytokine levels measured by ELISA.

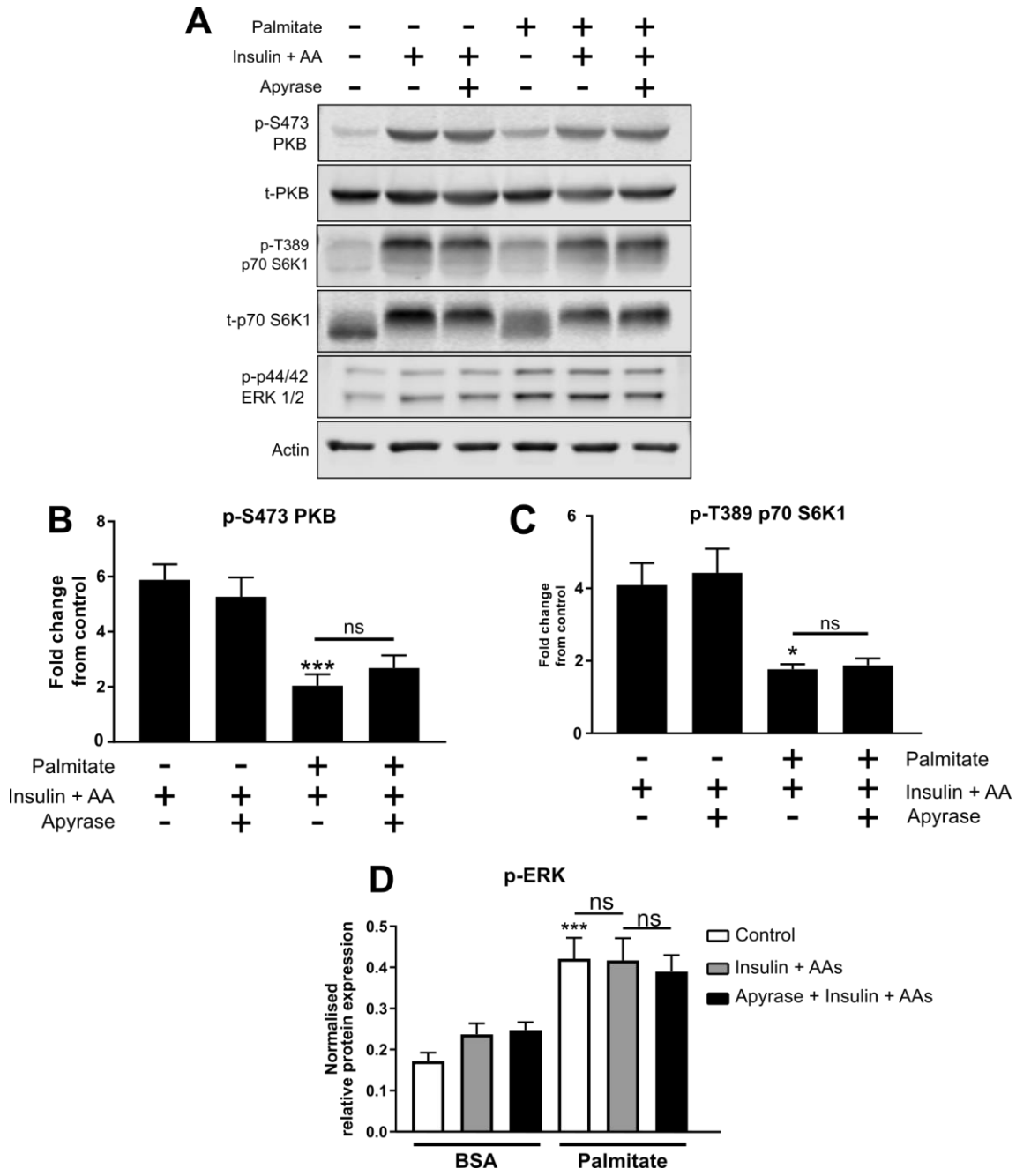



**Figure 3.7 Apyrase does not alter palmitate-induced insulin and anabolic resistance in C2C12 myotubes**

C2C12 myotubes treated for with palmitate (500 μM) or BSA (0.1 % w/v; 16 hours) ± apyrase (0.5 units/mL) and treated with insulin (20 nM) and AAs (3.34 Mm) for 1 hour. **A)** Representative immunoblots for PKB (pS473), total PKB, p70-S6K1 (pT389), p70-S6K1, ERK 1/2 (pThr202/Tyr204) and Actin. **B)** Densitometric analysis for phosphorylated PKB normalised to total PKB represented as the effect of palmitate and apyrase on insulin and AA-treated controls (fold change from insulin and AA treated cells) (***P<0.001 for palmitate against control and ns=not significant for the effect of apyrase on palmitate response; n=6). **C)** Densitometric analysis for phosphorylated p70-S6K1 normalised to total p70-S6K1 represented as in B (*P<0.05 for palmitate against control; n=6). **D)** Densitometric analysis for phosphorylated ERK normalised to actin (***P<0.001; n=6). All groups compared using One-way ANOVA with Bonferroni's multiple comparisons.

### 3.8 Intracellular calcium levels increase in response to ATP in a concentration-dependent manner in C2C12 myotubes

To test whether intracellular calcium was altered by ATP treatment in C2C12 myotubes, cells were acutely injected with ATP and real-time changes to $[Ca^{2+}]_i$ assessed using a fluorescent dye. ATP increased $[Ca^{2+}]_i$ in C2C12 myotubes in a concentration-dependent manner (Figure 3.8A), with significant incremental changes occurring from 30 μM to 200 μM ATP (n=6; $P<0.05$ to $P<0.001$ for 30 μM and 200 μM against control; Figure 3.8B).

### 3.9 Treatment with ATPγS does not impair anabolic signalling pathway activation by insulin or amino acids

C2C12 myotubes were treated with ATPγS (16 hours) to test the hypothesis that eATP may elicit anabolic blunting and pro-inflammatory cytokine release on muscle cells. Hydrolysis of palmitate-induced eATP with apyrase showed no effect on anabolic sensitivity or inflammatory cytokine secretion (Figure 3.6-7). Direct treatment with ATPγS (50 μM or 100 μM; 16 hours) did not alter the phosphorylation of PKB induced by insulin (20 nM; n=6; Figure 3.9A) and p70-S6K1 induced by AA treatment (3.34 mM; n=6; Figure 3.9B), ($P>0.05$ for 50 μM and 100 μM ATPγS against control).

### 3.10 Treatment with ATPγS attenuates levels of pro-inflammatory markers in C2C12 myotubes

Treatment with ATPγS (16 hours) significantly increased the levels of IL-6 from non-detectable at baseline to ~20 and ~40-fold higher with 50 μM and 100 μM, respectively (n=6; $P\leq0.01$ and $P<0.001$ for 50 μM and 100 μM respectively; Figure 3.10A). Levels of pro-inflammatory cytokine MIF were significantly reduced following 100 μM ATPγS treatment (n=6; $P\leq0.01$; Figure 3.10B). The expression of phospho-NF-κB (Ser536) (p65 subunit) was moderately but not significantly attenuated by ATPγS (n=6; Figure 3.10C). These data go against the hypothesis that ATPγS would upregulate multiple inflammatory markers in these cells, akin to palmitate.

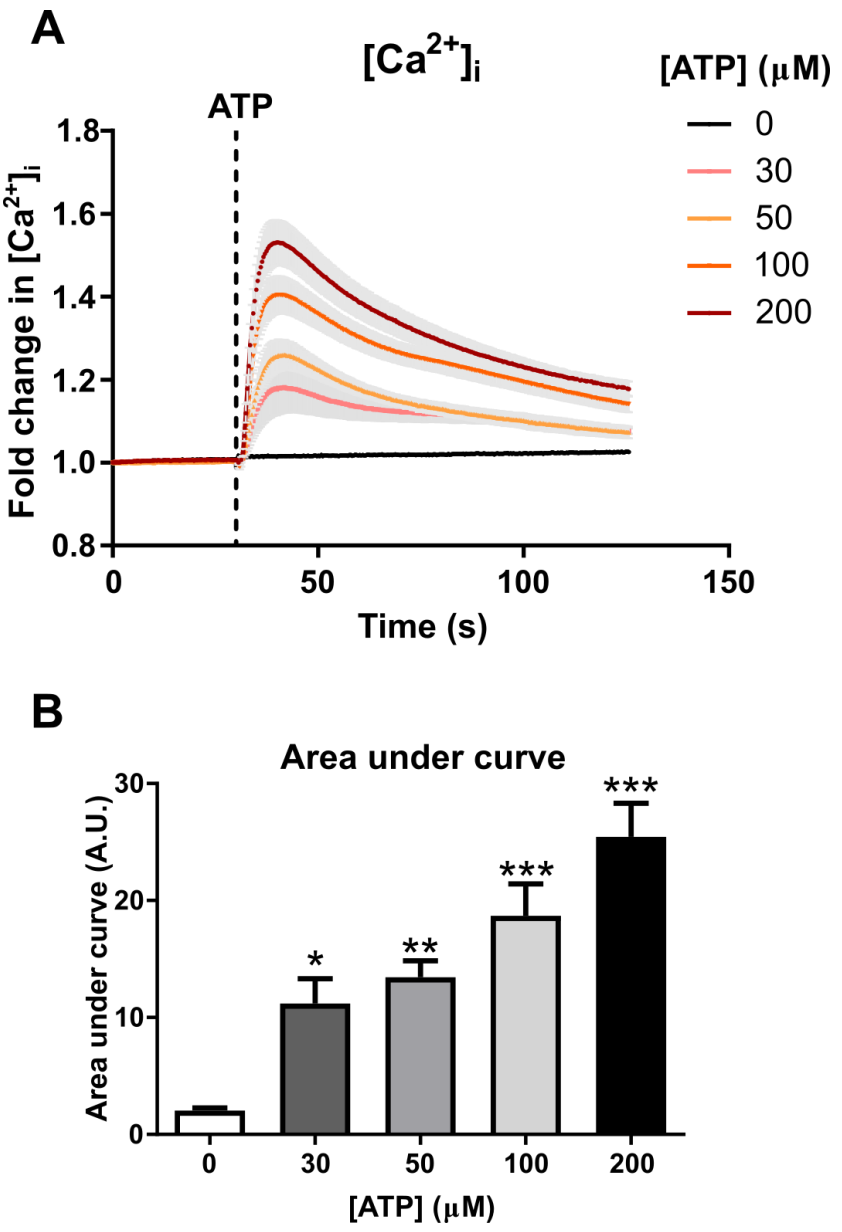



**Figure 3.8 Intracellular calcium levels increase in response to ATP in a concentration-dependent manner in C2C12 myotubes**

**A)** Representative intracellular calcium transients in response to increasing concentrations of ATP (0-200 µM) measured using fluorescent dye Fluo-4 in a plate based assay (n=6). **B)** Area under the curve analysis for data shown in A. ATP increased intracellular calcium in a concentration-dependent manner from 30 µM to 200 µM (One-way ANOVA with Bonferroni's multiple comparisons *P<0.05, **P<0.01, ***P<0.001; n=6). Solid line represents mean, grey shading represents SEM.

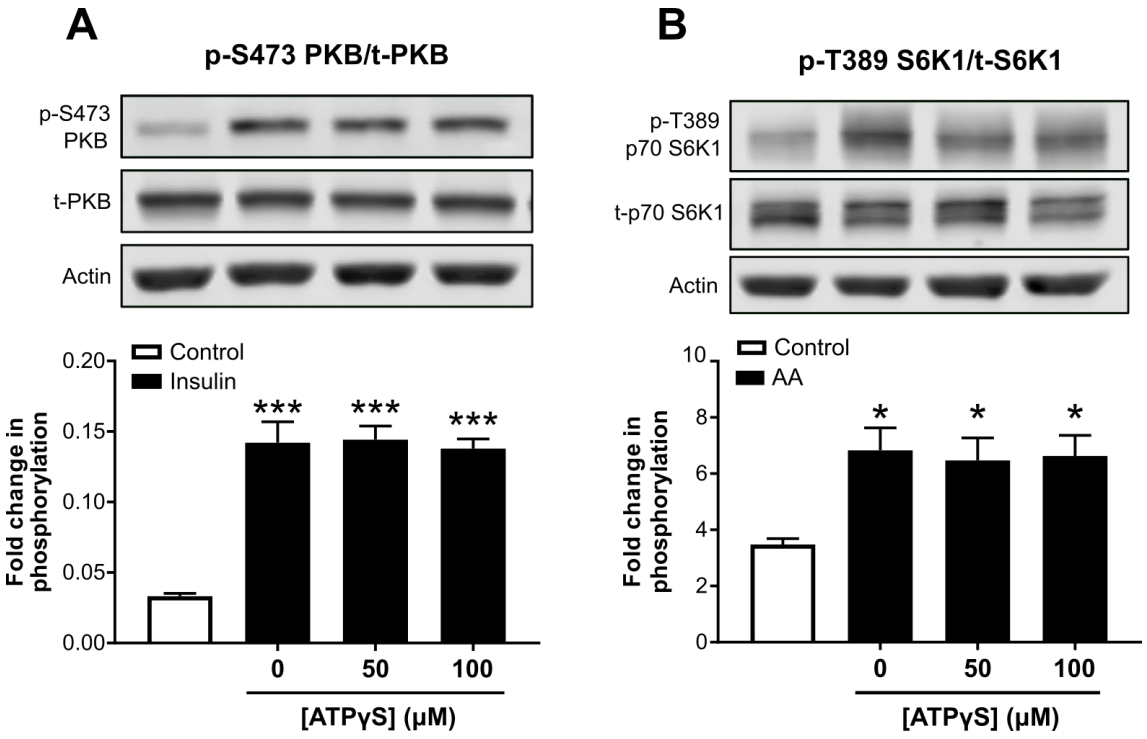



**Figure 3.9 Treatment with ATPγS does not alter insulin and amino acid-mediated anabolic signalling pathway activation in muscle cells**

Assessment of the impact of ATPγS treatment (16 hours) on the intracellular signalling response to insulin and amino acids through Western blotting in C2C12 myotubes. **A)** Representative immunoblot and densitometric analysis for phosphorylated PKB normalised to total PKB following treatment with 50-100 μM ATPγS (16 hours) and 1 hour insulin (20 nM) or control treatment (***P<0.001 for all insulin groups against control; n=6). **B)** Representative immunoblot and densitometric analysis for phosphorylated p70-S6K1 normalised to total p70-S6K1 in response to ATPγS treatment (16 hours) and 1 hour treatment with AAs (3.34 mM) (*P<0.05 for all AA-treated groups against control; n=6). Groups compared using One-way ANOVA with Bonferroni's multiple comparisons analysis.

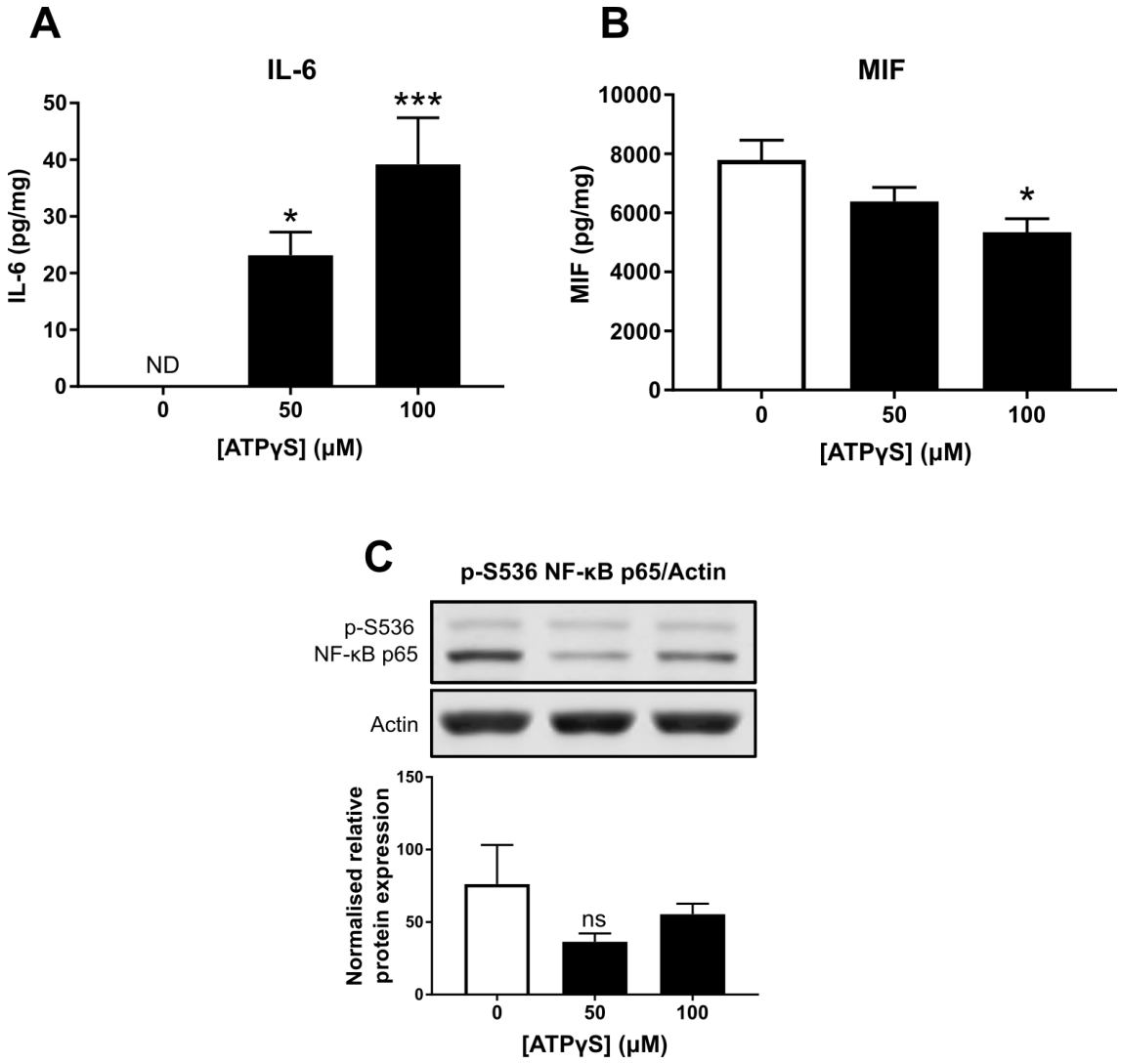



**Figure 3.10 Treatment with ATPγS attenuates pro-inflammatory markers in C2C12 myotubes**

**A)** ATPγS (16 hours) caused a concentration-dependent increase in the levels of IL-6 from non-detectable levels (ND) to concentrations ~20 and ~40-fold higher with 50 μM and 100 μM respectively (*P<0.05, ***P<0.001 against control; n=6). **B)** ATPγS (16 hours) concentration-dependently decreased levels of MIF, reaching statistical significance at 100 μM (*P<0.05 against control; n=6). **C)** Representative immunoblot and densitometric analysis showing mild attenuation (not statistically significant) of phosphorylated NF-κB (pS536) normalised to actin (n=6). All groups compared with One-way ANOVA with Bonferroni's multiple comparisons analysis.

### 3.11.1 ATPγS treatment leads to phosphorylation of anabolic signalling proteins in an insulin and amino acid-mimetic manner.

Acute treatment with ATPγS (100 μM; 15 minutes) significantly elevated the levels of phosphorylated PKB (n=5; P<0.05; Figure 3.11.1A) in the absence of insulin or AAs. At 50 μM and 100 μM ATPγS significantly increased the levels of phosphorylated p70-S6K1 (n=5; P<0.05 and P<0.01, respectively; Figure 3.11.1B). This suggests that eATP acts in an insulin and AA-mimetic manner to activate PI3K/PKB and mTOR signalling.

### 3.11.2 Acute effect of ATPγS on anabolic signalling is attenuated by broad spectrum but not P2X4R and P2X7R antagonists.

To test whether the acute ATPγS effect on PKB and S6K1 (seen in Figure 3.11.1) was P2R-mediated, C2C12 myotubes were treated with ATPγS (15 minutes) after pre-treatment with P2R antagonists (45 minutes). As anticipated, ATPγS (50 μM) significantly increased the levels of phosphorylated p70-S6K1 (n=6; P<0.0001 against control) and this was completely prevented by broad spectrum P2R antagonists PPADS (100 μM) and suramin (100 μM) (P<0.001 for PPADS and suramin against ATPγS control; Figure 3.11.2A,B). P2X4R antagonist 5-BDBD (5 μM) nor P2X7R antagonist A438079 (100 μM) modified the ATPγS-induced response (P>0.05 against ATPγS control). The levels of phosphorylated PKB were modestly, but not significantly, increased following ATPγS treatment (Figure 3.11.2A,C), consistent with earlier observation (Figure 3.11.1A). This modest effect was significantly prevented by PPADS pre-treatment (n=6; P<0.05 for PPADS against ATPγS control) and attenuated by suramin (Figure 3.11.2A,C). Phosphorylation of ERK, used as a positive control for P2R-mediated ATP effects (May *et al.*, 2006), was increased following ATPγS treatment (n=6; P<0.001 for ATPγS against control) and significantly prevented by both PPADS and suramin treatment but not 5-BDBD or A438079 (P<0.001 for both PPADS and suramin against ATPγS control; Figure 3.11.2A,D). PPADS and suramin decreased PKB phosphorylation to levels below those seen in controls. This supports the notion that tonic release of nucleotides maintains ‘resting’ signalling transduction via P2Rs (Lazarowski, Boucher and Harden, 2000).

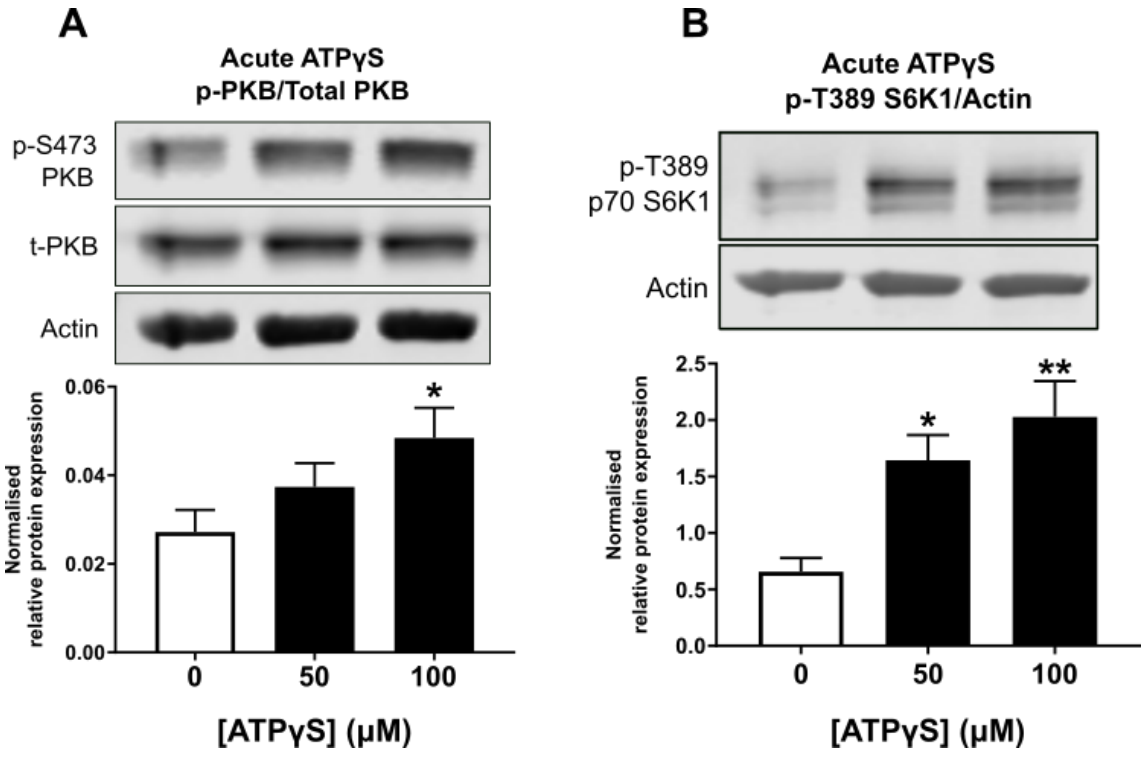



**Figure 3.11.1 ATPγS treatment leads to phosphorylation of anabolic signalling proteins in an insulin and amino acid-mimetic manner**.

Treatment of C2C12 myotubes with ATPγS (15 minutes). **A)** Representative immunoblots of PKB (pS473) and total PKB with densitometric analysis showing significant increase in phosphorylation after ATPγS treatment (100 μM; *P<0.05; n=5). **B)** Representative immunoblot of p70-S6K1 (pT389) and densitometric analysis for phosphorylated protein normalised to actin showing significant increase in response to 50 and 100 μM ATPγS (*P<0.05 compared to control; n=6). Groups compared with One-way ANOVA with Bonferroni's multiple comparison analysis.

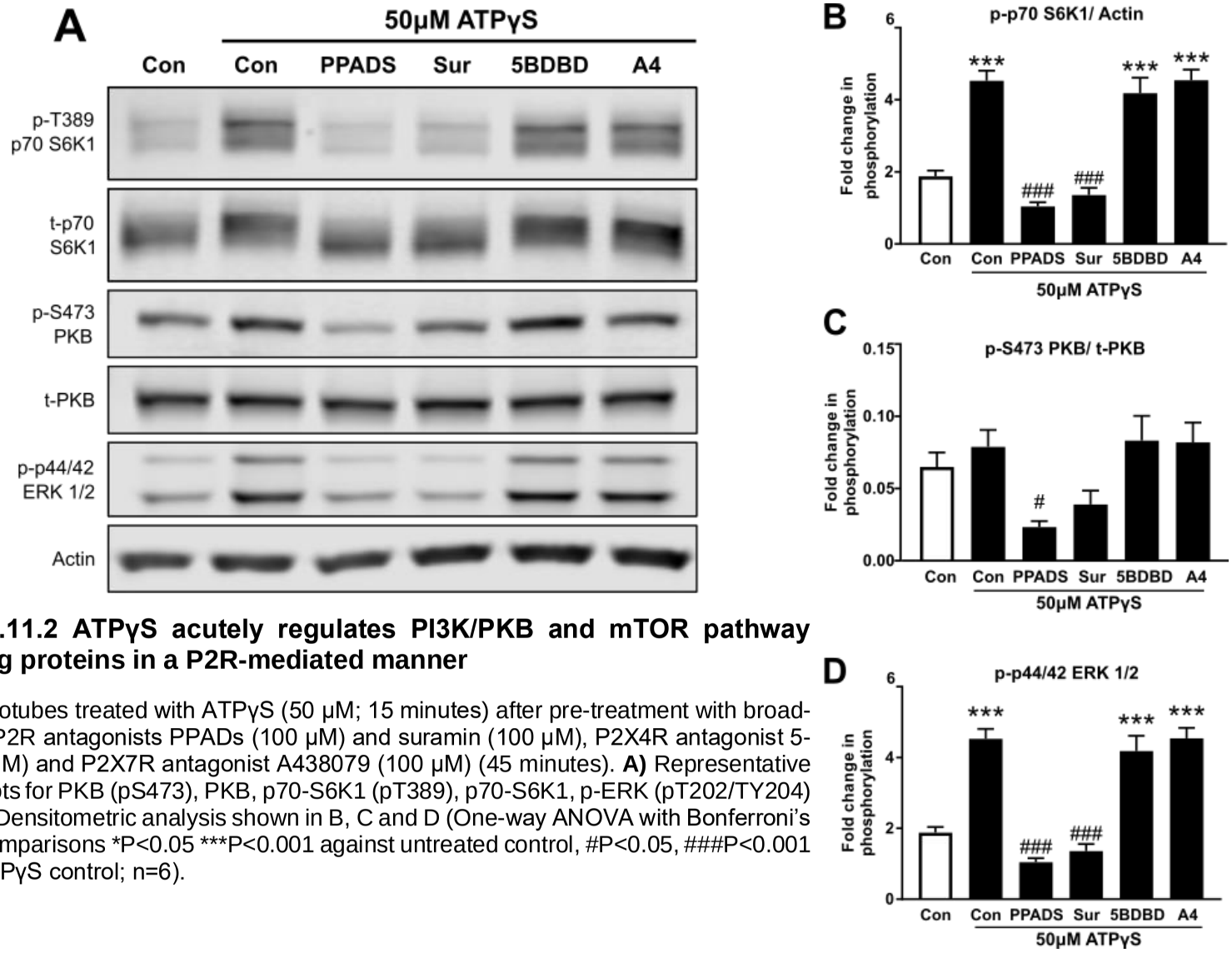



**Figure 3.11.2 ATPγS acutely regulates PI3K/PKB and mTOR pathway signalling proteins in a P2R-mediated manner**

C2C12 myotubes treated with ATPγS (50 μM; 15 minutes) after pre-treatment with broad-spectrum P2R antagonists PPADs (100 μM) and suramin (100 μM), P2X4R antagonist 5-BDBD (5 μM) and P2X7R antagonist A438079 (100 μM) (45 minutes). **A)** Representative immunoblots for PKB (pS473), PKB, p70-S6K1 (pT389), p70-S6K1, p-ERK (pT202/TY204) and actin. Densitometric analysis shown in B, C and D (One-way ANOVA with Bonferroni's multiple comparisons *P<0.05 ***P<0.001 against untreated control, #P<0.05, ###P<0.001 against ATPγS control; n=6).

### 3.12 ATPγS acutely enhances glucose uptake in an insulin-mimetic and concentration-dependent manner

The acute effects of ATPγS on the insulin signalling pathway led to the question of whether this would translate to an increase in insulin-independent glucose uptake into myotubes. This was tested by assessing the effect of acute ATPγS treatment on 2-DG uptake (as a proxy for glucose uptake) in C2C12 myotubes, using insulin treatment as a positive control. As anticipated, insulin (200 nM, 30 minutes) significantly enhanced glucose uptake into myotubes ($n=9$; $P<0.01$; Figure 3.12). In the absence of insulin, ATPγS (30 minutes) significantly increased glucose uptake in a concentration-dependent manner, with concentrations above 50 μM showing an effect similar or more potent than that observed after insulin treatment ($n=9$; $P\leq0.01$ and $P<0.001$ for 50 and 100 μM ATPγS, respectively, against control; Figure 3.12).

### 3.13 Palmitate alters insulin and ATPγS-mediated glucose uptake

To establish whether the ATPγS effect on glucose uptake was maintained after palmitate treatment C2C12 myotubes were treated with palmitate (500 μM; 16 hours) or BSA controls (0.1 %; w/v) and glucose uptake measured in response to insulin (200 nM) or ATPγS (100 μM), as above. Palmitate treatment prevented both insulin and ATPγS-mediated glucose uptake ($n=8$; $P<0.05$ for insulin against palmitate with insulin; $P<0.05$ for ATPγS against palmitate with ATPγS; Figure 3.13).

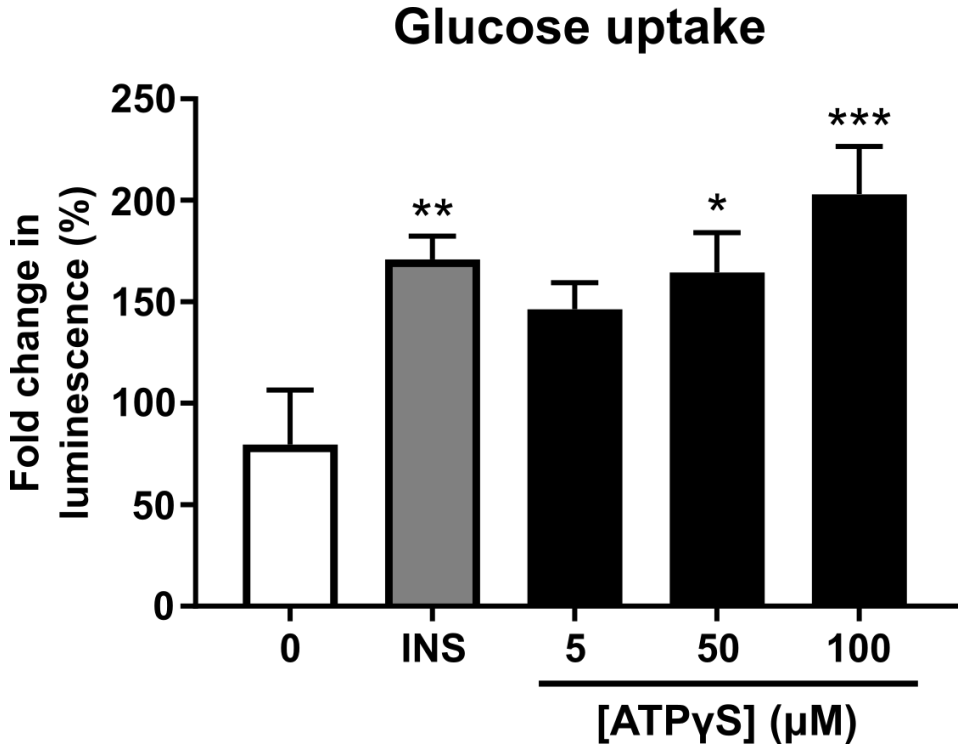



**Figure 3.12 ATPγS acutely enhances glucose uptake in an insulin-mimetic and concentration-dependent manner**

Glucose uptake in C2C12 myotubes pre-treated for 15 minutes with insulin and ATPγS following 15 minute incubation with 2-DG (100 μM) ± insulin and ATPγS, measured using luminescence based assay. Insulin (200 nM) significantly increased 2-DG uptake (**P<0.01) and this was matched and superseded with 50 and 100 μM ATPγS respectively. Data represented as fold change from control which is normalised to control mean (One-way ANOVA with Bonferroni's multiple comparisons analysis *P<0.05; ***P<0.001 for 50 and 100 μM ATPγS against control; n=9).

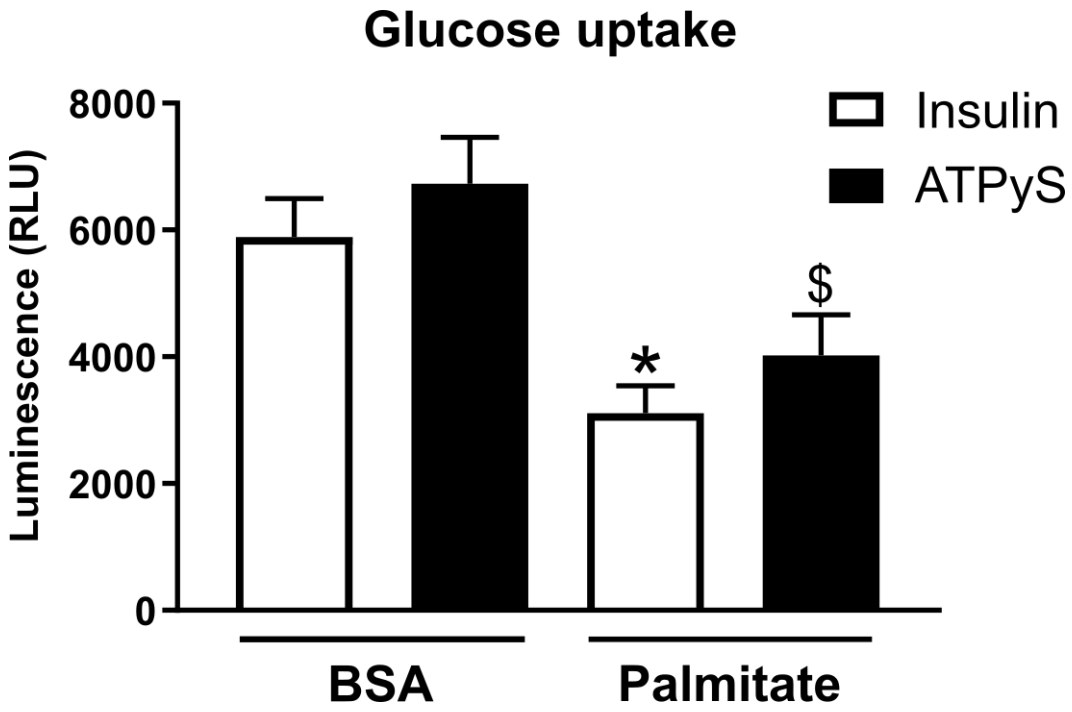



**Figure 3.13 Palmitate alters insulin and ATPγS-induced glucose uptake in C2C12 myotubes**

Glucose uptake measured after treatment with palmitate (16 hours) or BSA controls and insulin and ATPγS (30 minutes) in C2C12 myotubes. Uptake observed in BSA controls after 15 minute incubation with insulin (200 nM) or ATPγS (100 μM) and subsequent 15 minute incubation with 2-DG (100 μM) was significantly prevented by palmitate treatment (500 μM; 16 hours) (One-way ANOVA with Bonferroni's multiple comparisons analysis; *P<0.05 between insulin groups; $P<0.05 between ATPγS groups; n=8). Data represented as mean ± SEM for relative luminescence units (RLU).

### 3.14.1 Treatment with ATPγS (long-term) enhances glucose metabolism of C2C12 myotubes in a P2R-mediated manner

C2C12 myotubes were treated for a short (15 minutes) and longer period (16 hours) with ATPγS (with PPADS or suramin) to investigate whether the acute increase in glucose uptake was reflective of a P2R-mediated increase in glucose metabolism.

Glycolytic rate was estimated by extracellular acidification rate (ECAR) in response to ATPγS (100 μM; 16 hours) with or without suramin (100 μM) and acutely in response to glucose (5.5 mM), oligomycin (2 μM) and 2-DG (50 mM). In the absence of glucose, glycolytic rate was unchanged between treatments (n=22-24; $P>0.05$; Figure 3.14.1A,B). In response to 5.5 mM glucose injection, ATPγS treated cells showed significantly higher rates of glycolysis, and this was significantly attenuated by suramin (n=22-24; $P<0.05$ and $P<0.01$ for ATPγS against control and $P<0.05$ for ATPγS against suramin; Figure 3.14.1A,C).

### 3.14.2 ATPγS acutely increases glycolysis in a P2R-dependent manner distinct from the long-term effect

Glycolytic rate was measured prior to (baseline) and following acute injection with ATPγS (100 μM) ± PPADS (100 μM) or suramin (100 μM) and treatment effect assessed after injection of glucose (5.5 mM), oligomycin (2 μM) and 2-DG (50 mM). In the absence of glucose, acute injection of ATPγS significantly increased ECAR ($P<0.001$) and this response was blocked by PPADS but not suramin ($P<0.001$ for PPADS against ATPγS; n=22-24; Figure 3.14.2A,B). Suramin not only did not block the ATPγS response, but in the first measurement after injection (~ 8 minutes) showed a significantly higher effect on ECAR than ATPγS alone, albeit this was transient and quickly returned to levels matching the ATPγS response ($P<0.001$ for suramin against ATPγS; Figure 3.14.2A,C). Upon glucose addition, the rate of glycolysis was higher with ATPγS which was blocked by PPADS but not suramin ($P<0.05$ for ATPγS against control, $P\leq0.001$ for PPADS against ATPγS; Figure 3.14.2A,D). These data indicate that short and long-term ATPγS treatment regulates glucose metabolism with differential P2R activation.

### 3.15 Amino acid treatment increases extracellular ATP levels leading to changes in intracellular calcium in C2C12 myotubes

Preliminary observations suggested that insulin and AAs (combined) acutely increases the levels of eATP (data not shown). To test this and possible eATP-mediated responses, the levels of eATP and intracellular calcium were measured after treatment with insulin and/or AAs. Treatment with AAs (3.34 mM; 1 hour) moderately increased the levels of eATP in C2C12 myotubes, whereas insulin (50 nM) did not (n=3; P=0.07 for AA against control; Figure 3.15). As $[Ca^{2+}]_i$ increases in response to eATP in these cells (Figure 3.8), these were also treated acutely with AA (3.34 mM) and insulin (20 nM) to examine changes to $[Ca^{2+}]_i$. AA treatment (3.34 mM) significantly increased $[Ca^{2+}]_i$ which was not observed with insulin treatment, and blocked after co-treatment with insulin (P<0.01 against AA; Figure 3.15B,D). Interestingly, when AAs were injected after apyrase pre-treatment (0.5 units/mL; 60 minutes) the calcium response was dampened (P<0.05 for apyrase group against AA group), suggesting that the increase in calcium was, at least in part, mediated by eATP acting in an autocrine/paracrine manner (Figure 3.15B,D).

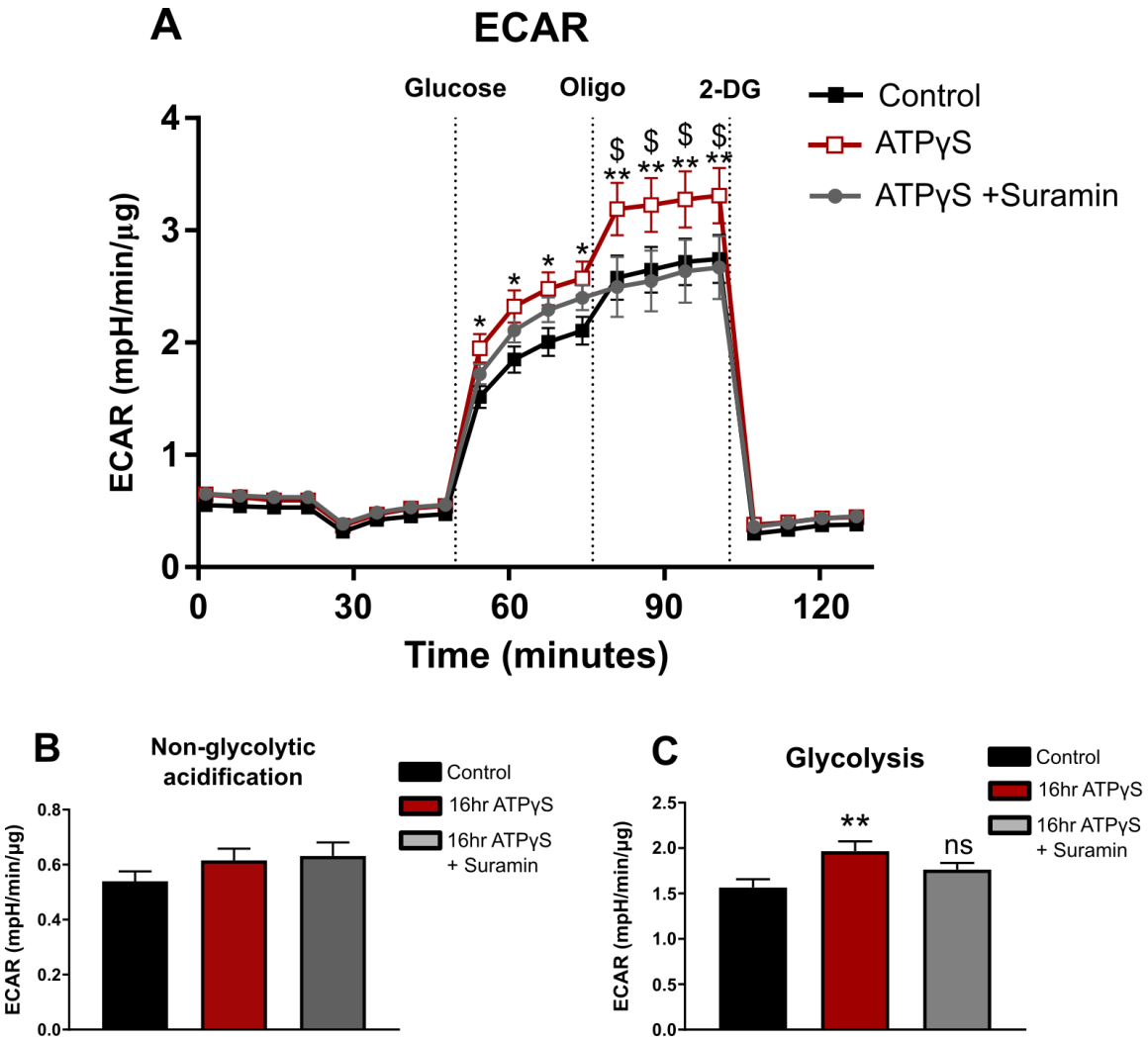



**Figure 3.14.1 ATPγS treatment enhances glycolysis in C2C12 myotubes which is attenuated by suramin**

**A)** Representative extracellular flux assay demonstrating changes to glycolytic rate as estimated by extracellular acidification rate (ECAR) in response treatment with ATPγS (100 µM; 16 hours) ± suramin (100 µM) or vehicle; as well as acute injection of glucose (5.5 mM), oligomycin (2 µM) and 2-deoxyglucose (2-DG, 50 mM) at the indicated points (Two-way ANOVA with repeated measures and Bonferroni's multiple comparisons analysis *P<0.05 and **P<0.01 for ATPγS against control and $P<0.05 for suramin against ATPγS; n=22-24). **B)** Non-glycolytic acidification demonstrating mean ECAR prior to glucose injection. **C)** Glycolysis measured as the change in ECAR following glucose injection compared to baseline levels (One-way ANOVA with Bonferroni's multiple comparisons analysis *P<0.05 for ATPγS against control). Suramin treatment returns ECAR to control levels (P>0.05 for suramin against control) abolishing ATPγS response.

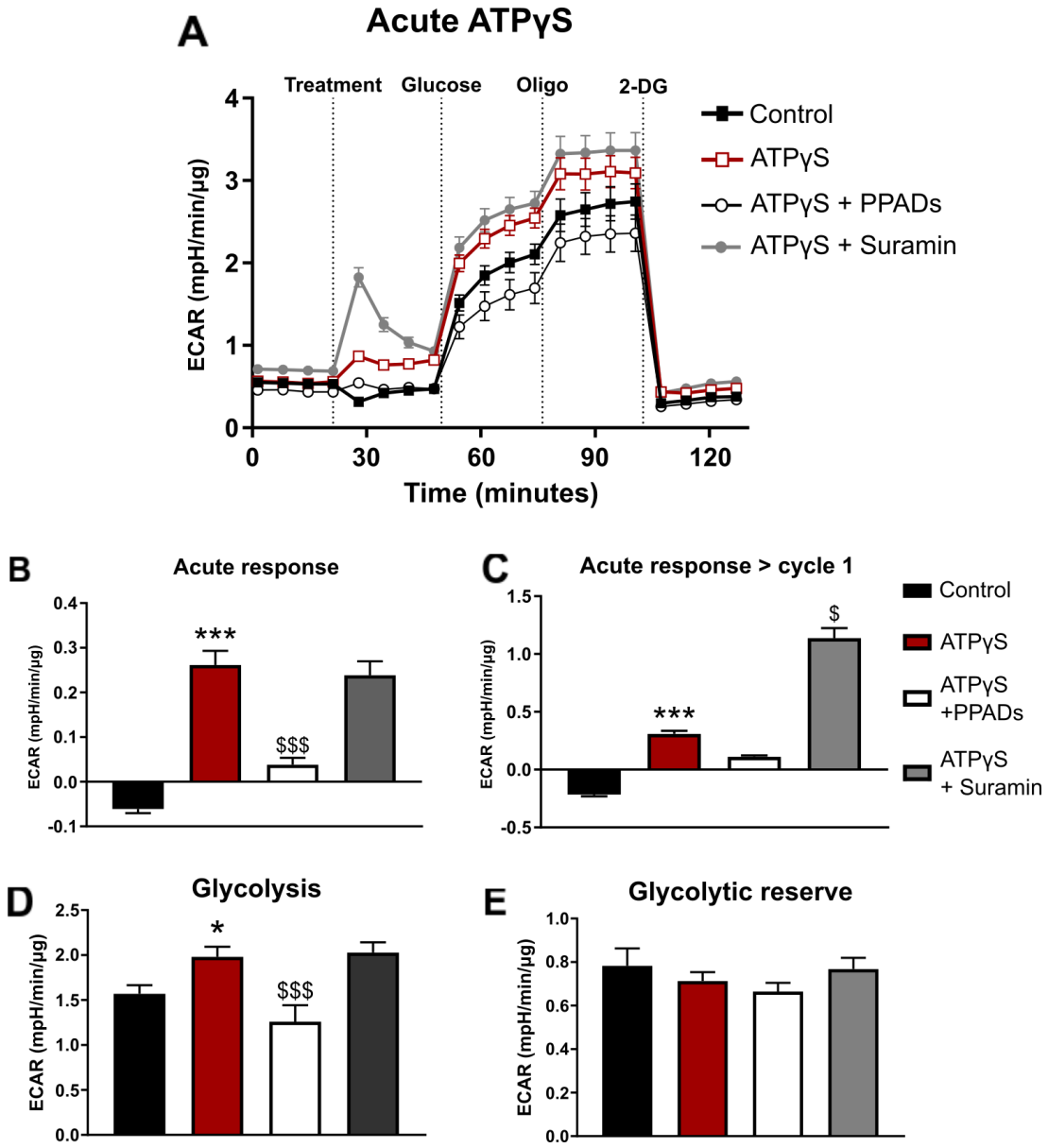



**Figure 3.14.2 Acute ATPγS treatment alters glycolytic rate in C2C12 myotubes in a P2R-regulated manner**

**A)** Representative extracellular flux assay demonstrating changes to glycolytic rate as estimated by extracellular acidification rate (ECAR) in response to acute injections of ATPγS (100 µM) ± suramin (100 µM), PPADs (100 µM) or vehicle; followed by acute injection of glucose (5.5 mM), oligomycin (2 µM) and 2-deoxyglucose (2-DG, 50 mM) at the indicated points (Two-way ANOVA with repeated measures ***P<0.001$_{(drug)}$, ***P<0.001$_{(time)}$, ***P<0.001$_{(interaction)}$ (n=22-24) **B)** Acute response to treatment measured by difference between last measurement before glucose and last baseline measurement (***P<0.001 against control $$$P<0.001 against ATPγS **C)** Acute response after one cycle (***P<0.001 against control $P<0.05 against ATPγS). **D)** Glycolysis measured as a difference between last measurement before oligomycin and last baseline reading (*P<0.05 against control $$$P<0.001 against ATPγS) **E)** Glycolytic reserve measured as oligomycin effect. One-way AVOVA with Bonferroni's multiple comparisons analysis.

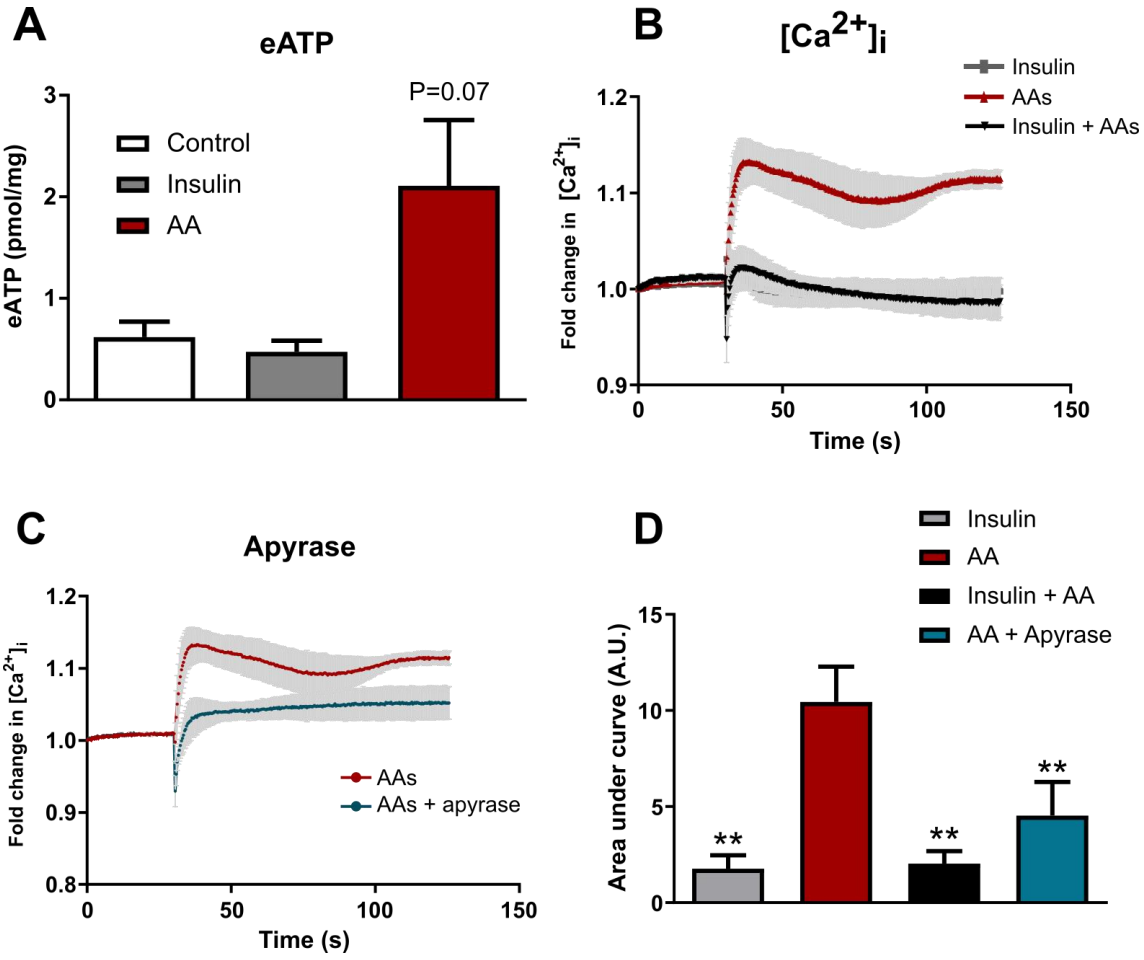



**Figure 3.15 Amino acid treatment of C2C12 myotubes increases extracellular ATP and intracellular calcium**

**A)** Change in eATP following AA (3.34 mM) and insulin (50 mM) treatment (1 hours) in C2C12 myotubes (One-way ANOVA with Bonferroni's multiple comparisons analysis P=0.07 for AAs against control). **B)** Representative curves of changes in intracellular calcium in response to AAs (3.34 mM), insulin (20 nM) or both measured using Fluo-4 in a plate based assay (n=3). **C)** Representative curves of changes in intracellular calcium in response to AAs (3.34 mM) (same data as in B) or following apyrase pre-treatment (0.5 units/mL; 1 hour). **D)** Area under the curve analysis for data shown in B and C (One-way ANOVA with Bonferroni's multiple comparisons **P<0.01 against AA; n=3).

# Discussion

In the present study, C2C12 myotubes were used to assess the potential involvement of the purinergic signalling system in mechanisms regulating insulin and anabolic resistance in SM. Palmitate treatment was used to induce insulin and anabolic resistance in these cells. Extracellular ATP was enhanced following palmitate treatment but modulation of eATP demonstrated that the nucleotide did not appear to regulate the FA-induced effects on intracellular signalling or inflammatory cytokine release. Interestingly, eATP appeared to show potential novel anti-inflammatory effects as well as insulin mimetic actions on glucose uptake and intracellular metabolism.

**Assessing anabolic sensitivity in C2C12 myotubes differentiated at physiological glucose concentration**

To assess the effect of elevated FA exposure separately from the effects of hyperglycaemia on insulin and anabolic resistance *in vitro,* C2C12 myotubes were differentiated under physiological glucose concentrations, 5.5 mM, unlike standard culture conditions where cells are maintained and differentiated at 25 mM glucose (hyperglycaemic). Here, across the 7 days of differentiation, cells demonstrated morphologically identical features from myotubes cultured at supraphysiological glucose conditions (Burattini *et al.*, 2004), showing alignment and fusion of myoblasts by day 3, elongation and myotube formation by day 5 and fully formed Y-shaped myotubes by day 7. Glucose concentration during differentiation of C2C12 myotubes has often been overlooked as a factor of experimental outcomes. Data concerning glucose concentration during differentiation are scarce and subject to debate. For instance, Grzelkowska-Kowalczyk and colleagues showed that differentiating myotubes in high glucose concentrations (15 mM) for three days led to a 2-fold decrease in myoblast fusion and decrease in myogenic transcription factor expression compared to euglycaemic controls (5 mM glucose) (Grzelkowska-Kowalczyk *et al.*, 2013). In contrast, Fulco and colleagues, who considered 25 mM glucose as euglycaemic and 5 mM glucose as glucose restriction, showed that differentiation at 5 mM glucose for 48 hours impaired myogenesis and activated AMPK, an important sensor of energy stress (Fulco *et al.*, 2008). These studies, however, provide no methodological details about the frequency of glucose replenishment in these

cells or difference between myotubes after full differentiation, which is not achieved until days 5-7 (Burattini *et al.*, 2004). Importantly for this study, glucose was replenished in fresh media every 24 hours during differentiation to avoid glucose depletion and experiments were conducted after 7 days, ensuring fully formed myotubes were always used.

Insulin and AAs represent important anabolic stimuli that regulate SM glucose metabolism and protein synthesis. Impaired insulin-stimulated glucose uptake and intracellular utilisation (insulin resistance) and AA-stimulated protein synthesis (anabolic resistance) are key contributing factors to loss of muscle mass and function in metabolic disease (Ferrannini *et al.*, 1996; Park *et al.*, 2009; Cruz-Jentoft *et al.*, 2010). Here, insulin and AA treatments were used to establish a SM cell model of anabolic sensitivity by assessing activation of PI3K/PKB and mTOR signalling pathways in C2C12 myotubes. Insulin treatment (20 nM, 60 minutes) increased phosphorylation of PKB at serine 473, an important site for full kinase activation (Sarbassov *et al.*, 2005), in a concentration-dependent manner. This is well-established and consistent with data showing a 2-3-fold increase in phosphorylation of PKB after insulin treatment (100 nM, 15 minutes) in C2C12 myotubes (Sun *et al.*, 2017; Vlavcheski and Tsiani, 2018) and *ex-vivo*, in insulin-infused soleus muscles (0.6-100 nM for 50 minutes) (Zheng and Cartee, 2016). In human SM, 3 hour insulin infusion increases PKB phosphorylation 5-fold (Cozzone *et al.*, 2008). Here, insulin also enhanced phosphorylation of p70-S6K1, consistent with previous findings (Conejo *et al.*, 2001) and augmented the AA-induced phosphorylation of p70-S6K1. AA alone (3.34 mM, 60 minutes) caused a 2-fold increase in p70-S6K1 phosphorylation at threonine 389, an mTOR regulated site indispensable for p70-S6K1 activation (Shimobayashi and Hall, 2014). The effect of AAs on p70-S6K1 phosphorylation is also well documented, particularly of EAAs, such as lysine, methionine, phenylalanine, tryptophan and threonine, which enhance phosphorylation of p70-S6K1 1.6-2-fold in C2C12 myotubes (2 mM, 30 minutes) and leucine which increases it 6-fold (Atherton *et al.*, 2010). These data helped establish a good model of anabolic sensitivity in mature C2C12 myotubes that was used to test the overarching hypothesis of this study.

**Palmitate treatment as a model of skeletal muscle metabolic impairment**

A model of FA-induced metabolic dysfunction was subsequently used to test this hypothesis. Insulin resistance, inflammatory cytokine production and mitochondrial dysfunction are characteristics of FA-induced metabolic dysfunction in SM and were used here as markers of a well-developed model (replicating earlier studies) to study the mechanisms regulating these features.

In agreement with the hypothesis, treatment with palmitate (500-750 μM; 16 hours) prevented the phosphorylation of PKB and p70-S6K1 induced by insulin and AAs (60 minutes). These findings concur with others showing that 24 hour treatment with palmitate (600 μM) suppressed PKB and p70-S6K1 phosphorylation in response to insulin treatment (100 nM for 15 minutes) (Yang *et al.*, 2013) and palmitate (400 μM; 6 hours) caused a 40 % decrease in insulin-stimulated PKB phosphorylation (100 nM for 10 minutes) in C2C12 myotubes (Sinha *et al.*, 2004). Importantly, at these concentrations, palmitate has been shown to cause IR in muscle cells without impairing cell viability, for up to 24 hours (Pimenta *et al.*, 2008; Yang *et al.*, 2013). Palmitate treatment also attenuated the insulin and AA effect on phosphorylation of p70-S6K1. This is likely a combination of blunted insulin signalling, as demonstrated by impaired PKB phosphorylation and previous findings (Yang *et al.*, 2013) and desensitisation to AA treatment after exposure to high concentrations of palmitate. Indeed, treatment with lipotoxic ceramides diminishes intracellular AA availability and attenuates insulin and AA-induced S6K1 phosphorylation (Hyde *et al.*, 2005) and intravenous lipid infusion in humans prevents the otherwise ~2-fold increase AA-stimulated protein synthesis (Stephens *et al.*, 2015). The effect of high concentrations of palmitate on C2C12 myotubes shown here provided a good model of FA-induced insulin and anabolic resistance.

Inflammation plays an important role in the development of SM IR, in part, via increased influx of FAs and inflammatory cytokines from AT (and SM itself). These induce SM inflammation and alter metabolism to promote IR (Wu and Ballantyne, 2017). For instance, palmitate treatment in L6 myotubes activates IKK/IκB/NF-κB signalling, resulting in a 30% decrease in insulin-stimulated glucose uptake (Sinha *et al.*, 2004).

Furthermore, conditioned media (CM) from palmitate-treated macrophages activates PKC*θ* and *ε* in L6 myoblasts (Samokhvalov *et al.*, 2009) causing reduced PI3K signalling and glucose uptake in GLUT4-overexpressing cells (Kewalramani *et al.*, 2011) and causes TLR2/4-mediated IR in L6 myotubes (Nguyen *et al.*, 2007). Elevated TNF-α levels present in the CM impaired insulin-stimulated glucose uptake in C2C12 myotubes via activation of p38 MAPK (Talbot *et al.,* 2014). Others have demonstrated attenuated insulin signalling in muscle cells from TNF-α-mediated activation of p38 MAPK, via IKK/IκB/NF-κB signalling (De Alvaro *et al.*, 2004).

Plasma TNF-α levels are elevated in severe inflammation and implicated in IR and diabetes (Swaroop *et al.,* 2012). TNF-α-induced AT IR (Atsumi *et al.*, 2007) and catabolic effects on muscle glucose metabolism (Benigni *et al.*, 2000) are mediated by macrophage migration inhibitory factor (MIF). Plasma MIF levels are upregulated in chronic inflammatory metabolic disorders including in obesity and T2D (Church *et al.*, 2005; Skurk *et al.*, 2005; Morrison and Kleemann, 2015) and the cytokine participates in HFD-induced IR, as demonstrated by improved glucose tolerance and protection from HFD-induced IR in MIF knockout mice (Finucane *et al.*, 2014). These observations, added to the ability of SM to release MIF (Miyatake *et al.*, 2014) led to the hypothesis that palmitate treatment would increase MIF levels in C2C12 myotubes. Indeed, palmitate treatment (16 hours) caused a 3-fold increase in MIF in these cells. MIF release from palmitate-treated muscle cells has been shown on one other occasion, in response to 18 hour treatment with 500 μM palmitate in C2C12 myotubes (Pillon *et al.*, 2014). Although the direct consequence of elevated MIF levels in this system remains unexplored, one report indicated that 24 hour treatment of liver and SM cells with recombinant MIF did not impact insulin signalling, whereas it significantly attenuated insulin-induced glucose uptake into adipocytes (Cui *et al.*, 2018). It is plausible that MIF is released from these cells as they are metabolically compromised, in a similar way that MIF is secreted during ischaemia (Dayawansa *et al.*, 2014), hypoxia (Simons *et al.*, 2011) and AA starvation (Chuang *et al.*, 2012). Further work is required to establish its origin and downstream effects.

In addition to MIF, palmitate treatment (16 hours) led to a significant increase in the extracellular levels of IL-6 in C2C12 myotubes.

IL-6 was one of the first established myokines, and has been extensively characterised for its paradoxical effects on inflammation (reviewed by Scheller *et al.*, 2011; Muñoz-Cánoves *et al.*, 2013). Transient and short-term action of muscle-derived IL-6 benefits SM hypertrophy, myogenesis and regeneration and contributes to whole-body glucose homeostasis, stimulating glucose production (Fischer, 2006; Wolsk *et al.*, 2010; Pedersen and Febbraio, 2012; Eckardt *et al.*, 2014). Conversely, long-lasting elevated circulating IL-6 is a feature of pro-inflammatory conditions and is associated with muscle wasting and IR (Kern *et al.*, 2001; Pradhan *et al.*, 2001; Rui *et al.*, 2002). Consistent with findings here, palmitate (600 μM for 24 hours) induced a 10-fold increase in IL-6 gene expression and increased release in C2C12 myotubes (Yang *et al.*, 2013) and this was reproduced by Jove and colleagues who showed that 16 hour treatment with palmitate (500 μM) caused a 3.5-fold increase in IL-6 mRNA in the same cells (Jové *et al.*, 2005). The latter also showed a 3-fold induction in extracellular IL-6 after palmitate treatment and both effects were PKC and NF-κB-dependent. As such, IL-6 release was used as a positive control for palmitate-induced responses in SM. The role of elevated IL-6 here, however, remains to be determined as the levels of the myokine were also increased following ATPγS treatment (in the absence of palmitate) in these cells (discussed below).

Mitochondrial respiration was also assessed, given the role played by mitochondrial dysfunction and increased mitochondrial oxidative stress in T2D and IR (Sergi *et al.*, 2019). Here, palmitate treatment (16 hours) at concentrations > 500 μM impaired basal oxygen consumption as well as ATP synthase activity and spare respiratory capacity in C2C12 myotubes. At lower concentration (250 μM), these parameters were increased. Increased FA availability is likely leading to increased rates of FAO in these cells. At 250 μM palmitate, FAO remains coupled to ATP synthesis (St-Pierre *et al.*, 2002) as basal OCR and ATP synthase activity were increased relative to control cells and these cells were able to respire maximally in response to exogenous uncoupling with FCCP. The oxidation of FAs generates ROS, such as superoxide, which in turn activate uncoupling protein (UCPs) that allow proton re-entry into the mitochondrial matrix, independently of ATP synthase (proton leak) (Andrews *et al.,* 2005; Busiello *et al.,* 2015).

Mitochondrial uncoupling, as well as intrinsic antioxidant activity by enzymes such as superoxide dismutases, control the levels of ROS within cells (Lambert and Brand, 2009). However, with excess FAO, ROS production increases to supersede the effect of antioxidant mechanisms and uncoupling, leading to build-up of lipotoxic metabolites, cellular oxidative stress (Cross *et al.*, 1987), mitochondrial damage and impaired insulin signalling (Anderson *et al.*, 2009). This suppresses mitochondrial respiration and ATP production, which was observed here in response to palmitate (> 500 μM) by reduced ATP synthase activity and impaired spare respiratory capacity. This is consistent with data showing increased ROS, mitochondrial DNA damage, activated JNK signalling and IR in palmitate-treated L6 myotubes (Yuzefovych *et al.,* 2010). The latter demonstrated these effects at a wider range of palmitate concentrations (100 μM to 1 mM). Assessing changes to markers of mitochondrial function and FAO such as activation of AMPK, ACC, lipotoxic metabolites, ROS and expression of UCPs would help clarify the concentration-dependent differences observed here.

The data discussed above demonstrate reduced anabolic sensitivity, enhanced inflammation and diminished mitochondrial function induced by high concentrations of palmitate during long-term exposure in C2C12 myotubes. This generated a good model with which to test the involvement of the purinergic signalling system in these responses.

**Contribution of purinergic signalling system to palmitate-induced IR and inflammation**

It was hypothesised that the effects of palmitate treatment on anabolic sensitivity and inflammation may be regulated by eATP, given its roles in inflammation and glucose metabolism. Here, palmitate treatment (500 μM, 16 hours) significantly increased the levels of eATP in C2C12 myotubes, which has only been reported once in muscle cells (Pillon *et al.*, 2014). Pillon and colleagues demonstrated that CM from palmitate-treated (500 μM, 18 hours) myotubes had potent monocyte chemoattractant properties, which were prevented by apyrase. Importantly, as SFAs are cytotoxic (Yang *et al.*, 2013) and ATP is released in response to cellular damage (Lazarowski *et al.,* 2003), the group eliminated cytotoxicity as a source of increased eATP showing that myotube viability was not affected using MTT reduction, lactate dehydrogenase and caspase-3 cleavage as markers.

Elevated eATP can originate from increased release or decreased extracellular degradation of the nucleotide (Robson *et al.*, 2001; Robson *et al.,* 2006). Future experiments would investigate the origin of palmitate-induced eATP seen here by inhibiting ATP release using pannexin (Pnx) and connexin (Cnx) channel blockers, measuring activity of endogenous ecto-nucleotidases and inhibiting extracellular hydrolysis by CD39 and CD73. It is plausible, however, given the work by Pillon and colleagues (Pillon *et al.*, 2014), that palmitate acting via TLR4-NF-κB upregulates Pnx3 channel expression to increase ATP release.

Here, apyrase prevented palmitate-dependent increase in eATP but did not alter baseline or palmitate-induced secretion of IL-6 or MIF, nor did it alter the blunted insulin and AA-dependent phosphorylation of PKB or p70-S6K1. This was also the case for palmitate-induced phosphorylation of ERK. Extracellular hydrolysis of ATP by apyrase may result in upregulated signalling via AMP and adenosine, and these can have opposing roles to ATP, particularly in inflammation and immune responses (Beldi *et al.*, 2008; Faas *et al.,* 2017). Furthermore, ATP may activate highly sensitive P2R in an autocrine manner (Ostrom *et al.,* 2000). This is observed in the vascular epithelium, where ATP is released due to endothelial damage (Lohman *et al.,* 2012). Treatment of endothelial cells with high concentrations of glucose and palmitate (24 hours), results in upregulated expression of P2X4R and P2X7R, release of inflammatory cytokines and upregulated IL-6 mRNA expression (Sathanoori *et al.*, 2015). The effect on cytokine release and IL-6 expression were prevented by P2X4R and P2X7R blockade (at 24 hours) but treatment with apyrase did not prevent these effects at 24 hours, only after 48 hours (Sathanoori *et al.*, 2015). This suggests that extracellular ATP may elicit P2R-mediated roles that are not negated by the use of apyrase alone. Subsequent experiments in this study therefore assessed the effect of direct and continuous treatment with eATP on anabolic sensitivity and inflammation.

**Effects of ATP treatment on skeletal muscle inflammation and metabolism**

Activation of P2XRs by ATP results in rapid $Ca^{2+}$ influx and downstream activation of MAPK, PKC and $Ca^{2+}$-calmodulin-dependent protein kinases (CAMK) (Ostrom, Gregorian and Insel, 2000; Cseri *et al.*, 2002).

Activation of P2YRs (dependent on activated G-protein) stimulates IP3-mediated release of $Ca^{2+}$ from intracellular stores, increases DAG to activate PKC, inhibits adenylyl cyclase and reduces cAMP levels (von Kugelgen and Harden, 2011; Erb and Weisman, 2012). Here, treatment of C2C12 myotubes with ATP resulted in a concentration-dependent increase in $[Ca^{2+}]_i$. This was consistent with $Ca^{2+}$ transients in primary mouse myotubes in response to ATP (180 μM), which were mediated via P2X4, 5, 7 and P2Y1 and 4 receptors (Deli *et al.*, 2007; Buvinic *et al.*, 2009). Extracellular nucleotide treatment in myotubes has demonstrated steep and rapid increases in $[Ca^{2+}]_i$, suggestive of P2XR activity (Cseri *et al.*, 2002) and increases in IP3, suggestive of P2YR activity (Häggblad and Heilbronn, 1987; Keresztes *et al.,* 1991). Pharmacological activation of P2Y1 in human myotubes also causes an IP3-mediated increase in $[Ca^{2+}]_i$ which results in ERK1/2 activation (May *et al.*, 2006). The $[Ca^{2+}]_i$ peaks observed here were rapid and transient, but remained concentration-dependently elevated, suggesting involvement of both P2XR and P2YRs. Receptor specific agonists would be used in future to identify the P2Rs mediating $[Ca^{2+}]_i$ changes in these cells and contribution of P2XRs assessed by using medium free of calcium.

In the absence of palmitate, ATPγS (16 hours) concentration-dependently increased and decreased the levels of IL-6 and MIF, respectively, in C2C12 myotubes. IL-6 and ATP are released at high levels during muscle contraction and exercise (Steensberg *et al.*, 2000; Pedersen and Fischer, 2007; Osorio-Fuentealba *et al.*, 2013). The expression of IL-6 in muscle is regulated by intracellular $Ca^{2+}$-mediated signalling involving $Ca^{2+}$-dependent phosphatase calcineurin (Allen *et al.*, 2010) and action of p38 MAPK and CAMK (Weigert *et al.*, 2007) and the cytokine regulates its own expression via JAK2/STAT3 signalling (Naka *et al.*, 1997; Schmitz *et al.*, 2000).

ATP-P2YR-mediated increase in $[Ca^{2+}]$ is also associated with elevated expression of IL-6 mRNA and activation of ERK and NF-κB pathways (Buvinic *et al.*, 2009). Buvinic and colleagues demonstrated that electrical stimulation (ES) of primary rat myotubes led to ATP release via Panx1 channels, which acted in an autocrine manner on P2XRs and P2YRs to increase $[Ca^{2+}]_i$; and treatment with exogenous ATP in these cells increased IL-6 mRNA (Buvinic *et al.*, 2009).

ES of primary myotubes and muscle fibres also increases eATP, which augments expression and extracellular levels of IL-6 in a P2R-IP3-$Ca^{2+}$-dependent manner (Bustamante *et al.*, 2014). As treatment with apyrase did not prevent the palmitate-induced increase in IL-6, in this study, it is plausible that ATPγS-mediated IL-6 release occurs via a separate mechanism. For example, in myotubes palmitate partly mediates IL-6 secretion by activation of the pro-inflammatory NF-κB pathway (Jové *et al.*, 2005). In this study, ATPγS decreased the phosphorylation of p65 NF-κB, suggesting that this pathway is unlikely to mediate IL-6 secretion. IL-6 is a pleiotropic myokine, demonstrating pro and anti-inflammatory effects in SM (Pedersen and Fischer, 2007; Muñoz-Cánoves *et al.*, 2013) so a better understanding is required of the functional implications of ATPγS-induced IL-6 secretion. Contrary to expectation, ATPγS treatment decreased the levels of pro-inflammatory cytokine MIF and decreased activation of NF-κB signalling pathway. Together with increased IL-6 (which may act in an anti-inflammatory manner), these data pose the question of whether continuous ATP exposure may be immunomodulatory in SM.

Although not fully characterised, ATP has demonstrated anti-inflammatory properties. In microglia, eATP inhibits the release of inflammatory cytokines from lipopolysaccharide(LPS)-primed cells (Ogata *et al.*, 2003), suppresses release of nitric oxide from BV-2 cells (Brautigam *et al.*, 2005) and decreases TNF-α and ROS levels in primary cells (Boucsein *et al.*, 2003). In human immune cells, ATP inhibits LPS-stimulated production of pro-inflammatory cytokines and up-regulates anti-inflammatory signals, mediated via P2Y11R (Vitiello *et al.*, 2012). Furthermore, ATP suppresses TNF-α and IL-12 in LPS-treated macrophages (Haskó *et al.*, 2000). To date, no anti-inflammatory roles of eATP on SM have been reported. A better understanding is therefore required of the role of eATP on SM inflammation, as, although direct treatment with ATPγS decreased MIF levels, apyrase treatment did not aggravate the palmitate-stimulated increase in MIF.

Independently of palmitate, ATPγS pre-treatment (16 hours) did not alter insulin-stimulated phosphorylation of PKB or AA-dependent increase in p70-S6K1 phosphorylation, suggesting that long-term exposure to eATP does not impair

anabolic sensitivity and almost certainly does not mediate the palmitate-induced effects on C2C12 myotubes.

Importantly, palmitate, eATP, IL-6 and MIF all participate in the regulation of glucose metabolism in SM, raising interesting questions about the relationship of these molecules to one another. For instance, acute treatment of C2C12 myotubes with ATPγS, better mimicking the transient and high levels of eATP resulting from muscle contraction and exercise, caused a concentration-dependent increase in the phosphorylation of PKB and p70-S6K1 in the absence of insulin and AAs. These responses were abolished by PPADS and suramin but not by P2X4R or P2X7R antagonists. The insulin-like actions of ATP were further expanded to show a concentration-dependent increase in glucose uptake in C2C12 myotubes that was comparable to the insulin effects. A similar insulin mimetic effect of eATP was demonstrated by Osorio-Fuentealba and colleagues (Osorio-Fuentealba *et al.*, 2013), who showed that eATP, released from ES or exogenously administered, promotes glucose uptake in L6 myotubes, primary myotubes and muscle fibres through mobilisation of GLUT4 via a PI3Kγ-PKB-AS160 signalling cascade. In their study, treatment of primary myotubes with ATP (100 μM, 15 minutes) caused a >2-fold increase in 2-NBDG uptake which was prevented by apyrase (2 units/mL; 30 minutes) and suramin (100 μM) (Osorio-Fuentealba *et al.*, 2013). Interestingly insulin-induced uptake was not blocked by apyrase or suramin, which taken together with observations that eATP did not alter INSR activation, suggests that eATP-induced glucose uptake is insulin-independent. Furthermore, akin findings in this chapter, ATP (100 μM) significantly enhanced PKB (S473) phosphorylation after 15, 30 and 60 minutes in muscle cells (Osorio-Fuentealba *et al.*, 2013).

Groups that demonstrated eATP-mediated glucose uptake in SM did not investigate which P2Rs may be activated (Osorio-Fuentealba *et al.*, 2013). Data presented here suggest that the ATPγS-induced increase in PKB and p70-S6K1 phosphorylation did not occur downstream of P2X4R or P2X7R but was mediated by P2YRs, as it was prevented by both PPADS and suramin. P2Y2R is the most likely candidate receptor as both antagonists show moderate affinity towards P2Y2R (Burnstock, 2005) and this is highly expressed in mature myotubes (Banachewicz *et al.*, 2005).

This would also concur with findings by Kim and colleagues, who demonstrated P2R-mediated glucose transport in C2C12 myotubes, suggested to occur via P2Y2, 4 or P2X1 (Kim *et al.*, 2002).

Interestingly, eATP regulation of p70-S6K1 in SM had not been described. Similar data were recently reported by Ito and colleagues (Ito *et al.,* 2018). The group demonstrated P2Y2R-mediated increase in p70-S6K1 phosphorylation (T389) in response to eATP treatment (> 10 μM; 30 minutes) in C2C12 myotubes. This anabolic response and eATP-induced increase in $[Ca^{2+}]_i$ were blocked by depletion of intracellular $Ca^{2+}$ and P2Y2R knockdown. ATP-mediated effects occurred by activation of a P2Y2R/PI3K/PLC/IP3R pathway, acting via class III PI3K (Vps34) and not class I (PI3K/PKB) which primarily regulates insulin signalling. Interestingly, data in this chapter showed AA-dependent increase in $[Ca^{2+}]_i$, which was blocked by apyrase; AA-mediated increase in eATP and increased p70-S6K1 phosphorylation in response to AA and ATPγS (independently). Given that AA signalling results in mTORC1 activation partly via Vps34 (Nobukuni *et al.*, 2005), it will be interesting to determine whether treatment with AAs regulates this pathway and whether AA-induced muscle hypertrophy requires ATP release.

The AA-mediated increase in eATP and $[Ca^{2+}]_i$ seen here was not mimicked by insulin; and co-treatment with insulin abolished the AA effect. The impact of insulin treatment on eATP has not been previously studied but it was hypothesised that insulin would increase $[Ca^{2+}]_i$, as calcium influx was believed to be necessary for insulin-stimulated glucose uptake into SM fibres (Lanner *et al.*, 2006), even though others have shown that insulin does not increase total $[Ca^{2+}]_i$ in various muscle models (Klip, Li and Logan, 1984; Cheung, Constantine and Bonventre, 1987). Data in this chapter suggests that insulin does not alter eATP release or $[Ca^{2+}]_i$. Although very sensitive methods were used in Lanner *et. al.* (Lanner *et al.*, 2006) to measure $[Ca^{2+}]_i$, their methodology relied on ES of fibres prior to measurements. Evidence from other groups (Kim *et al.*, 2002; Osorio-Fuentealba *et al.*, 2013), taken together with data presented here, suggests that eATP likely modulates the intracellular calcium levels during ES, so it is possible that the insulin effect observed by the group was partly eATP-mediated.

Although insulin itself does not alter $[Ca^{2+}]_i$, it may modulate the effect of other stimuli, such as AA. In future, more sensitive methods such as single cell ratiometric calcium imaging would be used to better understand this relationship.

In this study, treatment with ATPγS (15 minutes) enhanced glucose transport into myotubes in an insulin-independent manner. Extracellular ATP-mediated glucose uptake has been reported but, to date, no evidence exists of altered intracellular glucose metabolism in response to eATP in SM. Here, for the first time, ATPγS treatment (short and long-term) altered glycolytic rate in a P2R-dependent manner. Unexpectedly, ATPγS also increased extracellular acidification in the absence of glucose, which was enhanced by suramin, so further work is required to understand this response. The acute increase in glycolysis was blocked by PPADS but not suramin suggesting differential P2R regulation of these effects. This may also suggest that more acute effects may be regulated by P2XRs as PPADS selectively inhibit P2XR and only moderately antagonise P2YRs, while suramin antagonises P2YRs with higher affinity than P2XRs (Burnstock, 2018). In vascular endothelial cells exogenous ATP increases ECAR and upregulates the key glycolytic enzymes PFK-B3 and HK as well as GLUT-1 expression (Lapel *et al.*, 2017). This has not been observed in SM so future experiment would aim to assess the effect of eATP treatment on SM glycolytic machinery.

The effect of eATP on glucose metabolism may be indirectly mediated by autocrine and paracrine IL-6 and/or MIF signalling. For instance, IL-6 increases basal and insulin-stimulated glucose uptake in L6 myotubes and primary myotubes (Al-Khalili *et al.*, 2006) and increases glucose oxidation in human primary myotubes, mediated by activation of AMPK (Glund *et al.*, 2007), which also promotes FAO (Carey *et al.*, 2006). MIF inhibits insulin-stimulated glucose uptake into AT (Atsumi *et al.*, 2007) and is implicated in HFD-induced AT inflammation and IR in mice (Finucane *et al.*, 2014). Contrastingly, MIF deficiency (knockout) impairs glucose tolerance and hyperinsulinaemia in mice and MIF regulates TNF-α effects to increase glycolysis and lactate production in myotubes (Benigni *et al.*, 2000). More recently, MIF was shown to decrease insulin-mediated glucose uptake and phosphorylation of PKB in soleus muscle and AICAR-mediated glucose transport in extensor digitorum longus (EDL) muscle of mice (Miyatake *et al.*, 2014).

The relationship between IL-6, eATP and MIF may therefore be important in the regulation of SM glucose homeostasis. For example, Miyatake and colleagues demonstrated a 70 % reduction in MIF release following ES of C2C12 myotubes (Miyatake *et al.*, 2014). Although an acute effect of eATP on MIF secretion was not assessed here, it would be interesting to determine whether the decrease in MIF following contraction is caused by eATP and whether increased glucose transport requires inhibition of MIF release. An increase in intracellular ATP availability from upregulated glycolysis in these cells should also result in inhibition of AMPK (Jørgensen, Jensen and Richter, 2007) further enabling mTOR activation (seen here). AMPK activation was not yet investigated here, but others showed that treatment with eATP (100 μM; 30 minutes) did not alter AMPK phosphorylation (Thr172) in C2C12 myotubes (Ito *et al.,* 2018). MIF, on the other hand, regulates AMPK activation in a variety of tissues. In the heart, MIF (acting via CD74 receptors) promotes glucose uptake during hypoxia by activating AMPK, which protects cardiomyocytes during ischemia-reperfusion injury (Miller *et al.*, 2008) and MIF deficiency is believed to increase susceptibility to myocardial ischaemia in older cardiac patients (Ma *et al.*, 2010). MIF-CD74-mediated signalling also protects hepatocytes in metabolic models of liver injury (Heinrichs *et al.*, 2014) and regulates cardioprotective signalling mechanisms in a mouse model of T1D (STZ), via activation of AMPK, PKB and ERK (Tong *et al.*, 2010). Conversely, MIF-CD74 inhibits AMPK activation in lung adenocarcinoma cell lines, enhancing mTOR activation (Brock *et al.*, 2012) and decreases AICAR-mediated glucose uptake into muscle (Miyatake *et al.*, 2014). AMPK is an important whole body and cellular energy sensor, and it appears that MIF may also act as a cellular stress signal, as MIF levels are elevated during energetic stress (hypoxia, ischaemia, sepsis, inflammation) and seen here to increase after palmitate treatment, when anabolic and mitochondrial function are compromised. MIF was also upregulated after glucose deprivation in C2C12 myotubes (3 hours, 1 mM glucose compared to 5.5 mM; data not shown). In future, it will be interesting to elucidate the relationship between eATP and MIF (and possible AMPK activation) and how these might regulate SM glucose metabolism.

Insulin-independent mechanisms to upregulate glucose transport and utilisation have high therapeutic value, as these may help regulate glycaemia in insulin-deficient and resistant states. As acute stimulation with ATPγS demonstrated insulin-mimetic effects these treatments were conducted following palmitate treatment to assess whether insulin-independent glucose uptake would be sustained. Palmitate treatment impaired ATPγS-stimulated glucose uptake, to a similar extent that it inhibited insulin-dependent glucose uptake, suggesting a level of purinergic resistance. However, others have shown that eATP-mediated glucose uptake was preserved in insulin resistant SM fibres from HFD-fed mice (Osorio-Fuentealba *et al.*, 2013). A better understanding of purinergic resistance is therefore required, and in this study future experiments would aim to assess the effect of palmitate treatment on ATPγS-induced activation of anabolic signalling proteins and ATP-induced increase in $[Ca^{2+}]_i$. Interestingly, recent findings suggest that contraction protects C2C12 myotubes from palmitate-induced IR (Nieuwoudt *et al.*, 2017). In these cells, ES increases basal glucose uptake and PKB phosphorylation and electrically stimulating cells before palmitate treatment prevented the ~50 % decrease in insulin-stimulated glucose uptake and recovered insulin sensitivity and PKB response (Nieuwoudt *et al.*, 2017). It is plausible, given data shown here and by others (Jaimovich *et al.*, 2011; Osorio-Fuentealba *et al.*, 2013) that the effect of ES on basal insulin signalling is dependent on eATP arising from contraction. This may also represent a role for eATP as an exercise mimetic, acting as a stimulus for glucose uptake during increased energy demand.

**Summary and future work**

For the first time, treatment of C2C12 myotubes with a non-hydrolysable purinergic receptor agonist led to upregulated glucose transport coupled with an increase in glucose metabolism. Manipulating eATP by hydrolysis with apyrase or directly treating cells with ATPγS demonstrated that palmitate-induced effects on anabolic sensitivity and inflammation are unlikely to be mediated by eATP, as originally hypothesised. Instead, cells may release ATP in response to long-term SFA exposure in an effort to recover impaired glucose and protein metabolism, particularly when the requirement for glucose uptake is high, such as during exercise.

Further experimentation is required to elucidate the whole body metabolic effects of extracellular nucleotides and nucleosides. For instance, UTP, a potent P2Y2R agonist, promotes glucose uptake in C2C12 myotubes (100 μM, 20 minutes, 1.8-fold increase) in a similar manner to ATP (2-fold increase compared to vehicle) (Kim *et al.*, 2002). Furthermore, adenosine, acting via P1 receptors, increases insulin-stimulated glucose uptake in SM (Vergauwen, Hespel and Richter, 1994; Takasuga *et al.*, 1999; Liu *et al.*, 2001) and AT (Takasuga *et al.*, 1999) (reviewed by Koupenova and Ravid, 2013). Given the wide range and pattern of expression of P2Rs (Volonté *et al.*, 2006; Burnstock and Verkhratsky, 2009) and P1Rs (Sheth *et al.*, 2014) across different tissues and their differential affinity for multiple nucleotides and nucleosides, an *in vivo* approach will be more relevant to establish these effects going forward. In rodents, it would be possible to assess the effect of P2Y2R agonists and antagonists on glucose metabolism during hyperinsulinaemic-euglycaemic clamps, coupled with glucose tracers to assess insulin sensitivity and glucose metabolism in metabolic tissues. It would also be valuable to assess these responses following HFD-induced obesity and in models of diabetes to further elucidate the involvement of the purinergic signalling system on whole-body energy metabolism.

These advancements have the potential to become highly translatable to human health and disease as ATP is readily available as an oral supplement. Oral ATP supplementation has already demonstrated beneficial effects on exercise tolerance, and post-exercise hypertrophy and recovery (Wilson *et al.*, 2013; de Freitas *et al.*, 2018) so the added observation that it may decrease glycaemia and upregulate protein synthesis would make it a valuable therapeutic target and/or tool for conditions characterised by insulin and anabolic resistance.

# Chapter 4

# The effect of high-fat overfeeding on glucose and amino acid balance during disuse in humans

# Introduction

Adequate maintenance of SM mass is a key determinant of quality of life and metabolic health and is driven by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The inability of SM to appropriately synthesise new protein in response to anabolic stimuli such as protein and AA ingestion is termed anabolic resistance and represents an important contributing factor for the loss of muscle mass and function observed during ageing, disuse and critical illness (Rennie, 2009; Wall and van Loon, 2013; Wall *et al.*, 2015). This loss of muscle mass and strength is also aggravated and occurs more rapidly in metabolic disease, such as in T2D (Park *et al.*, 2006; Leenders *et al.*, 2013). Importantly, health/disease states where anabolic resistance is evident are also associated with reduced insulin sensitivity (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Alibegovic *et al.*, 2009; Dirks *et al.*, 2016), and it is believed that that lipid overflow contributes to the development of both phenomena, as discussed in Chapter 3. The relationship between insulin and anabolic resistance and the likely contribution of elevated FA availability to impaired AA metabolism and protein synthesis are incompletely understood and a better elucidation of these mechanisms is required to develop therapeutic approaches to improve metabolic health and quality of life.

It is clear from data demonstrated and discussed in Chapter 3, that lipid overflow impairs intracellular anabolic signalling responses to insulin and AA treatment in muscle cells. However, it remains unclear whether intracellular impairments arise as a consequence of diminished substrate delivery to SM, particularly considering that the vasodilatory response to insulin is diminished in ageing (Meneilly *et al.*, 1995) and microvascular function becomes impaired in T2D and exacerbates muscle loss (Groen *et al.*, 2014). The temporal regulation of these phenomena is also not completely understood, and given the important anabolic role of insulin in stimulating MPS (Bennet and Rennie, 1991; Biolo *et al.*, 1995), it is important to establish whether anabolic resistance is partly mediated by the loss of insulin anabolism in insulin resistant conditions. The association between lipid-induced IR and anabolic resistance has been seen across different models. For example, in mice (Anderson *et al.*, 2008) and rats (Masgrau *et al.*, 2012), diet-induced obesity impairs activation of MPS in response to feeding, in a manner that is

associated with chronic intramuscular lipid infiltration (Masgrau *et al.*, 2012). Furthermore, compared to lean individuals, obese women demonstrate impaired whole-body protein anabolic responses to hyperinsulinaemia and hyperaminoacidaemia (Chevalier *et al.*, 2005), and MPS in response to these stimuli is negatively related to whole-body fat mass in humans (Guillet *et al.*, 2004). However, although associated, the contribution of lipid-induced IR to anabolic resistance remains unclear. Independently from overfeeding or lipid infusions, SM disuse also leads to loss of muscle mass, associated with increased body fat (Ferrando *et al.*, 1996; Brooks *et al.*, 2008), loss of strength (Rudrappa *et al.*, 2016) and the development of IR (Mikines *et al.*, 1991; Biensø *et al.*, 2012). Therefore, experimental models of disuse can be used to investigate the development of insulin and anabolic resistance (which may be partly mediated by impaired lipid homeostasis) separately from lipid overflow.

Muscle disuse, which can result from physical inactivity during critical illness, recovery from trauma or chronic inactive behaviour, has been extensively modelled experimentally by strategies including bed rest (systemic) and forearm or leg immobilisation (local) (Rudrappa *et al.*, 2016). In otherwise healthy humans, long periods of disuse (10-42 days) lead to a rate of muscle loss of ~0.5-0.6 % per day (reviewed in (Phillips *et al.,* 2009; Wall and van Loon, 2013) and disuse-induced atrophy is accompanied by decreased functional capacity (Deitrick, 1948; Gibson *et al.*, 1987), basal metabolic rate (Haruna *et al.*, 1994), insulin sensitivity (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Dirks *et al.*, 2016) and glucose tolerance (Yanagibori *et al.*, 1994; Dirks *et al.*, 2016). In bed rest studies, reduced glucose tolerance and insulin sensitivity is seen after only 3 days (Lipman *et al.*, 1972; Yanagibori *et al.*, 1994; Dirks *et al.*, 2016) and these become significantly impaired after 7-9 days, as seen during hyperinsulinaemic-euglycaemic clamps in healthy humans (Lipman *et al.*, 1972; Kenny *et al.*, 2017). Bed rest represents a clinically relevant model, reflecting disuse in periods such as hospitalisation, but does not eliminate the systemic effects contributing to muscle atrophy, such as systemic inflammation (Drummond *et al.*, 2013; Mutin-Carnino *et al.*, 2014), vascular dysfunction (Hamburg *et al.*, 2007), impaired hormonal homeostasis (Choukèr *et al.*, 2013; Guerra *et al.*, 2014) and whole-body insulin sensitivity (Bergouignan *et al.*, 2011).

A model of local inactivity, such as leg or forearm immobilisation, makes it possible to better isolate the mechanisms regulating muscle function while individuals remain active and maintain habitual food intake (or consume controlled diets as experimental interventions). Leg immobilisation for 7 days leads to an approximate 15-25 % impairment in muscle glucose uptake (Richter *et al.*, 1989) and after 14 days leads to a ~30 % impairment in protein synthetic response to protein ingestion (Wall, Snijders, *et al.*, 2013). Changes to MPS during bed rest and limb immobilisation have been observed without apparent increases in MPB (Gibson *et al.*, 1987; Phillips *et al.,* 2009), suggesting that atrophy during disuse is primarily mediated by an impairment in MPS in response to anabolic stimuli (Ferrando *et al.*, 1996; Symons *et al.*, 2009).

The mechanisms of disuse-induced insulin and anabolic resistance remain unclear but it is believed that alterations in lipid metabolism, particularly accumulation of intramyocellular lipid and lipotoxic metabolites may contribute to the development of these impairments. Indeed, prolonged periods of disuse (> 4 weeks) are associated with IMCL accumulation (Manini *et al.*, 2007; Cree *et al.*, 2010) and shorter periods of disuse have demonstrated changes to DAG metabolism and PKC signalling (Dirks *et al.*, 2016, 2018), which are directly implicated in the development of IR (Bosma *et al.*, 2012). Independently of disuse, increase in lipid availability by direct intravenous infusion of lipid not only directly impairs insulin sensitivity (Kruszynska *et al.*, 2002) but also inhibits AA-induced increase in MPS in healthy men (Stephens *et al.*, 2015). It, therefore, remains to be understood whether disuse brings about impairments in insulin and anabolic resistance by altering lipid metabolism (in a manner known to induce IR) and whether lipid overflow *per se* (or IR) alters AA metabolism to drive loss of muscle mass.

The study presented in this chapter addresses these questions by aiming to determine the effect of lipid availability on the development of inactivity-induced insulin and anabolic resistance. This was assessed by applying the arterialised venous-deep venous (AV-V) forearm balance method prior to and following 2 and 7 days of forearm immobilisation in young healthy males.

Dietary intake was controlled during immobilisation and participants consumed a eucaloric control diet or hypercaloric high-fat diet (providing an additional 50 % energy from fat) to better understand the role of lipid overflow *per se* on the development of insulin and anabolic resistance during disuse. A local model of inactivity (forearm immobilisation) was used as it induces muscle disuse in an isolated limb whilst only moderately impacting daily living (less invasive than leg immobilisation) and experimental test days were conducted after 2 and 7 days of immobilisation to establish whether the substantial IR seen following 7 days of disuse (Dirks *et al.*, 2016) is already present after 2 days. It was hypothesised that the 2.5 fold increase in dietary fat (during HFD) would exacerbate the early development of insulin resistance by diminishing insulin-stimulated glucose uptake following mixed meal drink consumption and immobilisation would impair AA balance across the forearm in a manner that was also accelerated by high-fat overfeeding.

# Results

Thirteen healthy young males (age 25 ± 2 years) were subjected to 7 days of forearm immobilisation under one of two dietary interventions, controlled eucaloric diet (CON) or hypercaloric diet providing 50 % excess energy from fat (HFD). Eligible participants underwent a baseline visit where the AV-V sampling method was employed alongside measures of blood flow to access substrate utilisation prior to intervention. Subjects returned to the lab for a casting visit where forearm immobilisation and dietary intervention were initiated. After 2 and 7 days of immobilisation, participants returned to the lab for experimental days. Detailed study schematics, test day schematics and participant characteristics before the start of the study can be found under Methods (2.3).

## 4.1 Dietary intervention

The two experimental groups did not differ in any of the participant characteristics prior to the start of the study. Habitual dietary intake was not different between participants in eucaloric diet (CON; n=8) or high-fat diet (HFD; n=5) groups (Figure 4.1A; P>0.5 for all parameters). Compared to habitual diet, controlled diets during immobilisation both showed lower protein and higher CHO content (in percentage energy, % en, for both, P<0.01 and $g \cdot d^{-1}$ CHO for HFD P≤0.01). Caloric intake in HFD was 1.5 fold higher than in CON (P<0.001), which was primarily attributed to a 2.5 fold increase in fat content ($g \cdot d^{-1}$; P<0.001), particularly saturated fat (3.6 fold higher in HFD compared to CON; P<0.001). During immobilisation dietary protein content was matched between groups (1.2 ± 0.0 $g \cdot KgBW \cdot d^{-1}$; P>0.05) so that protein intake did not differ between diets. To maintain the target macronutrient composition of the diets, the volume of the mixed meal drink (Ensure Plus, Abbott Nutrition) taken at t= 0 on baseline, day 2 and day 7 was adjusted so that 0.2 g fat, 0.3 g protein and 1.1 g CHO ($g \cdot KgBW \cdot d^{-1}$) were ingested in both groups. Participants in the CON diet maintained body mass throughout the immobilisation period, whereas this significantly increased in the HFD group (1.1 Kg ± 0.45 for day 7 against baseline; P<0.05; Figure 4.1B) suggesting that individuals in CON groups maintained energy balance whereas individuals in the HFD groups were on positive energy balance, as expected.

**A**

| | CON (n=8) | | HFD (n=5) | |
|---|---|---|---|---|
| | **Habitual** | **Immobilisation** | **Habitual** | **Immobilisation** |
| **Energy (Kcal)** | 2782 ± 228.8 | 2835 ± 103.2 | 2572 ± 371.8 * | 4207 ± 138.4 # |
| **Protein ($g \cdot kgBW^{-1} \cdot d^{-1}$)** | 2 ± 0.2 | 1.2 ± 0.0 | 2 ± 0.3 | 1.2 ± 0.0 |
| **Protein ($g \cdot d^{-1}$)** | 117 ± 15.7 | 90.0 ± 7.0 | 111 ± 22.0 | 81.7 ± 1.6 |
| **Carbohydrates ($g \cdot d^{-1}$)** | 317 ± 20.2 | 365.4 ± 12.3 | 268 ± 29.7 * | 369.6 ± 11.4 |
| **Fat ($g \cdot d^{-1}$)** | 107 ± 12.7 | 104.8 ± 4.1 | 116 ± 20.3 * | 258.8 ± 9.9 # |
| **Protein (En%)** | 16 ± 1.1* | 12.7 ± 0.7 | 17 ± 1.4 * | 7.8 ± 0.3 |
| **Carbohydrate (En%)** | 46 ± 1.7* | 51.8 ± 0.7 | 43 ± 1.7 * | 35.2 ± 0.1 |
| **Fat (En%)** | 35 ± 1.9 | 33.4 ± 0.7 | 40 ± 1.3 * | 55.4 ± 0.4 |

**B**

| | CON (n=8) | HFD (n=5) |
|---|---|---|
| **Baseline** | 74.3 ± 5.5 | 67.7 ± 1.55 |
| **Day 2** | 74.0 ± 5.48 | 67.9 ± 1.53 |
| **Day 7** | 73.8 ± 5.4 | 68.8 ± 1.44 |
| **Day 7 – Baseline** | -0.5 ± 0.47 | 1.09 ± 0.45 |
| **P-value** | 0.33 | 0.04 |

**Figure 4.1 Dietary intake and body weight prior to and during 7 day immobilisation**

**A)** Dietary intake prior to and during 7 day immobilisation period for young healthy male volunteers (n=13) allocated to eucaloric controlled diet (CON; n=8) or high-fat diet providing 50 % excess energy from fat (HFD; n=5). Parameters of habitual food intake were taken from self-reported 3-day food diaries prior to the start of the study and controlled diet during immobilisation was calculated and provided by the research team. **P<0.05 for habitual diet relative to controlled diet; #P<0.05 for CON against HFD during immobilisation (unpaired t-test). **B)** Body weight (Kg) prior to and after 2 and 7 days of immobilisation. No change in CON groups after 7 days (n=5; P>0.05) but significant increase in HFD group after 7 days (n=5; P<0.05) assessed using unpaired t-tests between day 7 and baseline.

### 4.2 Forearm muscle function is impaired following 7 days of immobilisation

Forearm muscle strength was assessed using a hand-held dynamometer prior to and after 7 days of immobilisation and represented as percentage change in strength (Kg) from pre-immobilised period. Muscle strength was comparable at baseline between CON and HFD groups and no changes were observed in the non-immobilised arm after immobilisation. In the casted arm, immobilisation decreased strength by 17.4 ± 3.4 % and 17.5 ± 2.1 % in CON and HFD groups, respectively, demonstrating an effect of disuse on function which was not exacerbated by HFD ($P_{(day)}<0.001$, $P_{(intervention)}>0.05$; Figure 4.2).

### 4.3 Glucose balance across the forearm is altered by immobilisation but not exacerbated by HFD

Arterialised plasma glucose levels were measured prior to and after consumption of a mixed meal drink (at t=0) before and after 2 and 7 days of immobilisation (Figure 4.3A,B). No differences were observed in fasting glucose levels across immobilisation for either CON or HFD groups ($P>0.05$) where fasting arterialised venous glucose in CON was 4.4 ± 0.06 mmol/L at baseline, 4.2 ± 0.16 mmol/L on day 2 and 4.38 ± 0.1 mmol/L on day 7 and in HFD was 4.4 ± 0.15 mmol/L at baseline, 4.6 ± 0.19 mmol/L on day 2 and 4.5 ± 0.19 mmol/L on day 7. Mixed meal drink consumption significantly increased arterialised venous plasma glucose in both CON (n=8; Figure 4.3A) and HFD (n=5; Figure 4.3B) ($P_{(time)}<0.001$ for both) at all days. In CON group significant changes from fasting glucose levels were seen in the first 120 minutes at baseline (peaked at 40 minutes 6.6 ± 0.2 mmol/L), between 20-60 minutes at day 2 (peak at 40 minutes 6.6 ± 0.2 mmol/L) and between 40-140 minutes at day 7 (peak at 40 minutes 6.1 ± 0.2 mmol/L), with levels returning to fasting concentrations at baseline and day 2 but remaining elevated at day 7 ($P<0.05$ for 180 against 0 on day 7; Figure 4.3A). In HFD group no significant changes from fasting concentrations were observed at baseline (peaked between 40-60 minutes 5.9 ± 0.4 mmol/L) and day 2 (peaked between 40-60 minutes at 5.4 ± 0.3 mmol/L) ($P>0.05$) with the time effect being attributed to a significant increase in glucose after 20 minutes at day 7 (peak at 20 minutes 5.8 ± 0.3 mmol/L) ($P<0.05$; Figure 4.3B). No changes were observed in total area under the curve indicating that the same total amount of glucose was available across all days (insets Figure 4.3A-B).

The difference between arterialised venous and venous blood glucose was assessed to infer on glucose balance across the forearm during immobilisation. Combined analysis of CON and HFD groups at baseline and day 7 demonstrated that mixed meal drink consumption increased AV-V glucose balance ($P_{(time)}$<0.001), whereas this effect was attenuated by immobilisation ($P_{(day)}$<0.01) demonstrating a time*day interaction ($P_{(interaction)}$<0.001). Fasting AV-V difference was not altered by immobilisation or diet (average fasting AV-V in CON across all days = 0.09 ± 0.03 compared to HFD = 0.17 ± 0.03). Postprandial AV-V difference decreased during immobilisation ($P_{(day)}$<0.05) but no effect of diet was observed. In the CON group, postprandial AV-V difference peaked at 40 minutes in baseline (0.88 ± 0.2 for 40 minutes compared to fasting 0.15 ± 0.07 $mmolL^{-1}$; P>0.05 baseline) and day 2 (0.5 ± 0.08 for 40 minutes compared to fasting 0.04 ± 0.01 $mmol^{-1}$ ; P<0.01) but this increase was not observed after 7 days (0.3 ± 0.2 $mmolL^{-1}$ at 40 minutes compared to 0.1 ± 0.04 $mmolL^{-1}$ at fasting; P>0.05; Figure 4.3C). This is also reflected when taking into account total postprandial AV-V glucose balance calculated as the area under the curve for the 180 minute postprandial period, which demonstrated a 67.28 % decrease after 2 days (P<0.01) and additional 12 % decrease after 7 days of immobilisation (P<0.05 for day 2 and 7 vs baseline; P>0.05 day 7 vs day 2; Figure 4.3E). In the HFD group there was a significant increase in AV-V balance after 60 minutes at baseline (1.1 ± 0.1 at 60 minutes compared to 0.2 ±0.06 $mmolL^{-1}$ at fasting; P≤0.01) and after 40 minutes on day 2 (0.5 ± 0.04 at 40 minutes compared to 0.13 ± 0.05 at fasting; P<0.05) but no differences were seen at day 7 (P>0.05) (Figure 4.3D). Across the postprandial period, total AV-V glucose balance decreased by 56.6 % after 2 days (P<0.05 against baseline) and further 12% between days 2 and 7 (P>0.05 compared to day 2; Figure 4.3E). These data suggest that the balance of glucose across the forearm was impaired after 2 days of immobilisation irrespective of consumption of a eucaloric or HFD, and this was not significantly aggravated by an additional 5 days of disuse.

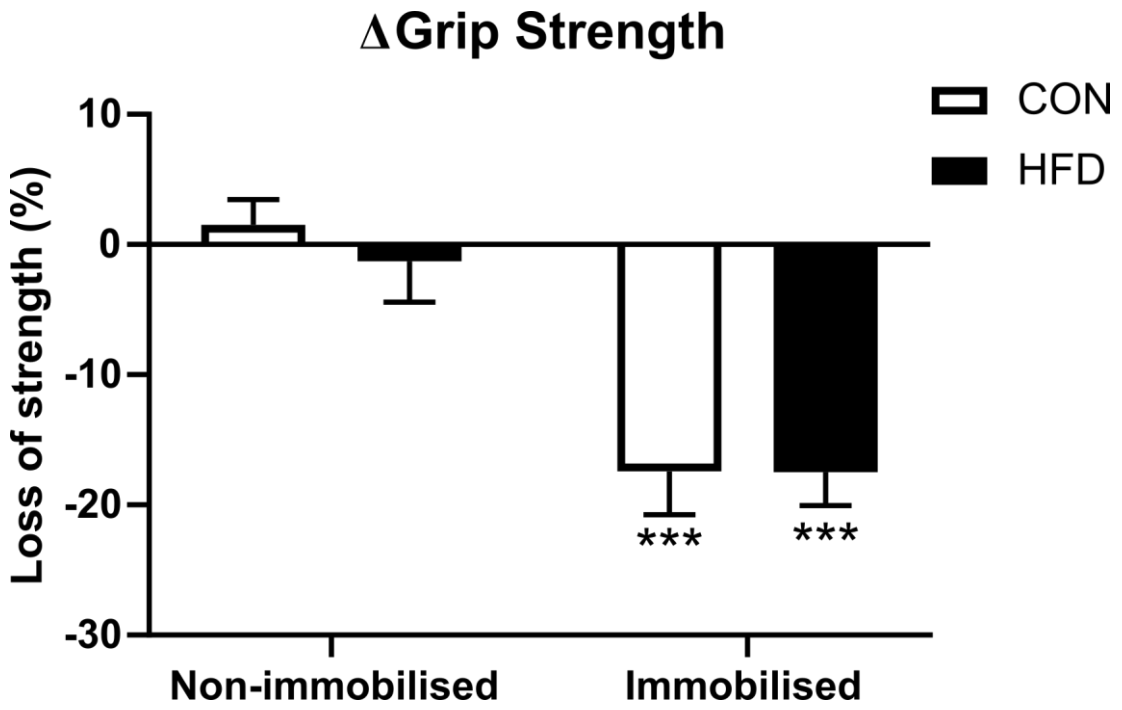



**Figure 4.2 Functional impairment following immobilisation is not exacerbated by HFD**

Mean (± SEM) percentage loss in grip strength following 7 days of forearm immobilisation in healthy, young males in either eucaloric controlled diet (CON; n=8) or high fat diet (HFD; n=5) groups. Data analysed as percentage change from pre-immobilisation for both immobilised and non-immobilised forearms with two-way ANOVA (time x intervention) showing significant immobilisation effect (***P<0.001) but no difference between interventions (P>0.05).

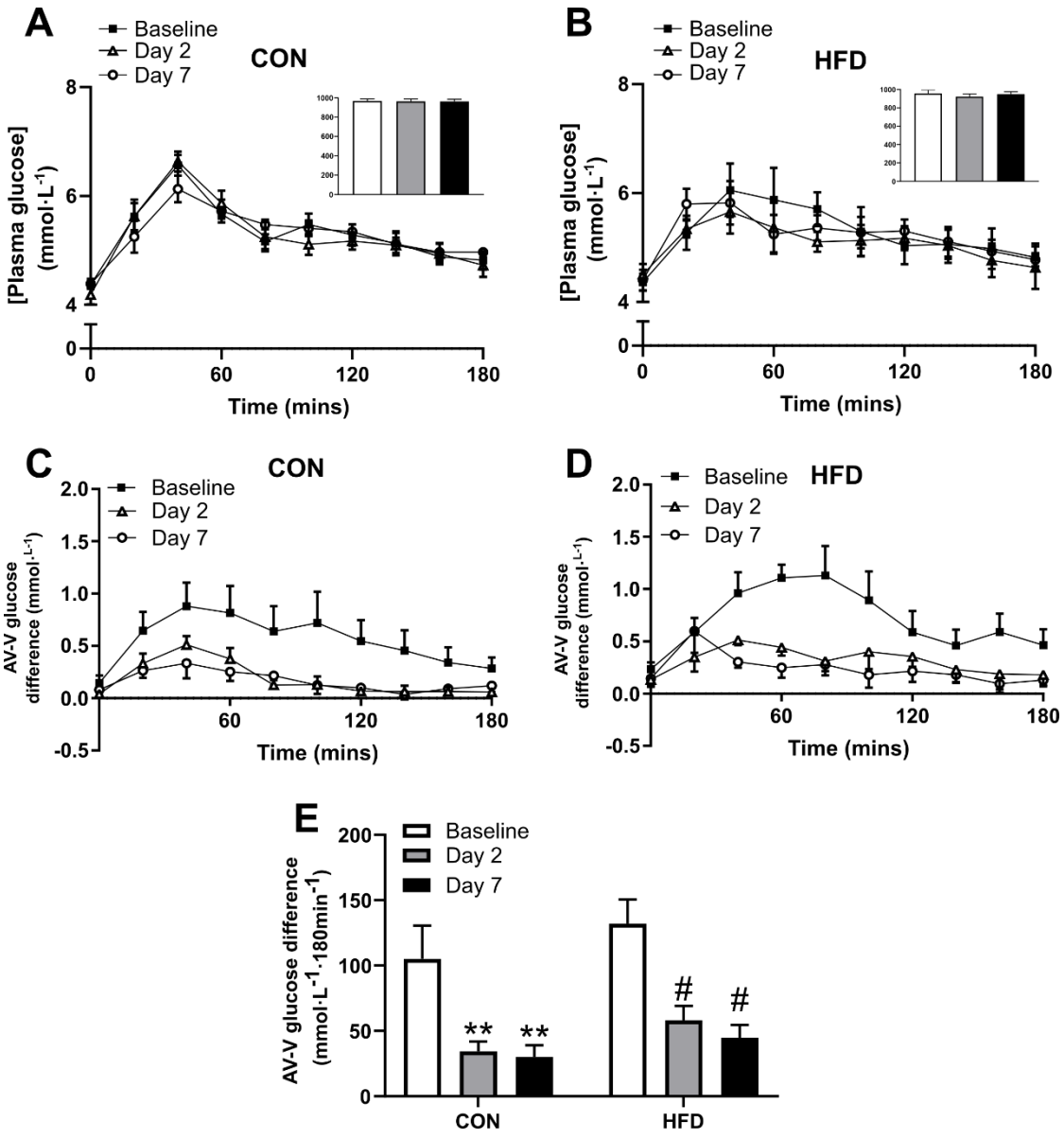



**Figure 4.3 Arterialised-venous deep-venous forearm glucose balance is impaired following immobilisation but not exacerbated by HFD**

Arterialised venous glucose concentration at baseline and following 2 and 7 days of immobilisation in healthy young males fed a controlled eucaloric diet (CON; n=8) **(A)** or a high fat diet providing 50 % excess energy from fat (HFD; n=5) **(B)** assessed during 180 minutes after consumption of a mixed meal drink (t=0). Significant time effect in CON and HFD ($P_{(time)}$<0.001) with no change caused by immobilisation ($P_{(day)}$>0.05), two-way repeated measures ANOVA with Bonferroni's multiple comparisons. Insets in A and B represent total area under the curve (AUC) for the 180 minute postprandial period. Arterialised-venous deep-venous (AV-V) forearm glucose balance at baseline and following 2 and 7 days of immobilisation for subjects in CON **(C)** or HFD **(D)** groups and representative AUC for the 180 minute postprandial in **E**. Significant effect of drink consumption and immobilisation in CON ($P_{(time)}$<0.01, $P_{(day)}$<0.05) and HFD ($P_{(time)}$<0.01, $P_{(day)}$<0.05) with two way ANOVA with repeated measures. **E)** AUC was significantly lower at day 2 and day 7 compared to baseline in CON (**P<0.01) and day 2 and day 7 compared to baseline in HFD (#P<0.05); two-way ANOVA with Bonferroni's multiple comparisons.

### 4.4 Forearm immobilisation for 7 days does not alter brachial artery blood flow under eucaloric or HFD.

To achieve a true measure of net glucose balance, the Fick's Principle was applied (Methods 2.4.7.1). Brachial artery assessments were made prior to and during 180 minute postprandial period following mixed meal drink consumption before and after 2 and 7 days of forearm immobilisation (Figure 4.4). Immobilisation did not alter brachial artery area (Figure 4.4A,B), velocity (Figure 4.4C,D) or blood flow (Figure 4.4E,F), calculated as the product between area and velocity, in CON (A,C,E) or HFD (B,C,D) groups ($P_{(day)}>0.05$ for all parameters). In CON group, a time effect was observed in area (A), velocity (C) and blood flow (E) ($P_{(time)}<0.05$) but no differences from fasting levels were detected with multiple comparisons. The same was the case for velocity (D) and blood flow (F) in HFD group but no time effect was observed on arterial area (B) ($P_{(time)}>0.05$). There was a trend towards higher blood flow in the HFD group across immobilisation ($P_{(day)}=0.08$; Figure 4.4F).

### 4.5 Forearm glucose uptake is impaired following immobilisation but not exacerbated by HFD

Forearm glucose uptake (FGU) was calculated as the product of the AV-V glucose balance (Figure 4.3C,D) and brachial blood flow (Figure 4.4E,F). In CON group, as blood flow was unchanged during immobilisation, FGU closely resembles AV-V balance (Figure 4.5A). Significant time ($P_{(time)}<0.01$) and immobilisation ($P_{(day)}<0.05$) effects were observed in CON (Figure 4.5A). Across the 180 minute postprandial period, total glucose uptake calculated as total area under the curve was reduced by 57.5 % after 2 days and by a further 43 % by day 7 in CON ($P<0.05$ and $P<0.01$ for day 2 and 7 vs baseline, respectively; Figure 4.5C). In the HFD group, a significant time effect was seen ($P_{(time)}<0.05$) but the effect of immobilisation was not significant ($P_{(day)}>0.05$; Figure 4.5B). Total postprandial glucose uptake decreased by 38.5% after 2 days and an additional 15 % by day 7 in the HFD group (Figure 4.5C) but these effects did not reach statistical significance. No difference was observed between diets ($P>0.05$ HFD compared to CON; Figure 4.5C).

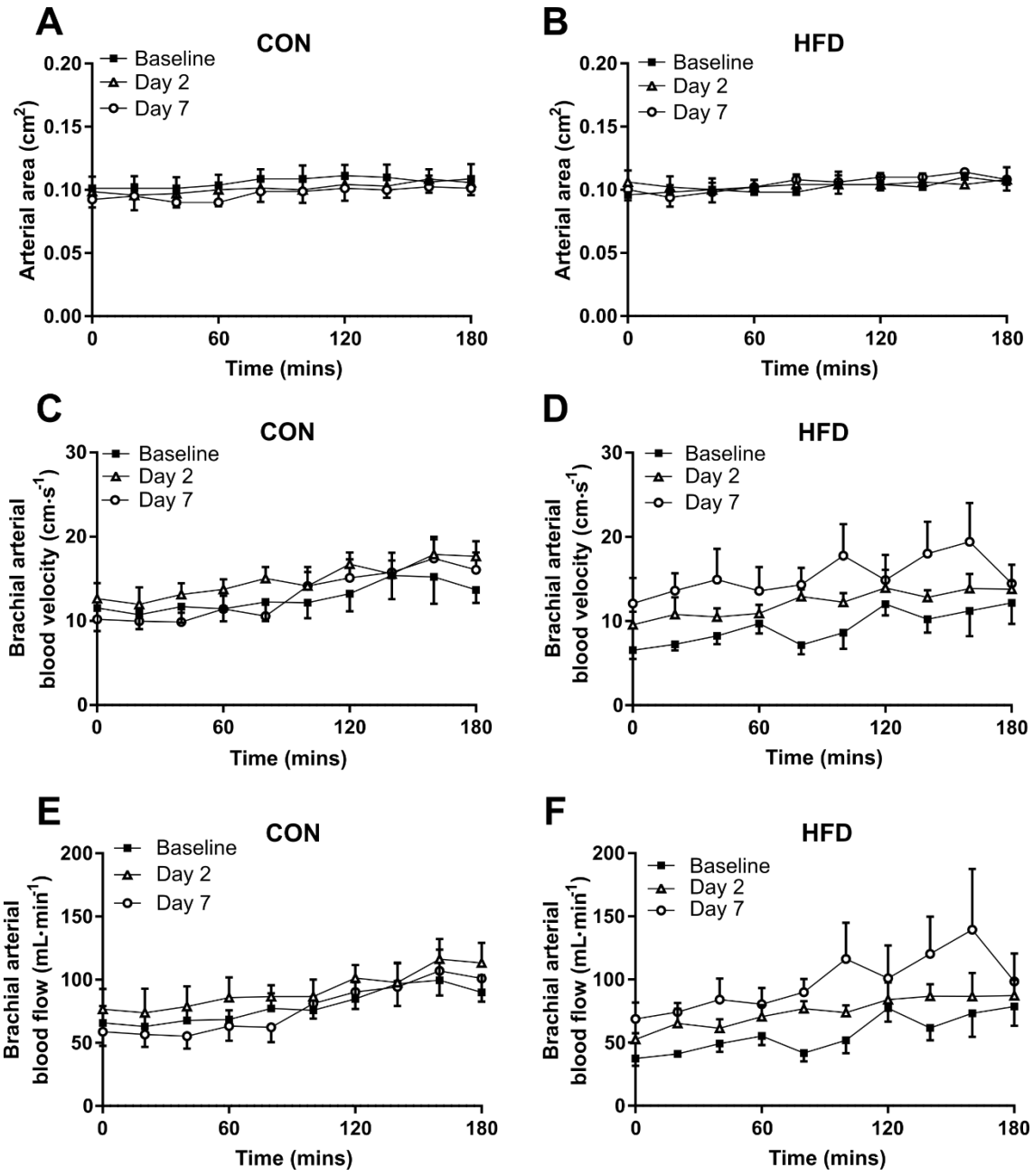



**Figure 4.4 Brachial arterial area, velocity and blood flow are not altered by immobilisation, irrespective of energy balance.**

Brachial artery area **(A, B),** blood velocity **(C,D)** and blood flow **(E,F)** following mixed meal drink consumption (at t=0) prior to and following 2 and 7 days of forearm immobilisation in young healthy males (n=13), measured using Doppler Ultrasound. During immobilisation participants were subjected to eucaloric controlled diet (CON; n=8) **(A,C,E)** or high-fat diet providing 50 % excess energy from fat (HFD; n=5) **(B,D,F)**. All parameters except HFD arterial area **(B)** showed a time effect ($P_{(time)}<0.05$) but no interaction with intervention by two-way ANOVA with repeated measures.

## 4.6 Serum insulin concentrations and calculated whole body insulin sensitivity are not altered by forearm immobilisation under eucaloric or HFD

Insulin was measured in serum from arterialised blood samples prior to and post immobilisation. Mixed meal drink consumption significantly increased insulin concentrations in both CON ($P_{(time)}<0.001$; Figure 4.6A) and HFD ($P_{(time)}<0.001$; Figure 4.6B) groups but no effect of immobilisation or diet were observed ($P_{(day)}>0.05$ and $P_{(intervention)}>0.05$ for both; Figure 4.6A,B). Total serum insulin across the postprandial period was not altered by immobilisation or diet ($P>0.05$ for day and intervention; Figure 4.6C). Matsuda index was calculated to infer whole-body insulin sensitivity, reflecting rate of glucose disposal into insulin sensitive tissues, primarily SM (Defronzo, 1999) and the homeostatic model assessment of insulin resistance (HOMA-IR) was used as a proxy for IR (primarily hepatic) and β-cell function (Matthews *et al.*, 1985). Matsuda (Figure 4.6D) and HOMA-IR (Figure 4.6E) indices did not reveal a significant effect of immobilisation or dietary intervention on insulin sensitivity ($P_{(day)}>0.05$ and $P_{(intervention)}>0.05$).

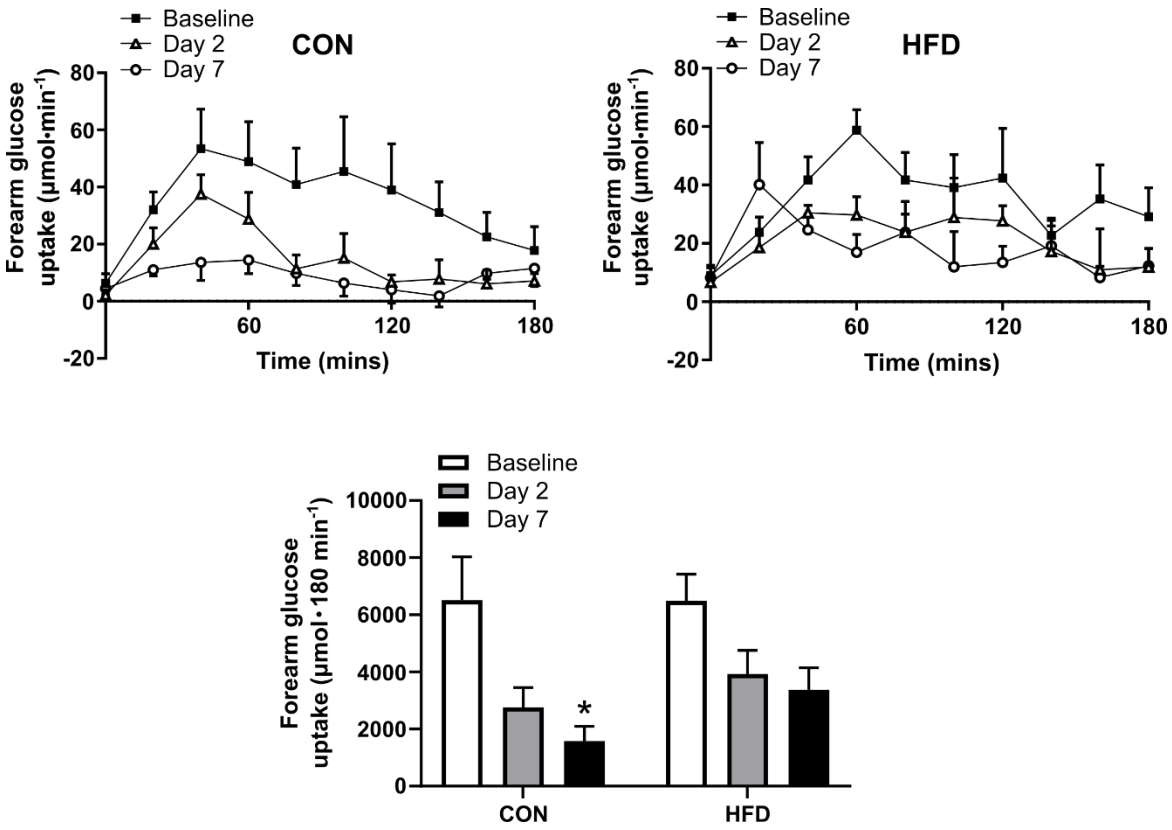



**Figure 4.5 Forearm glucose uptake is impaired following immobilisation but not exacerbated by HFD**

Forearm glucose uptake (FGU) at baseline and following 2 and 7 days of forearm immobilisation from young healthy males fed an eucaloric controlled diet (CON; n=8) **(A,C)** or high fat diet providing 50 % excess energy from fat (HFD; n=5) **(B,C)**. FGU was calculated as the product of the AV-V difference in glucose and brachial arterial blood flow. **C)** Total forearm glucose uptake calculated as area under the curve from A and B over the 180 minute postprandial period. Significant effect of immobilisation ($P_{(day)}<0.05$) but diet ($P_{(intervention)}>0.05$ for total FGU. *$P<0.05$ against baseline in CON.

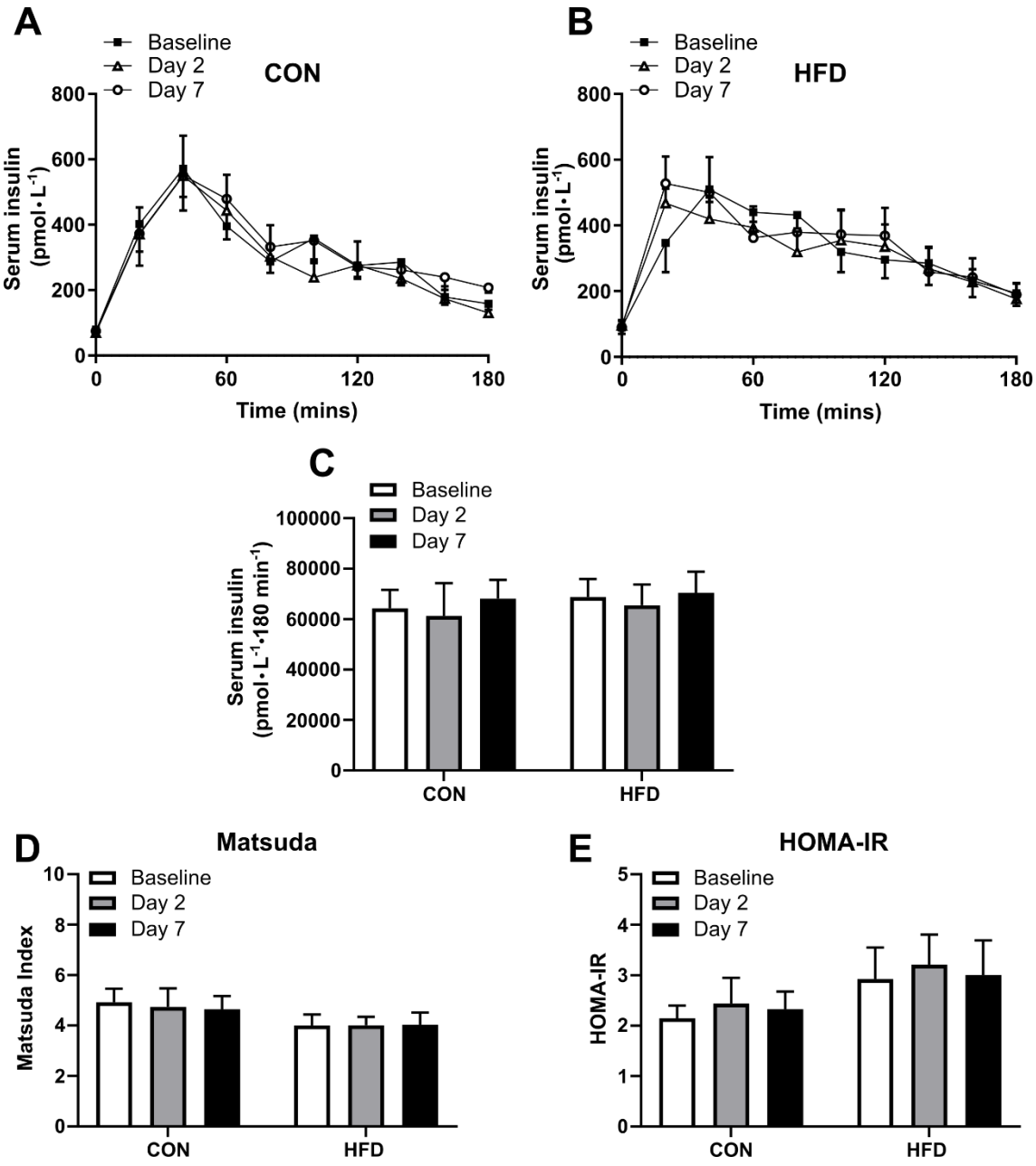



**Figure 4.6 Insulin levels and insulin sensitivity are not altered by forearm immobilisation under eucaloric or HFD**

Arterialised-venous (AV) serum insulin concentrations at baseline and following 2 and 7 days of forearm immobilisation in healthy young males fed a eucaloric control diet (CON; n=8; **A**) or a high-fat diet providing 50% excess energy from fat (HFD; n=5; **B**). **C)** Total area under the curve (AUC) for the 180 minute postprandial period following ingestion of a mixed meal drink in CON and HFD groups. No effect of immobilisation or diet on insulin concentrations ($P_{(day)}>0.05$, $P_{(intervention)}>0.05$) with two-way ANOVA with Bonferroni's multiple comparisons. **D)** Matsuda index **E)** Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). No differences in insulin sensitivity indices.

### 4.7 Immobilisation did not alter leucine and phenylalanine availability under eucaloric or HFD

Leucine and phenylalanine concentrations were measured in arterialised-venous and venous plasma samples before and after immobilisation in CON and HFD groups. Mixed meal drink consumption significantly altered the levels of arterialised plasma leucine in CON ($P_{(time)}<0.001$; n=8; Figure 4.7A) and HFD ($P_{(time)}<0.05$; n=5; Figure 4.7B) and phenylalanine in CON ($P_{(time)}<0.001$; n=8; Figure 4.7C) and HFD ($P_{(time)}<0.001$; n=5; Figure 4.7D). Neither immobilisation nor dietary intervention significantly altered arterialised leucine ($P_{(day)}>0.05$, $P_{(intervention)}>0.05$; Figure 4.7E) or phenylalanine ($P_{(day)}>0.05$, $P_{(intervention)}>0.05$; Figure 4.7E) concentrations. As blood flow was not altered by immobilisation or diet (Figure 4.4) these data indicate that AA delivery into the forearm was not impaired by immobilisation.

### 4.8 Net amino acid balance is altered by immobilisation and HFD

Impairments in AA uptake may be a driving mechanism for anabolic resistance. The net balance of leucine and phenylalanine were therefore measured to assess the impact of immobilisation and high-fat overfeeding on AA uptake. Leucine balance across the forearm was calculated by normalising the AV-V difference in AA concentration to blood flow in the fasting period and in the first 60 minutes following mixed meal ingestion. An effect of immobilisation and diet was seen in fasting net leucine balance ($P_{(day)}<0.05$, $P_{(intervention)}<0.05$) which was attributed to a significantly lower net balance in HFD compared to CON after immobilisation ($P<0.05$ for day 7 between HFD and CON; Figure 4.8A). The negative fasting net leucine balance seen before immobilisation (Figure 4.8A) was acutely reversed by mixed meal drink consumption in both CON and HFD (Figure 4.8B). Mixed meal drink consumption did not revert negative fasting balance after immobilisation in CON or HFD groups. Positive net balance seen at baseline in the postprandial period was significantly altered by immobilisation ($P_{(day)}\leq0.01$). This was attributed to a significant difference in leucine balance after immobilisation in CON group ($P<0.05$ for day 7 vs baseline in CON; n=8; Figure 4.8B).

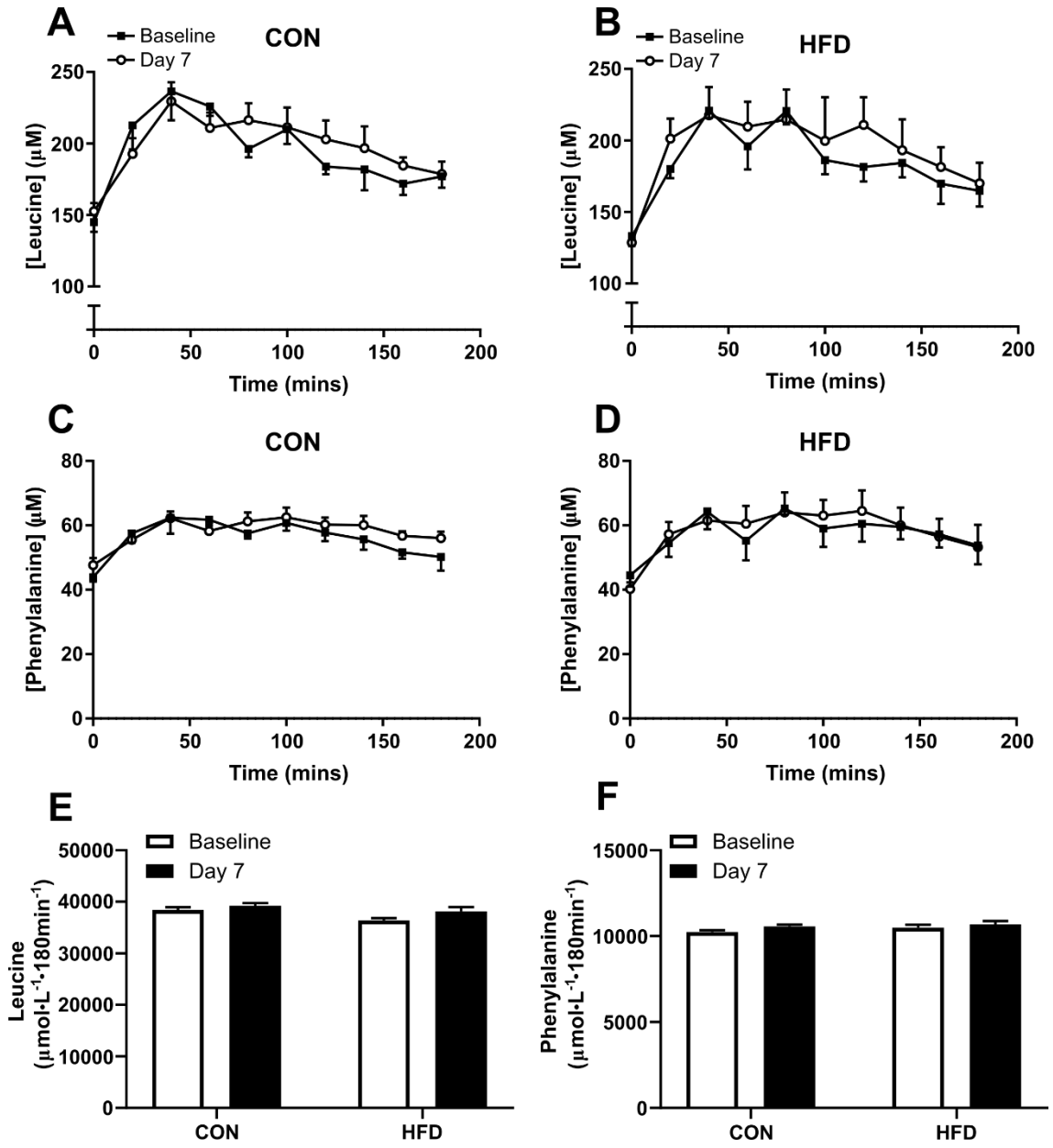



**Figure 4.7 Immobilisation did not alter leucine and phenylalanine availability under eucaloric or HFD**

Arterialised-venous (AV) plasma leucine concentrations at baseline and following 2 and 7 days of forearm immobilisation in healthy young males fed a eucaloric control diet (CON; n=8; **A**) or a high-fat diet providing 50% excess energy from fat (HFD; n=4; **B**) and phenylalanine in CON **(C)** and HFD **(D)**. Total area under the curve (AUC) in the 180 minute postprandial period following ingestion of a mixed meal drink in CON and HFD groups before and after immobilisation for leucine **(E)** and phenylalanine **(F)**. No effect of immobilisation or diet on amino acid concentrations ($P_{(day)}$>0.05, $P_{(intervention)}$>0.05) with two-way ANOVA with Bonferroni's multiple comparisons.

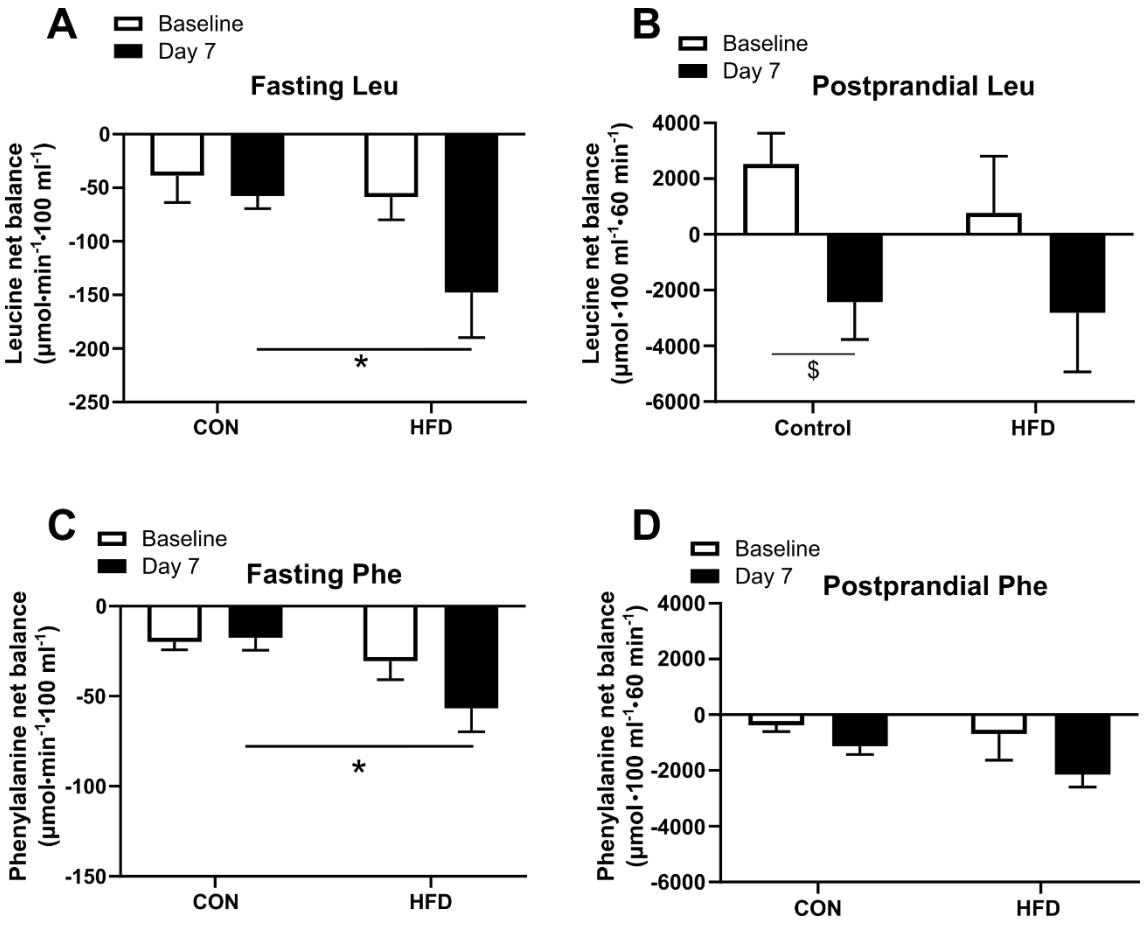



**Figure 4.8 Net amino acid balance is altered by immobilisation and HFD**

Net balance of leucine (Leu) calculated as the product of AV-V difference in plasma leucine concentration and blood flow in the fasting period (**A)** and calculated as total area under the curve for the first 60 minutes following ingestion of mixed meal drink **(B)** prior to (baseline) and after 7 days of forearm immobilisation in young healthy males fed a eucaloric controlled diet (CON; n=8) or a high fat diet providing 50 % excess energy from fat (HFD; n=4). **C)** Fasting net balance in plasma phenylalanine (Phe) levels before and after immobilisation in CON and HFD groups. **D)** Net balance of phenylalanine calculated as area under the curve for the first 60 minutes following mixed meal drink ingestion before and after immobilisation in CON and HFD group. Significantly immobilisation and diet effect in fasting leucine balance ($P_{(day)}<0.05$, $P_{(intervention)}<0.05$), diet effect in fasting Phe ($P_{(intervention)}<0.05$), and immobilisation in postprandial net balance of Leu and Phe ($P_{(day)}<0.05$) with two-way ANOVA with Bonferroni's multiple comparisons. *P<0.05 for HFD against CON, $P<0.05 for day 7 against baseline.

# Discussion

It is well-established that excessive lipid availability brings about IR in SM cells (*in vitro)*, acting via mechanisms described in the main introduction to this thesis and discussed in Chapter 3. In Chapter 3, treatment of skeletal muscle cells with high concentrations of palmitate led to loss of anabolic sensitivity, demonstrated as blunted activation of intracellular signalling proteins in the insulin/PKB and mTOR/S6K1 intracellular signalling pathways in response to acute treatment with insulin and mixed EAAs. These data suggest that lipid overflow induces insulin and anabolic resistance by impairing intracellular signalling. However, it is not known whether these lipid-induced intracellular impairments arise as a consequence of reduced substrate (glucose, AAs) delivery *in vivo* nor whether loss of the anabolic effect of insulin (in IR conditions) drives anabolic resistance.

To study insulin and anabolic resistance in humans, a model of SM disuse was used here, as physical inactivity (modelled by bed rest and limb immobilisation disuse models) induces insulin (Bergouignan *et al.*, 2011) and anabolic resistance (Symons *et al.*, 2009; Breen *et al.*, 2013; Tanner *et al.*, 2015) and causes muscle atrophy. Furthermore, it has been shown in experimental models of disuse that prolonged (> 4 weeks) bed rest or limb suspension causes substantial IMCL accumulation, ranging from 15 % to 75 % increase in intramyocellular lipid content (Manini *et al.*, 2007; Cree *et al.*, 2010). As lipid infusion in humans directly induces insulin resistance (Kelley *et al.*, 1993; Stephens *et al.*, 2015), and muscle lipid accumulation is negatively correlated with insulin sensitivity, this has in the past been hypothesised to be a likely driver of disuse-induced IR. However, such overt accumulation of lipid is not apparent after 5-7 days of disuse (Wall *et al.*, 2015; Dirks *et al.*, 2016), suggesting that IMCL accumulation *per se* does not participate in the rapid development of IR. Independently of triglyceride accumulation, changes in DAG metabolism (Dirks *et al.*, 2016) and PKCθ gene expression have been observed in the first week of disuse (Dirks *et al.*, 2018), and DAG-PKC signalling directly contributes to the development of IR (Bosma *et al.*, 2012). It remains unknown, however, whether these changes drive insulin and anabolic resistance and whether a positive energy balance driven by fat overfeeding would exacerbate the effects seen by

disuse. By investigating the development of these disuse-induced metabolic impairments in energy balance and lipid overflow (by high fat overfeeding), the role of lipid *per se* can be investigated. As such, participants in this study were subjected to 7 days of forearm immobilisation and fed a controlled eucaloric diet or high-fat diet providing excess 50 % energy from fat during immobilisation.

Data in this chapter demonstrated that forearm immobilisation caused both functional and metabolic impairments to forearm muscle tissue. Grip strength, used as a functional output, decreased ~17 % after 7 days of immobilisation, irrespective of energy balance. The magnitude of this change is equivalent to that seen after 21 days of forearm immobilisation (17-20 %) (Kitahara *et al.*, 2003; Miles *et al.*, 2005; Matsumura *et al.*, 2008), suggesting that functional decline is already present at 7 days and is not exacerbated by a longer period of immobilisation. To study *in vivo* muscle metabolism, the forearm AV-V balance technique was applied, which is a well-established method to measure glucose uptake across the forearm (Lipman *et al.*, 1970, 1972; Jackson *et al.*, 1987; Richter *et al.*, 1989) and other metabolites (Forster *et al.*, 1972; Abumrad *et al.*, 1981; Sonnenberg and Keller, 1982) and enables simultaneous sampling of arterialised venous and deep venous blood draining directly from the forearm muscle tissue (Jackson *et al.*, 1987).

In this study, it was demonstrated for the first time that disuse-induced muscle IR is evident after only two days of immobilisation, manifested by > 50 % decrease in insulin-stimulated glucose uptake to the forearm muscle bed, which was not aggravated by an additional 5 days of immobilisation. Contrary to the hypothesis, these effects were not altered by lipid overflow from HFD. In addition, it was demonstrated for the first time that immobilisation did not impair postprandial-mediated increase in blood flow. The effect of 7 days of immobilisation on glucose uptake seen here is in agreement with others demonstrating 30-40 % decrease in insulin sensitivity following one week of disuse (Mikines *et al.*, 1991; Alibegovic *et al.*, 2009; Dirks *et al.*, 2016; Dirks *et al.*, 2019). Work by Dirks and colleagues suggests that disuse-induced IR occurs between day 1 and 3 of muscle disuse, and plateaus thereafter. One day of bed rest is not sufficient to significantly affect whole-body insulin sensitivity, irrespective of energy balance (Dirks *et al.*, 2018),

which is likely a protective mechanism to resist the deleterious metabolic effects of short periods of physical inactivity, such as during sleep. However, by day 3 of bed rest, insulin sensitivity diminishes (Yanagibori *et al.*, 1994) and after 5-7 days glucose disposal declines by ~ 30 %, assessed during hyperinsulinaemic-euglycaemic clamps (Dirks *et al.*, 2016). Here, the systemic effects of bed rest models are eliminated as forearm immobilisation induces muscle disuse in an isolated limb. As this was used in combination with the AV-V balance technique (sampling blood draining directly from the forearm muscle bed) these data suggest that removal of muscle contraction leads to a more rapid decline in muscle glucose uptake (compared to bed rest) likely due to an immediate loss of substrate requirement. This also expands on current knowledge by confirming earlier suggestions that bed-rest induced whole-body IR occurs primarily in SM (Stuart *et al.*, 1988) and supports the use of the forearm immobilisation model to quantify glucose uptake as a direct measure of muscle insulin sensitivity. It is intriguing that the additional 5 days of disuse here did not cause more substantial impairments, particularly as muscle loss with immobilisation is substantially decreased at the earlier stages but continues at a reduced rate for an extended period of time (Wall and van Loon, 2013). It is plausible that motor unit recruitment is diminished early on due to reconditioning and that this precedes atrophy (Kawakami *et al.*, 2001; Deschenes *et al.*, 2002; de Boer *et al.*, 2007; Farthing *et al.,* 2009) or that changes to intracellular delivery/signalling occur early on and become rate-limiting so that no further aggravation is seen. Understanding the time course of development of these impairments is critical, however, as the first few days bring about the greatest loss of muscle mass and strength (primarily driven by anabolic resistance) (White, Davies and Brooksby, 1984; LeBlanc *et al.*, 1992; Ferrando *et al.*, 1995; de Boer *et al.*, 2007).

Blood flow and arterialised glucose concentration measured here were not altered by immobilisation, suggesting that the haemodynamic effect of insulin in the postprandial period to increase blood flow (ie. substrate delivery) to insulin sensitive tissues was not altered (Laakso *et al.*, 1990). However, insulin also controls microvascular perfusion and recruitment so it is possible that muscle perfusion is altered despite the unaltered arterial blood flow and glucose availability.

For example, insulin elicits its effects on muscle protein synthesis partly via endothelial-dependent vasodilation which enhances nutritive flow and mTORC1 signalling (Timmerman *et al.*, 2010) so it is possible that IR is seen at the endothelial level to impair glucose delivery. It is also plausible that impaired glucose uptake arises from blunted glucose transport into muscle, as others have demonstrated that 7 days of bed rest, which resulted in a 22 % decline in insulin sensitivity, reduced muscle GLUT4, HK and PKB expression and blunted insulin-induced PKB and glycogen synthase activation (Biens*ø et al.*, 2012). It would be important to establish whether these changes are already present after 2 days of immobilisation by applying the forearm cast model combined with muscle biopsies.

Despite the significant increase in dietary fat intake in the high-fat overfed group (~260 g fat per day compared to 100 g per day in CON), disuse-induced decline in glucose uptake was comparable between diets. This was corroborated by comparable HOMA-IR values between dietary interventions and no effect of HFD on Matsuda index, as a proxy for peripheral insulin sensitivity. These data suggest that, contrary to the hypothesis, high-fat overfeeding did not impact insulin sensitivity of the immobilised muscle tissue or systemically. This effect is unlikely to originate from lack of adherence to the diet from participants as the ~1 Kg body mass gain during HFD is in line with previous observations (Parry *et al.*, 2017). Although most reports indicate that high-fat overfeeding induces IR after 3-7 days (Wulan *et al.,* 2014; Hulston *et al.,* 2015; Lundsgaard *et al.*, 2017), others have demonstrated no effect of HFD on insulin sensitivity (Adochio *et al.*, 2009; Brøns *et al.*, 2009). The contribution of dietary fat to total energy intake in the hypercaloric diet given here (55 % en fat) is fractionally lower than fat contents of diets used previously (average ~ 65 %) (Bachmann *et al.*, 2001; Hulston *et al.,* 2015; Lundsgaard *et al.*, 2017; Parry *et al.*, 2017), which may contribute to the lack of HFD-induced IR seen here. In addition, protein and CHO total dietary content were matched between HFD and CON groups here to prevent metabolic impairments arising from other macronutrient changes during disuse. This may contribute to the discrepancy with other studies, for example, demonstrating decreased whole-body and leg glucose disposal following 3 days of increased fat (75 % en fat) but not CHO availability (Lundsgaard *et al.*, 2017) and increased

markers of IR such as reduced CHO oxidation and increased FFAs after HFD (75 % fat) compared to isocaloric diet (Constantin-Teodosiu *et al.*, 2012).

In addition to impaired glucose homeostasis, short-term disuse (5-7 days) leads to rapid development of muscle atrophy and loss of functional strength in both young (Wall *et al.*, 2014) and old individuals (Dirks *et al.*, 2014). For example, one-legged knee immobilisation for 5 days caused a ~1.4 % decline in muscle mass concomitant with ~9% decline in muscle strength (M. L. Dirks *et al.*, 2014). Atrophy developed over longer periods of disuse (> 10 days) is also associated with reduced functional capacity (Deitrick, 1948; Gibson *et al.*, 1987) and basal metabolic rate (Haruna *et al.*, 1994). Muscle mass is maintained by a balance between MPS and MPB and evidence suggests that disuse-induced atrophy primarily derives from impaired postprandial response to anabolic stimuli to promote MPS (anabolic resistance), as opposed to increased MPB rates (Gibson *et al.*, 1987; Phillips *et al.,* 2009). The mechanisms regulating the development of anabolic resistance remain poorly understood but contributing factors may include systemically impaired protein digestion (Ferrando *et al.*, 1996; Pennings, Koopman, *et al.*, 2011), AA absorption (Pennings, Boirie, *et al.*, 2011; Pennings, Koopman, *et al.*, 2011), and microvascular perfusion (Rasmussen *et al.*, 2006; Hamburg *et al.*, 2007; Timmerman *et al.*, 2010), and/or local impairments in AA uptake (Drummond *et al.*, 2010) or intracellular signalling (Cuthbertson *et al.*, 2005; Fry *et al.*, 2011). In addition, disuse impairs metabolic flexibility (Kelley and Mandarino, 2000) and leads to IMCL accumulation (Cree *et al.*, 2010; Bergouignan *et al.*, 2011) and given that anabolic resistance features in conditions marked by IR, such as T2D, ageing and disuse, it is possible that lipid-induced IR may contribute to the development of anabolic resistance. As such, AA metabolism was also investigated in this study to assess whether immobilisation would alter AA balance and the effect of high-fat overfeeding on this response.

Data in this chapter demonstrated that 7 days of forearm immobilisation did not alter arterialised concentration of the EAAs leucine and phenylalanine in the fasting or postprandial period. Mixed meal ingestion transiently increased the concentrations of these AAs, as expected, which along with increased circulating

insulin postprandially is sufficient for maximal protein anabolism (Greenhaff *et al.*, 2008; Moore *et al.*, 2009; Witard *et al.*, 2014). In addition, as blood flow was maintained throughout immobilisation, these data demonstrate for the first time that the delivery of leucine and phenylalanine to the forearm muscle bed was not impaired by immobilisation, regardless of energy balance. This is consistent with data demonstrating that the postprandial increase in plasma AA concentrations following 20 g bolus of dietary protein (meal like quantity) is not altered following 14 days of leg immobilisation (Wall, Snijders, *et al.*, 2013); and suggests that systemic digestion, absorption and AA availability in the muscle vicinity are unlikely to be the main mediator behind disuse-induced anabolic resistance.

As muscle tissue was not collected, it was not possible to measure intracellular signalling/processes/energy stores to gain a better insight into the mechanisms regulating AA metabolism during disuse. However, net balance of leucine and phenylalanine were measured before and after immobilisation in the postabsorptive and postprandial periods. The net balance approach has been developed to assess simultaneous rates (combined with tracers) of protein synthesis and breakdown across tissues and has made important contributions to the study of protein metabolism (Cheng *et al.*, 1985; Barrett *et al.*, 1987). In addition, the AV-V method has been validated as primarily reflecting SM metabolism across leg/forearm, with minor contribution from tissues such as fat and skin (Macdonald, 1999; van Hall *et al.*, 1999). Using deep-venous cannulae also minimises the contribution from non-muscle compartments to AV-V differences in AA concentrations (Macdonald, 1999; van Hall *et al.*, 1999). Leucine and phenylalanine are EAAs, i.e., they cannot be synthesised *de novo* so these AAs are only available from dietary AA intake (intestinal absorption of dietary AA) or endogenous protein breakdown. Therefore, measuring the balance of an AA that is minimally metabolised (transaminated or oxidised) in SM, such as phenylalanine (Van Hall *et al.,* 1999), provides a direct measure of the balance of synthesis and breakdown occurring across the forearm (in this study). In addition to phenylalanine, leucine net balance was assessed. The rate of leucine uptake corresponds to synthesis as well as oxidation (as leucine is transaminated in muscle) so net balance of leucine alone cannot be used to estimate rates of MPS.

However, leucine is a potent anabolic AA, as it alone can act to stimulate MPS (Atherton *et al.*, 2010) and increasing concentrations of leucine regulate the amplitude of the anabolic response to protein ingestion (Breen and Phillips, 2011). Therefore, a change in leucine net balance would have important functional implications for SM during disuse.

In this study, fasting (postabsorptive) leucine and phenylalanine balance were reduced after immobilisation during HFD but not controlled diet. During fasting, the rates of protein breakdown supersede those of synthesis to result in net muscle protein loss whereas in the postprandial response to food intake, increased AA availability (Tipton *et al.*, 1999), mainly leucine (Wall, Hamer, *et al.*, 2013), stimulates MPS (and inhibit breakdown) resulting in a positive net balance. The more negative fasting AA balance seen here after high-fat overfeeding may, therefore, be the result of reduced postabsorptive MPS or enhanced MPB. It is also possible that the moderate increase in blood flow seen during immobilisation in the HFD exacerbated this effect, as net balance takes into account the AV-V difference and blood flow. Immobilisation did not alter postabsorptive balance during controlled eucaloric diet. Some evidence suggests that postabsorptive MPS is impaired by immobilisation, manifested by ~40 % decrease (5 days single-leg immobilisation) (Wall *et al.*, 2016) and 27 % decrease (14 days single-leg immobilisation) in myofibrillar protein synthesis rate (Glover *et al.*, 2008) and by others in different experimental disuse models (Gibson *et al.*, 1987; Ferrando *et al.*, 1996; Paddon-Jones *et al.*, 2006; de Boer *et al.*, 2007). The decline in basal MPS appears to occur during the initial stages of disuse (de Boer *et al.*, 2007; Wall and van Loon, 2013). MPB is experimentally difficult to measure and therefore seldom reported (Symons *et al.*, 2009), so measurements of components of the proteolytic pathways are used as markers of breakdown. Such markers appear to also increase transiently in the early stages of disuse (Tesch *et al.*, 2008) and not change after 1-2 weeks (Abadi *et al.*, 2009). Although some changes to postabsorptive MPS/MPB have been documented, others have failed to see changes to postabsorptive MPS with disuse (bed rest) (Drummond *et al.*, 2012) so a better understanding is still required of the effect of disuse and lipid overflow on postabsorptive AA metabolism.

Leucine balance increased in response to mixed meal drink consumption before immobilisation, as expected, but it was demonstrated here for the first time that immobilisation impaired this postprandial response to protein/AA ingestion (from mixed meal drink consumption). It was also demonstrated for the first time that postprandial net leucine balance diminishes in the absence of atrophy (likely preceding and therefore driving atrophy) and is not substantially altered by HFD. Although this is not a direct measure of the rate of MPS or MPB, net balance provides a direct measure of the net change in synthesis (and oxidation) relative to breakdown, as a postprandial increase in leucine concentration (as seen here) drives MPS (Leenders and van Loon, 2011; van Loon, 2012), offsetting negative balance. Disuse-induced anabolic resistance has been demonstrated by others. For example, hyperaminoacidaemia-induced whole-body MPS rates are impaired following 14 days of bed rest (Biolo *et al.*, 2002, 2004); AA-induced MPS is delayed and attenuated after 14 days of single-leg immobilisation (in young males) (Glover *et al.*, 2008); and 5 days of single-leg immobilisation reduces postprandial MPS rates by 50 % (in response to 25 g protein) (Wall *et al.*, 2016). However, an effect of immobilisation on postprandial net leucine balance has not been reported, particularly in a forearm immobilisation model. Data in this chapter, therefore, provide important evidence to support the concept that the removal of contraction appears to represent a very potent stimulus that rapidly attenuates intracellular demand for glucose and highly anabolic AAs, to an extent that it is not altered by diet. The follow-up data from this study (e-publication ahead of print Dirks *et al.*, 2019), demonstrates that non-esterified fatty acid (NEFA) balance increases with disuse (likely driven by increased fatty acid uptake into muscle) irrespective of lipid overfeeding. It is therefore plausible that disuse maximises insulin and anabolic resistance (relatively rapidly following loss of contraction) via impaired lipid metabolism, independently of diet. Cell data from Chapter 3 (and by others discussed in Chapter 3) suggests that lipid overflow induces these metabolic impairments in muscle and, independently from disuse, lipid overflow has demonstrated detrimental effects on anabolic sensitivity. For example, increased lipid availability by acute intralipid infusion, not only decreases glucose disposal by 20 % but also prevents the 2.2-fold increase in mixed MPS rate in response to AA (21 g) ingestion (Stephens *et al.*, 2015).

Without a HFD non-immobilised control group in the present study, it is not possible to clarify whether HFD alone would alter insulin and anabolic resistance to the same extent, but in this study, the effect of disuse appears to supersede that of diet by causing substantial impairment to glucose and AA metabolism.

The systemic and cellular mechanisms that drive disuse-induced insulin and anabolic resistance or mediate lipid-induced anabolic resistance remain unclear. Given that, here, immobilisation did not alter arterialised concentrations of glucose and EAAs, nor blood flow, but balance of both glucose and leucine were significantly impaired by immobilisation, local mechanisms such as delivery to intracellular pools and intracellular signalling are most likely governing these impairments. For instance, AA uptake is partly regulated by expression of AA transporters such as LAT1, SNAT2, CD98 and PAT1, all of which become upregulated in response to EAA ingestion (Drummond *et al.*, 2010, 2011). The postprandial increase in expression of SNAT2 and LAT1 is blunted following 7 days of bed rest (Drummond *et al.*, 2012) but no change in mRNA expression is observed following 5 days of leg immobilisation (Wall *et al.*, 2016) leading to inconclusive evidence for an impairment in AA uptake following disuse. Protein and AA ingestion also upregulate mTOR pathway signalling (Glover *et al.*, 2010; Drummond *et al.*, 2012; Wall *et al.*, 2016) and immobilisation (5 days single-leg) significantly impairs postprandial activation of mTOR and 4E-BP1 (Wall *et al.*, 2016). Independently of disuse, lipid-induced anabolic resistance is associated with impaired postprandial 4E-BP1 activation and increased muscle LCFA-CoA and acylcarnitine content (Stephens *et al.*, 2015), which are markers of incomplete lipid oxidation known to be associated with the development of IR (Figure 1.6). It is also clear from data presented in Chapter 3 that lipid overflow impairs intracellular responses to insulin and AA treatment. It remains to be established, however, whether increased expression of lipotoxic intermediates and blunted activation of these intracellular signalling pathways is already seen after two days of forearm immobilisation. It would also be interesting to assess changes to AA balance in the first two days of disuse to assess the temporal differences between insulin resistance (in terms of loss of insulin-stimulated glucose uptake) and anabolic resistance.

If postprandial net balance of a potent anabolic AA such as leucine is not altered after two days, it is possible that insulin resistance precedes anabolic resistance and contributes to the loss of response to AAs. IR is a plausible driver of anabolic resistance as insulin directly enhances protein synthesis by activation of mTORC1 (Proud, 2002) and stimulates endothelial vasodilation by increasing capillary recruitment and microvascular volume, which increases nutritive flow to SM (Rasmussen *et al.*, 2006).

**Summary and future work**

Taking advantage of a minimally invasive methodology to study *in vivo* human muscle metabolism, this study demonstrated that short-term forearm immobilisation leads to rapid and severe muscle IR, leading to substantial impairment in insulin-stimulated glucose uptake after only two-days. As this was not exacerbated by further immobilisation or HFD, it is likely that removal of muscle contraction *per se* is the main contributor to disuse-induced IR. Furthermore, this study also demonstrated for the first time that postprandial balance of a potent anabolic AA such as leucine was decreased following 7 days of immobilisation, suggesting that AA uptake or synthesis (and oxidation) are altered by disuse. It is not possible to conclude at this time that intracellular signalling responses to mixed meal drink consumption were impaired by immobilisation, as muscle tissue was not collected, nor is it possible to conclude whether IR preceded anabolic resistance, as data for AA balance after two days are not available. However, it is possible to speculate that removal of muscle contraction leads to a rapid decline in energy substrate demand by skeletal muscle (possibly due to intracellular negative feedback loops resulting in diminished substrate uptake) and that this is, at least in part, driven by increased expression of lipotoxic metabolites in muscle (likely derived from increased NEFA uptake). Elucidating such mechanisms can be challenging *in vivo* but similarly to the translation seen between Chapter 3 and this chapter it is possible to use *in vitro* data to better understand the mechanisms driving the changes seen *in vivo*.

A better understanding of disuse-induced anabolic resistance can be gained by assessing the rates of postabsorptive and postprandial MPS and MPB during immobilisation.

This can be achieved by the use of stable isotope tracers of AAs such as phenylalanine (L-phenylalanine, RING-D5, 99 %) and leucine (L-leucine, 1-13C, 99 %). This technique was applied to this study during the baseline days and after 7 days of immobilisation, however, due to the small sample size presented here, the data were too underpowered to be able to draw conclusions from it. It would be interesting, in future experiments, to apply this technology prior to and following two days of forearm immobilisation in order to assess alterations in synthesis and breakdown rates of AAs. Furthermore, as it appears that NEFA uptake by muscle is increased during disuse, it would be interesting to adopt stable isotope FA tracers to better understand the dynamic changes in lipid metabolism occurring during disuse and HFD.

In conclusion, given the rapid and substantial impairments seen after a short period of disuse, which appears to occur regardless of energy balance, strategies to maintain metabolic and functional health should aim to incorporate a level of muscle contraction (Wall and van Loon, 2013; Dirks, Wall and van Loon, 2018) during periods of inactivity and improve lipid handling to diminish accumulation of intramyocellular lipotoxic moieties.

# Chapter 5

# Regulation of blood glucose by pharmacological activation of AMPK in Sprague-Dawley rats

# Introduction

In healthy individuals, glucose metabolism is regulated by neuroendocrine processes in an interplay between the pancreas, SM, liver, AT and the brain; acting to maintain glycaemia within a physiological range (DeFronzo, 1988). Impaired glucose homeostasis is a predominant feature of diabetes and maintaining blood glucose is a daily challenge for individuals with diabetes. This is especially true in T1D and advanced/insulin-treated T2D, given the increased risk of iatrogenic hypoglycaemia (Cryer, 2008).

The physiological defence against hypoglycaemia termed the counterregulatory response (CRR), acts to prevent further decreases in blood glucose levels. Firstly, insulin secretion is suppressed in response to a drop within the physiological range. As blood glucose levels drop further, α-cell glucagon secretion is increased followed by adrenaline release from the adrenal medulla (Dagogo-Jack *et al.,* 1993). In diabetes, severe hypoglycaemia and recurrent exposure to hypoglycaemic events lead to impairment of the CRR (Hedrington *et al.*, 2010) as well as loss of awareness of hypoglycaemia (IHA) and may cause severe brain damage and death (McCrimmon and Sherwin, 2010). Impaired counterregulation occurs as a result of previous exposure to hypoglycaemia (Cryer and Heller, 1991) decreased suppression of insulin secretion (exogenously administered), impaired glucagon secretion and attenuated sympathoadrenal response, causing delayed adrenaline release and symptom awareness (Taborsky, Ahrén and Havel, 1998; Mundinger *et al.*, 2015). Blunted glucagon release in response to hypoglycaemia is a major contributor to increased frequency of hypoglycaemia in T1D (Garber *et al.*, 1976; Rizza *et al.*, 1979; Dagogo-Jack *et al.,* 1993; Segel *et al.,* 2002) and, therefore, an important therapeutic target to explore.

AMPK has emerged as an important component of cellular and whole-body energy sensing over the past 20 years. The enzyme is allosterically activated by AMP (and ADP) during energy stress (such as hypoglycaemia) due to increased [AMP]/[ATP] ratios (Xiao *et al.*, 2007; Oakhill, Scott and Kemp, 2012). Activation by AMP further stimulates AMPK activation by enhancing its phosphorylation by upstream kinases, such as liver kinase B1 (LKB1) (Hawley *et al.*, 2003; Gowans

*et al.*, 2013) and calcium ($Ca^{2+}$)-calmodulin dependent protein kinase kinase-β (CAMKKβ) (Woods *et al.*, 2005) at threonine 172 residue, a site required for full kinase activation (Hawley *et al.*, 1996; Xiao *et al.*, 2007). Binding of ADP and AMP to AMPK also protects the enzyme from dephosphorylation by phosphatases such as PP2A and PP2C (*in vitro)* (Davies *et al.*, 1995; Hawley *et al.*, 1995; Gowans *et al.*, 2013). Once activated, AMPK acts to suppress anabolic and promote catalytic processes to increase energy availability (Hardie, 2007). Activated AMPK targets signalling proteins involved in pathways regulating protein synthesis (Inoki *et al.,* 2003), glycolysis (Marsin *et al.*, 2000; Bando *et al.*, 2005; Sakamoto and Holman, 2008), lipid metabolism (Carling, Zammit and Hardie, 1987) and mitochondrial homeostasis (Herzig and Shaw, 2018) (functions summarised in Figure 1.4). AMPK activation is also important in whole-body hormone and nutrient sensing (Hardie *et al.,* 2016; Carling, 2017).

Control of whole-body metabolism by AMPK is, at least in part, mediated by its actions in the brain (Minokoshi *et al.*, 2004). Brain plasma glucose levels are lower than peripheral levels and maintained at a narrower range (0.5-2.5 mmol/L) (Gruetter *et al.*, 1992). Deviations from this range are sensed by populations of glucose-sensing neurones such as GE and GI neurones (Song *et al.*, 2001) in various neuroendocrine brain regions, such as the hypothalamus and hindbrain (Levin *et al.*, 2004). Other glucose sensors are present in the hepatoportal vein, gut, carotid body and oral cavity and these constitute important entry points relaying neuroendocrine information to the brain (Adachi *et al.*, 1984; Watts and Donovan, 2010). Neuronal firing rate increases in GE and GI neurones as glucose levels increase and decrease, respectively. In GE neurones, an increase in glucose availability generates more intracellular ATP, leading to closure of $K_{ATP}$ channels, cellular depolarisation, calcium influx and initiation of action potential firing, akin GSIS in β-cells (Anand *et al.*, 1964; Mountjoy and Rutter, 2007; McTaggart *et al.,* 2010). During hypoglycaemia, however, reduced ATP production leads to $K_{ATP}$ channel opening, silencing these neurones. This response is believed to be an important transducing signal to trigger the CRR, as inhibition of VMH $K_{ATP}$ with sulfonylureas attenuates counterregulation (Evans *et al.*, 2004); whereas opening of the channel using diazoxide can amplify the CRR in healthy and counterregulatory-impaired rats (Evans *et al.*, 2004; McCrimmon

*et al.*, 2006). *In vitro* evidence from glucose-sensing hypothalamic neuronal cells further suggests that this response is AMPK-dependent, as genetically knocking down AMPK-α2 blunts the cells' response to low glucose (Beall *et al.*, 2012).

Direct pharmacological activation of AMPK in the VMH using an AMP mimetic, AICAR, increases the response to hypoglycaemia due to increased HGP, observed during hyperinsulinaemic-hypoglycaemic clamps in rats (McCrimmon *et al.*, 2004). This was also observed in a rodent model of T1D, the BB rat, in acute and recurrent hypoglycaemia (Fan *et al.*, 2009). In the latter, the glucagon response to hypoglycaemia was also amplified following AMPK activation. Furthermore, knockdown of VMH AMPK-α2 in rats caused a 60% and 40% reduction in glucagon and adrenaline, respectively, during hyperinsulinaemic-hypoglycaemic clamp studies, leading to impaired HGP and whole-body glucose uptake (McCrimmon *et al.*, 2008). Importantly, although AMPK is activated in response to acute hypoglycaemia, shown *in vitro* in a model of GE neuron (Beall *et al.*, 2012) and primary GI neurones (Murphy *et al.*, 2009), recurrent glucoprivation via injection of 2-DG into rats diminishes AMPK activation in response to subsequent insulin-induced hypoglycaemia (Alquier *et al.*, 2007). Together, these data suggest that central AMPK activation in response to hypoglycaemia is an important signal whose suppression may lead to defective counterregulation following recurrent hypoglycaemia.

Activation of AMPK in neuroendocrine sensory brain regions is also important for appetite regulation. Anorexigenic stimuli, which reduce food intake, such as insulin, leptin, exercise, hyperglycaemia and a shift from fasted to fed state all decrease AMPK activity. (Andersson *et al.*, 2004; Minokoshi *et al.*, 2004). Contrastingly, orexigenic, or appetite-promoting hormones, such as ghrelin or adiponectin, increase AMPK activity in the hypothalamus (Andersson *et al.*, 2004; Kola *et al.*, 2005). Pharmacologically or genetically enhancing AMPK and its activity in the hypothalamus stimulates food intake (Andersson *et al.*, 2004; Minokoshi *et al.*, 2004), whereas expressing dominant-negative AMPK mutants in this region attenuates glucoprivation-induced (with 2-DG) food intake in rats (Kim *et al.*, 2004). Furthermore, knockdown of AMPK-α2 in POMC and NYP-AgRP neurones of mice leads to altered neuronal glucose sensing and whole-body metabolism (Claret *et al.*, 2007).

In SM, AMPK activation promotes glucose disposal and enhances insulin sensitivity to lower hyperglycaemia (Musi and Goodyear, 2003), whereas direct activation of α-cell AMPK stimulates glucagon secretion (Leclerc *et al.*, 2011; Sun *et al.*, 2015). It is therefore important to differentiate the effect of central versus peripheral activation of AMPK at differing blood glucose levels. Pharmacological targeting of brain AMPK has often relied on compounds with low specificity (such as AICAR) or weak brain permeability (such as metformin) and these have, thus far, been directly injected into the brain. Novel AMPK activators have since been developed by companies such as Rigel Pharmaceuticals (CA, USA), who have collaborated in this project by providing two such compounds, R481 and R419. R481 is an indirect, metformin-like AMPK activator, which exhibits a positive brain:plasma distribution within 30 minutes of oral administration to mice (unpublished).

In this chapter, R481 was used to test the hypothesis that increased AMPK activation via peripheral administration of a brain permeable activator would amplify the CRR to hypoglycaemia, in healthy rats. AMPK activity in response to R481 was assessed *in vitro*, in GT1-7 glucose-sensing hypothalamic neurones, and the compound was subsequently used *in vivo*, in healthy Sprague-Dawley rats, where it was hypothesised that it would promote food intake and enhance the defence against hypoglycaemia.

# Results

## 5.1 R481 activates AMPK in GT1-7 hypothalamic neurones

To test the effect of R481 on AMPK activation and activity, *in vitro,* GT1-7 glucose-sensing hypothalamic neurones were treated with R481 (0-50 nM) for 30 minutes and changes to the AMPK signalling pathway assessed using Western blotting. R481 treatment significantly increased the levels of phosphorylated AMPK at threonine 172, in a concentration-dependent manner (Fig. 5.1.1A,B). R481 at 10 nM increased AMPK phosphorylation 2.63 ± 0.33 fold relative to control and reached a 10.17 ±1.99 fold increase at 50 nM (n=6; $P<0.01$ for both; Figure. 5.1.1B).

To determine whether changes to AMPK phosphorylation were accompanied by changes to AMPK activity, phosphorylation of a downstream substrate, ACC, was assessed. R481 treatment concentration-dependently increased phosphorylation of ACC (serine 79) from a 2.27 ±0.34 fold increase with 10 nM to 4.71±0.73 fold with 50 nM (n=8; $P<0.01$ for both; Figure. 5.1.1A,C).

AMPK is activated by an increase in the [AMP]/[ATP] ratio but despite activating AMPK, R481 did not alter the intracellular levels of ATP (measured using luminescence based plate assay), even at concentrations 10-fold higher than those required to activate AMPK in these cells (n=6; Figure. 5.1.2).

## 5.2 R481 did not alter feeding *ad libitum* or in response to fasting or hyperinsulinaemia

The effect of R481 on feeding behaviour was assessed in *ab libitum*, fasting-induced and hyperinsulinaemia-induced feeding studies. Healthy male Sprague-Dawley rats (250-300 g) were treated with R481 (20 mg/Kg; i.p.) or vehicle (HPMC) at the start of the dark-phase and food intake measured at 1, 2 and 4 hours post-injection. R481 had no effect on feeding at any time point ($P>0.05$, Vehicle n=4; R481 n=4; Figure 5.2A). Following a 16 hour (dark-phase) fast, healthy male Sprague-Dawley rats (300-350 g) were treated with R481 (5 mg/Kg; orally) or vehicle (HPMC) followed by insulin (0.75 U/Kg) one hour later. Food was re-introduced one hour after insulin injection and intake measured at 1 and 4 hours. R481 had no effect of insulin-induced feeding ($P>0.05$, Vehicle n=5; R481 n=5; Figure 5.2B). A third cohort of rats (250-300 g) was fasted for 16 hours

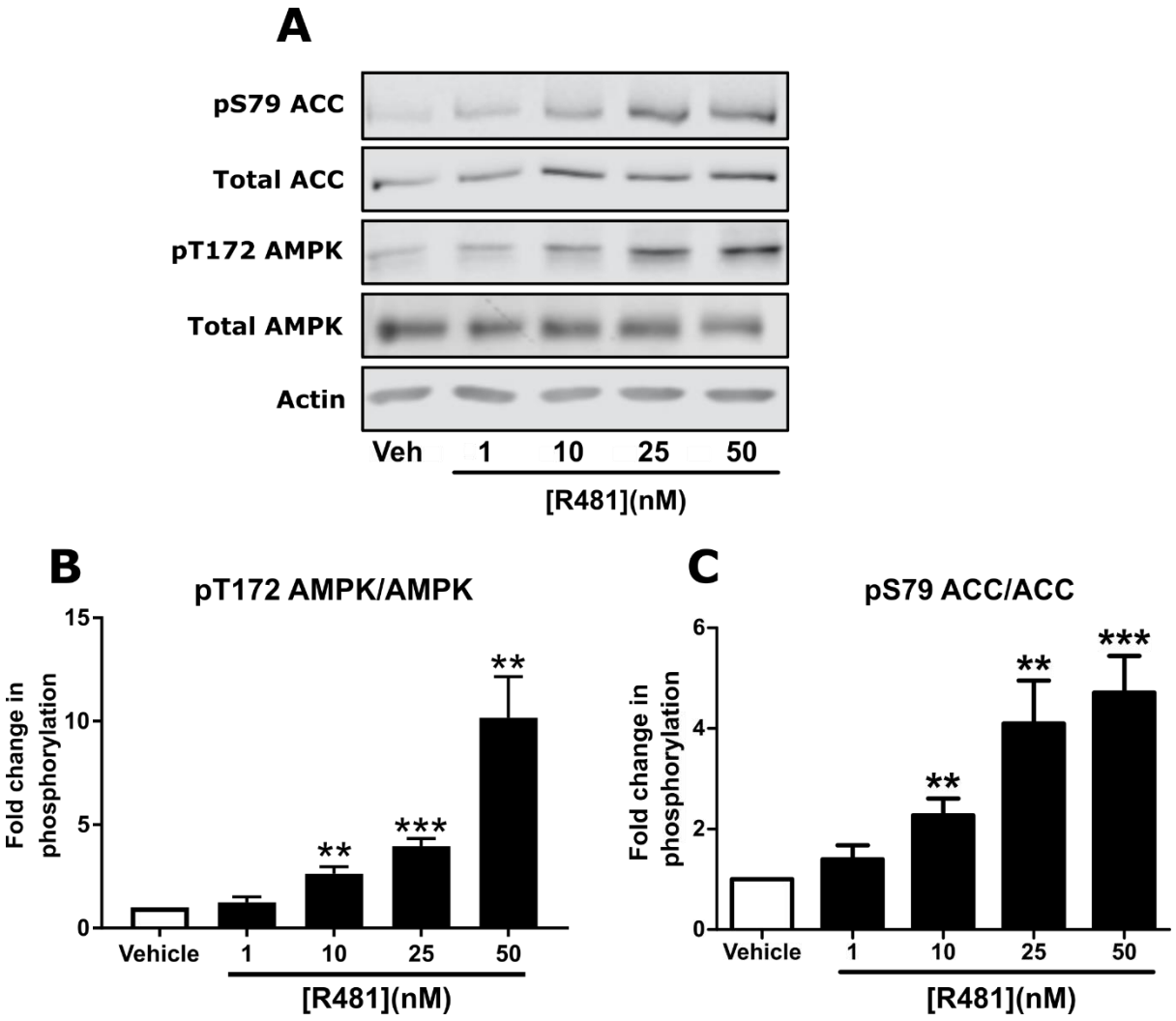



**Figure 5.1.1 R481 increases AMPK phosphorylation and activity in GT1-7 neurones**

GT1-7 mouse hypothalamic neurones treated with increasing concentrations of R481 for 30 minutes. **A)** Representative immunoblots for AMPK (pT172), AMPK, ACC (pS79), ACC and Actin. Densitometric analysis of the mean pooled data for phospho-AMPK normalised to total AMPK shown in **B** (n=6) and phospho-ACC normalised to total ACC in **C** (n=8) (**P<0.01; ***P<0.001; One-sample t-test in comparison to control).

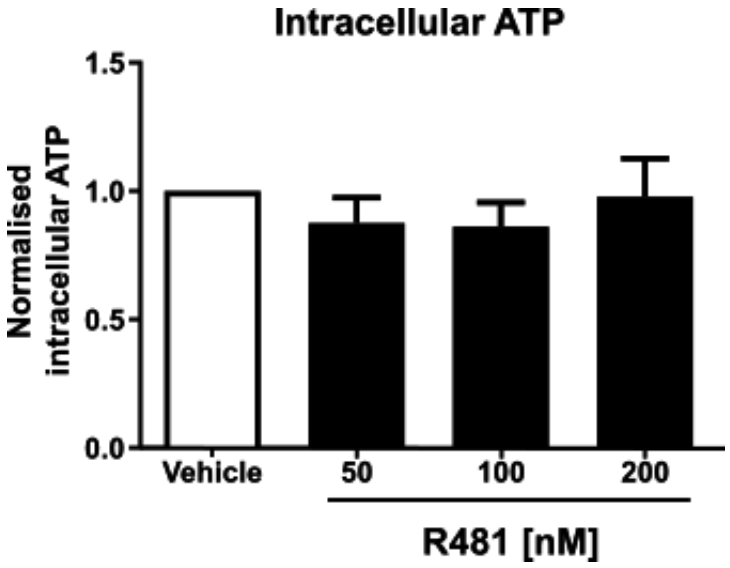



**Figure 5.1.2 R481 treatment does not alter intracellular ATP levels in GT1-7 neurones**

Intracellular ATP levels of GT1-7 mouse hypothalamic neurones treated for 30 minutes with increasing concentrations of R481 (0-200 nM), measured using a luminescence based assay (ATPLite) (n=6).

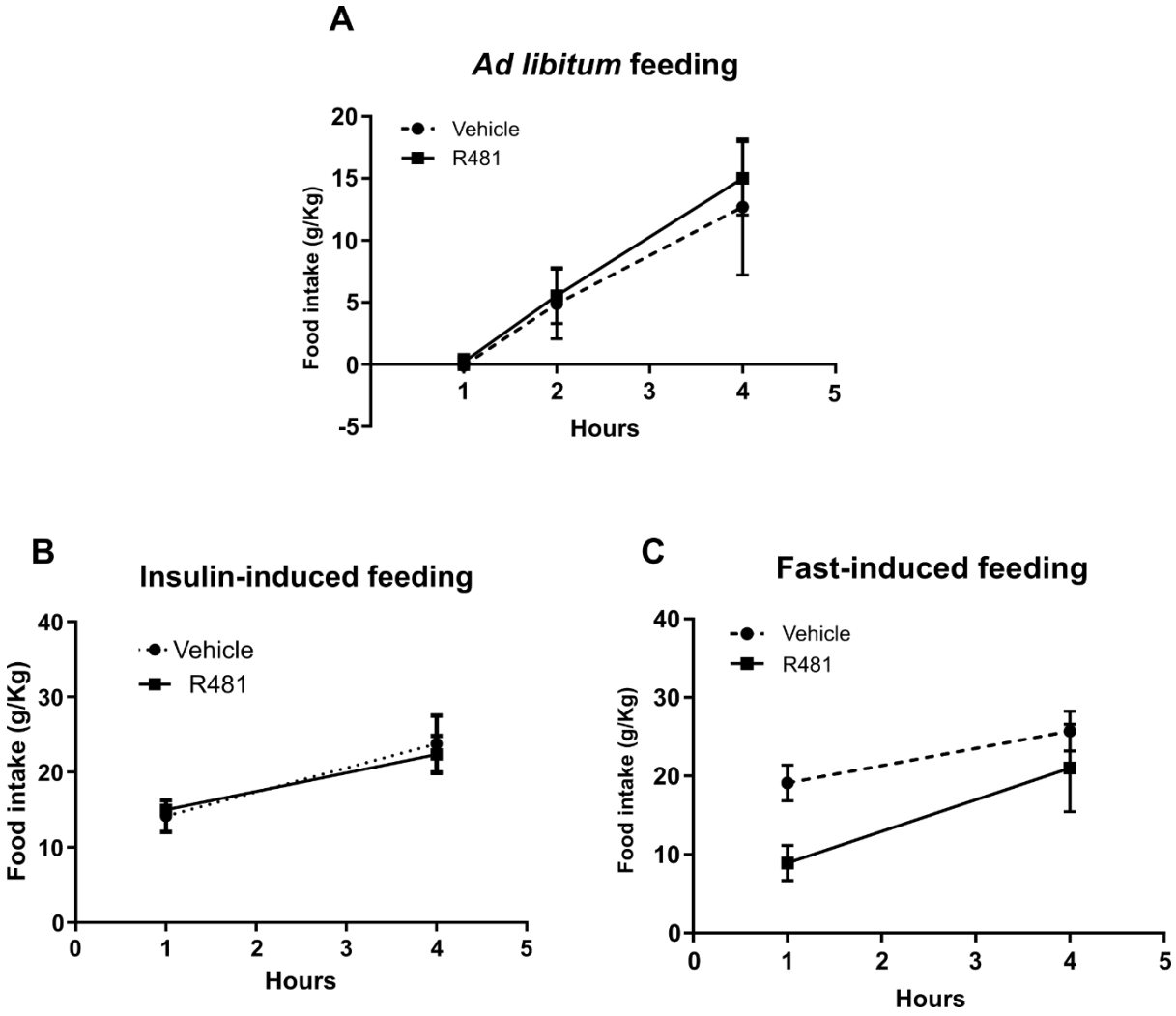



**Figure 5.2. R481 did not alter feeding ad libitum or in response to fasting or hyperinsulinaemia**

**A)** R481 (20 mg/Kg; i.p.) was administered at the start of the dark-phase and food intake measured 1, 2, 4 and 24 hours following injection (Vehicle n=4; R481 n=4). **B**) R481 (5 mg/Kg; orally) was administered one hour prior to insulin injection (0.75 U/Kg; i.p.), food re-introduced after 60 minutes and intake measured 1, 4 and 24 hours later (Vehicle n=5; R481 n=5). **C)** Rats were administered R481 (20 mg/Kg; i.p.) following 16 hour fast and food introduced immediately following injection and measured 1, 4 and 24 hours later (Vehicle n=9; R481 n=9); Two-way ANOVA with repeated measures, no significant drug effect.

and treated with R481 (20 mg/Kg; i.p.) or vehicle (HPMC), food introduced immediately after and food intake measured after 1 and 4 hours to assess the effect of the drug on fasting-induced feeding. R481 moderately, but not significantly, decreased food intake after 1 hour, which normalised after 4 hours (Vehicle n=9; R481 n=9; Figure. 5.2C).

### 5.3 R481 attenuates the insulin-induced drop in blood glucose

To assess the effect of R481 treatment on insulin-induced hypoglycaemia, rats (300-350 g) were fasted (16 hours) and treated with R481 (5 mg/Kg; orally), followed by insulin (0.75 U/Kg; i.p.) one hour later and blood glucose measured after 30 minutes. Basal blood glucose levels were not different between R481 and vehicle-treated rats (Figure. 5.3A), but the drug caused a modest yet significant attenuation of the drop in blood glucose following insulin treatment, compared to vehicle controls (Vehicle n=9; R481 n=9; $P<0.05$, unpaired two-tailed t-test; Figure 5.3B).

### 5.4 R481-induced increase in peak glycaemia is attenuated by AMPK inhibitor SBI-0206965 and ANS blocker hexamethonium

Glucose tolerance tests (2 g/Kg glucose; i.p.) were undertaken to assess the effect of R481 on glucose tolerance and clearance. SBI-0206965, an AMPK inhibitor, was used to test the dependency of the R481 effect on AMPK activation. Healthy, male Sprague-Dawley rats (200-300 g) were fasted (16 hours) and treated with SBI-0206965 (3 mg/Kg; i.p.) or vehicle (DMSO; 0.5 % DMSO v/v) for 30 minutes before being administered R481 (5 mg/Kg; i.p.) or vehicle (HPMC) and glucose (2 g/Kg; i.p.). Blood glucose was measured from the tail vein prior to (t=0) and after 15, 30, 60 and 120 minutes of R481/glucose injection. R481 significantly increased peak glucose excursion compared to SBI-0206965 treated controls (SBI-0206965 n=6; R481 n=10; R481+SBI-0206965 n=8; $*P<0.05_{(drug)}$, $***P<0.001_{(time)}$, $***P<0.001_{(interaction)}$, with Bonferroni's multiple comparisons analysis, **P<0.01, ***P<0.001 for R481 against vehicle at 15 and 30 minutes respectively), and this was attenuated by AMPK inhibitor SBI-0206965 ($P<0.05 for R481 versus R481+SBI-0206965; Figure. 5.4.1A). R481 enhanced peak glycaemia without altering glucose clearance, denoted by comparable total area under the curve measured between groups (P>0.05; One-way ANOVA; Figure. 5.4.1B).

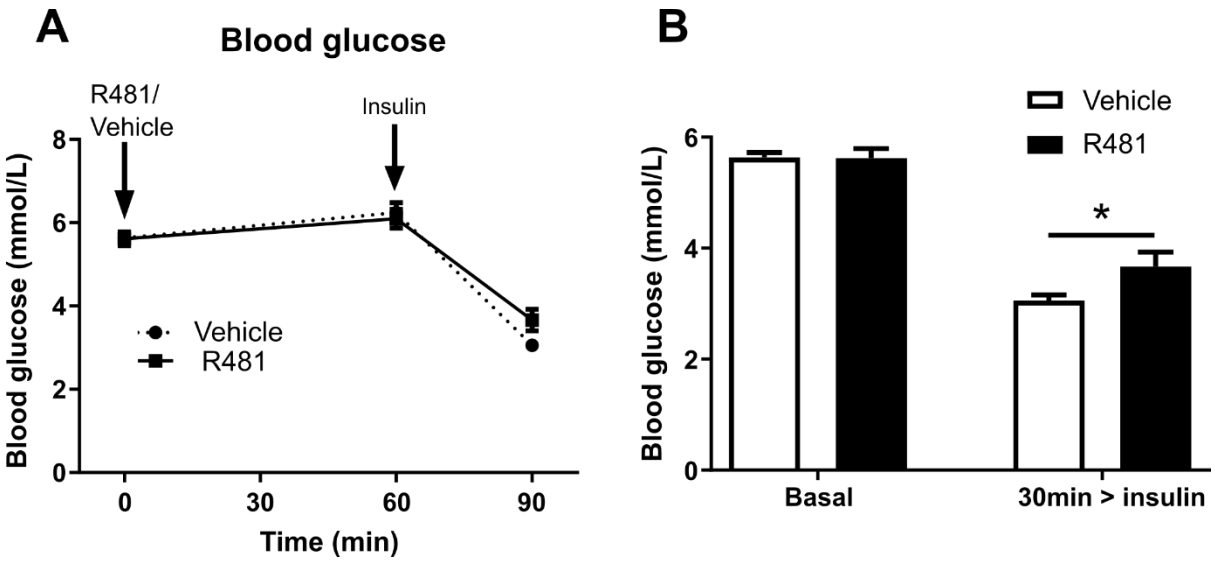



**Figure 5.3. R481 attenuates insulin-induced decrease in blood glucose**

Effect of R481 treatment on acute insulin-induced hypoglycaemia **A)** Male Sprague-Dawley rats were treated with R481 (5 mg/Kg; orally) following a 16 hour fast and 60 minutes later injected with a single dose of insulin (0.75 U/Kg; i.p.), 30 minutes after which blood glucose was measured. Basal glucose did not differ between groups but R481 attenuated the insulin-mediated drop in blood glucose after 30 minutes, shown in **B** (Vehicle n=9; R481 n=9; *P<0.05, Two-way ANOVA with Bonferroni's multiple comparisons).

To test whether R481 elicited the effects on peak glycaemia via activation of the autonomic nervous system (ANS), glucose tolerance tests were performed using a non-brain permeable homologue of R481, R419, and R481 ± ANS blocker hexamethonium. Healthy male Sprague-Dawley rats (200-300 g) were randomly assigned into five groups: vehicle (HPMC) (n=6), vehicle with hexamethonium (50 mg/Kg) (n=3), R419 (20 mg/Kg) (n=6), R481 (20 mg/Kg) (n=6) or R481 with hexamethonium (n=4). Hexamethonium was administered to fasted rats (16 hours; i.p.) 30 minutes before designated drug and glucose (2 g/Kg; i.p.) and blood glucose measured from the tail vein before (t =0) and 15, 30, 60 and 120 minutes post-injection (Figure. 5.4.2A,B). R481 significantly increased peak glucose response to glucose administration compared to vehicle controls ($P<0.01$, $P<0.001$ at 15 and 30 minutes, respectively for R481 against vehicle; Figure 5.4.2B) with no significant effect on glucose clearance, as blood glucose normalised to levels akin those of vehicle-treated animals after 120 minutes ($P>0.05$ for R481 against vehicle at 120 minutes). The increase in peak glycaemia by R481 was completely prevented by treatment with hexamethonium ($P<0.01$, $P<0.001$ at 15 and 30 minutes, respectively for R481 against R481 with hexamethonium). R419 did not significantly alter glucose levels compared to vehicle-treated rats ($P>0.05$; Figure 5.4.2A,B).

## 5.5 R481 raises fasting glycaemia independently of changes to insulin secretion and does not alter glucose infusion rate during hyperinsulinaemic-euglycaemic clamp

Subsequent experiments aimed to assess the effect of R481 on insulin sensitivity and endogenous insulin secretion. This was assessed during hyperinsulinaemic-euglycaemic clamps, performed in healthy male Sprague-Dawley rats (250-300 g) ~14 days after surgical implantation of carotid artery and jugular vein catheters (surgeries at Charles River, Margate, UK). Arterial and venous catheters were exteriorised by vascular access buttons, which enabled continuous infusion and pain-free blood sampling from conscious, freely moving rats. Fasted (16 hours) rats (200-350 g) were treated with R481 (20 mg/Kg; i.p.) or vehicle (HMPC) and 60 minutes later infused with insulin (20 mU/Kg/min) and glucose (50 % dextrose; w/v), marking the start of the clamp (t=0). Blood glucose target at steady state was 5.5 mmol/L (Study design in Figure 5.5A).

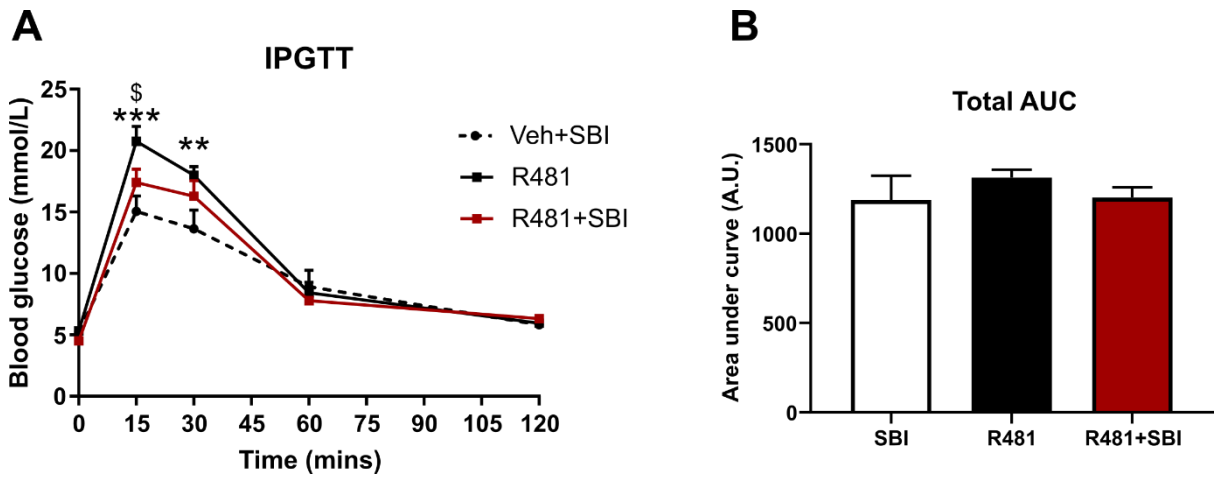



**Figure 5.4.1 Increase in peak glycaemia with R481 is attenuated by AMPK inhibitor SBI-0206965**

Glucose tolerance tests assessing the effect of R481 on glycaemia ± AMPK inhibition SBI-0206965 in Sprague-Dawley rats. **A)** After 16 hour fast, rats were administered SBI-0206965 (SBI; 3 mg/Kg; i.p.) or vehicle for 30 minutes, followed by R481 (5 mg/Kg; i.p.) or vehicle (HPMC) and glucose (2 g/Kg; i.p.) simultaneously (at t= 0). Blood glucose was measured prior to R481 treatment (t= 0) and after 15, 30, 60 and 120 minutes from tail vein samples using a glucometer (SBI-0206965 n=6; R481 n=10; R481+SBI0206965 n=8); Two-way ANOVA with repeated measures $*P_{(drug)}<0.05$, $***P_{(time)}<0.001$, $***P_{(interaction)}<0.001$, and Bonferroni's multiple comparisons analysis, **P<0.01, ***P<0.001 for R481 against vehicle, $P<0.05 for R481 versus R481+SBI-0206965. **B)** Total area under the curve (AUC) for data shown in A; P>0.05, One-Way ANOVA.

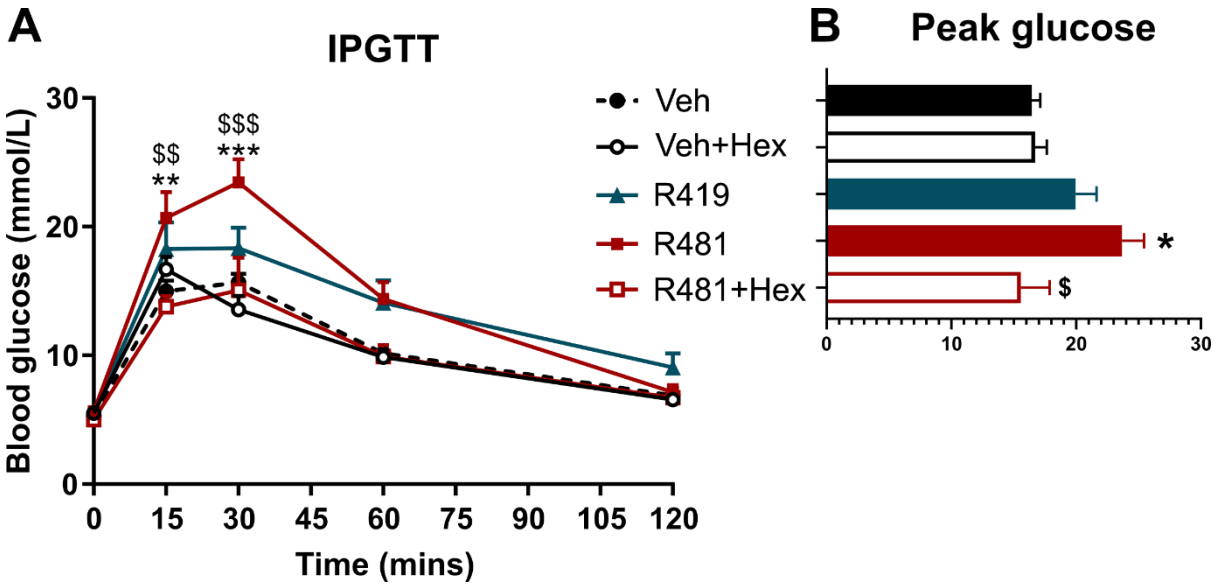



**Figure 5.4.2 R481 activates the autonomic nervous system to raise glycaemia**

Glucose tolerance test assessing the effect of ANS blockade on R481-induced glycaemia. **A)** Healthy male Sprague-Dawley rats were assigned to: vehicle (HPMC) (n=6), vehicle with hexamethonium (Veh + Hex, 50 mg/Kg; n=3), R419 (20 mg/Kg; n=6), R481 (20 mg/Kg; n=6) or R481 with hexamethonium (R481 + Hex; n=4). Hex or vehicle (saline) (i.p.) was administered to fasted (16 hours) animals 30 minutes before R481/R419 or vehicle (HPMC) + glucose (2 g/Kg; i.p.) and blood glucose measured before R481/R419 injection and 15, 30, 60 ad 120 minutes later. R481 significantly increased peak glucose response compared to control, which was prevented by hexamethonium. Two-way ANOVA with repeated measures, $^{**}P_{(drug)}<0.01$, $^{***}P_{(time)}<0.001$, $^{***}P_{(interaction)}<0.001$, with Bonferroni's analysis **P<0.01, ***P<0.001 for R481 against vehicle; $$P<0.01, $$$P<0.001 for R481 against R481+Hex**. B)** Hex significantly prevents ($P<0.05 R481+Hex Vs R481) R481-induced increase in peak glycaemia (*P<0.05 for R481 VS vehicle).

Blood glucose levels at baseline were slightly elevated in R481 treated animals (7.1 ± 0.6 mmol/L at t= -60 to 7.5 ± 0.2 mmol/L at t= 0), but decreased slightly in vehicle treated animals (7.2 ± 0.2 mmol/L at t= -60 to 6.3 ± 0.2 mmol/L at t= 0). This generated a significant relative increase in blood glucose at the start of the clamp in R481 treated rats ($P<0.05$; n=8; Figure 5.5B). Despite this difference, blood glucose profiles were well-matched between R481 and vehicle treated rats during the clamp ($P_{(drug)}>0.05$; Figure 5.5B). There was no difference in GIR between treatments (Figure 5.5C). To assess changes to insulin secretion, C-peptide levels were measured throughout the clamp. C-peptide levels decreased over the course of the clamp, but did not differ between vehicle and R481 treated animals at any time point (Vehicle n=7; R481 n=6; $*P_{(time)}<0.001$, $P_{(drug)}>0.05$; Figure 5.5D).

## 5.6 R481 delays exogenous glucose requirements during hyperinsulinaemic-hypoglycaemic clamps

To determine for the first time whether peripheral administration of a brain permeable AMPK activator, R481, could amplify the CRR to hypoglycaemia, hyperinsulinaemic-hypoglycaemic clamps were performed. Healthy male Sprague-Dawley rats (200-300 g) were purchased with pre-implanted catheters and vascular access buttons, as above, from Charles River (Margate, UK). Clamps were performed ~ 14 days following surgery and target blood glucose at nadir was 3.0 mmol/L (study design Figure. 5.6A). Fasted rats (16 hours) were treated with R481 (20 mg/Kg; i.p.) or vehicle (HMPC) and 60 minutes later clamp started. A bolus dose of insulin (80 mU/Kg/min) was administered for 10 minutes immediately followed by a continuous infusion at 20 mU/Kg/min concomitantly with variable 20% dextrose (w/v) for a total of 90 minutes. Target blood glucose was achieved during the last 30 minutes of the clamp (Vehicle= 2.98 ± 0.06 mmol/L; R491= 3.042 ± 0.12 mmol/L), and glucose profiles were well matched between R481 and vehicle treated animals (Figure 5.6B). R481 significantly delayed exogenous glucose requirements, denoted by decreased GIR (Vehicle n=9; R481 n=9) $***P_{(time)}<0.001$, $*P_{(drug)}<0.05$, $*P_{(interaction)}<0.05$; Figure 5.6C). R481 significantly increased peak glucagon levels, seen at t= 30 ($P<0.05$; Vehicle n=9; R481 n=9; Figure 5.6D-E).

Plasma adrenaline levels increased over the course of the clamp, in response to hypoglycaemia, but were not altered by R481 treatment (Vehicle n=8; R481 n=8; Figure 5.6F-G).

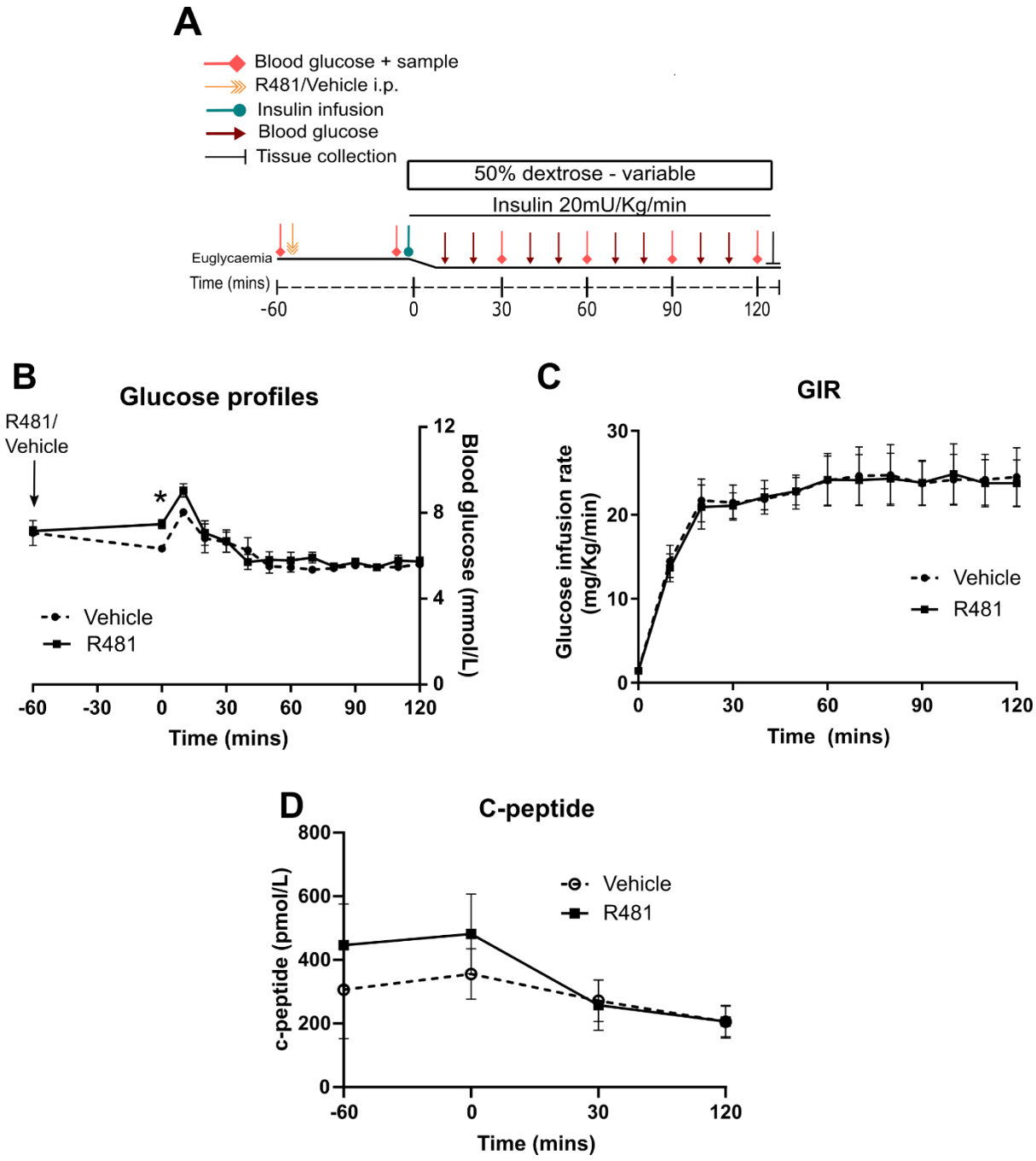



**Figure 5.5 R481 does not alter glucose infusion rate during hyperinsulinaemic-euglycaemic clamp or alter endogenous insulin secretion**

Hyperinsulinaemic-euglycaemic clamps in healthy male Sprague-Dawley rats ~14 days following surgical implantation of carotid artery and jugular vein catheters. **A)** Study design. **B)** Blood glucose profiles during clamp measured from tail vein samples with glucometer (Vehicle n=8, R481 n=8). No overall drug effects $P_{(drug)}>0.05$; mixed-effects analysis of repeated measures, but *P<0.05 for R481 against vehicle at t= 0 using Bonferroni's post-hoc test. **C)** Glucose infusion rate (GIR; mg/Kg/min) during the clamp using a 50% dextrose solution (Vehicle n=8; R481 n=8). **D)** Plasma C-peptide measured by ELISA (Vehicle n=8; R481 n=7).

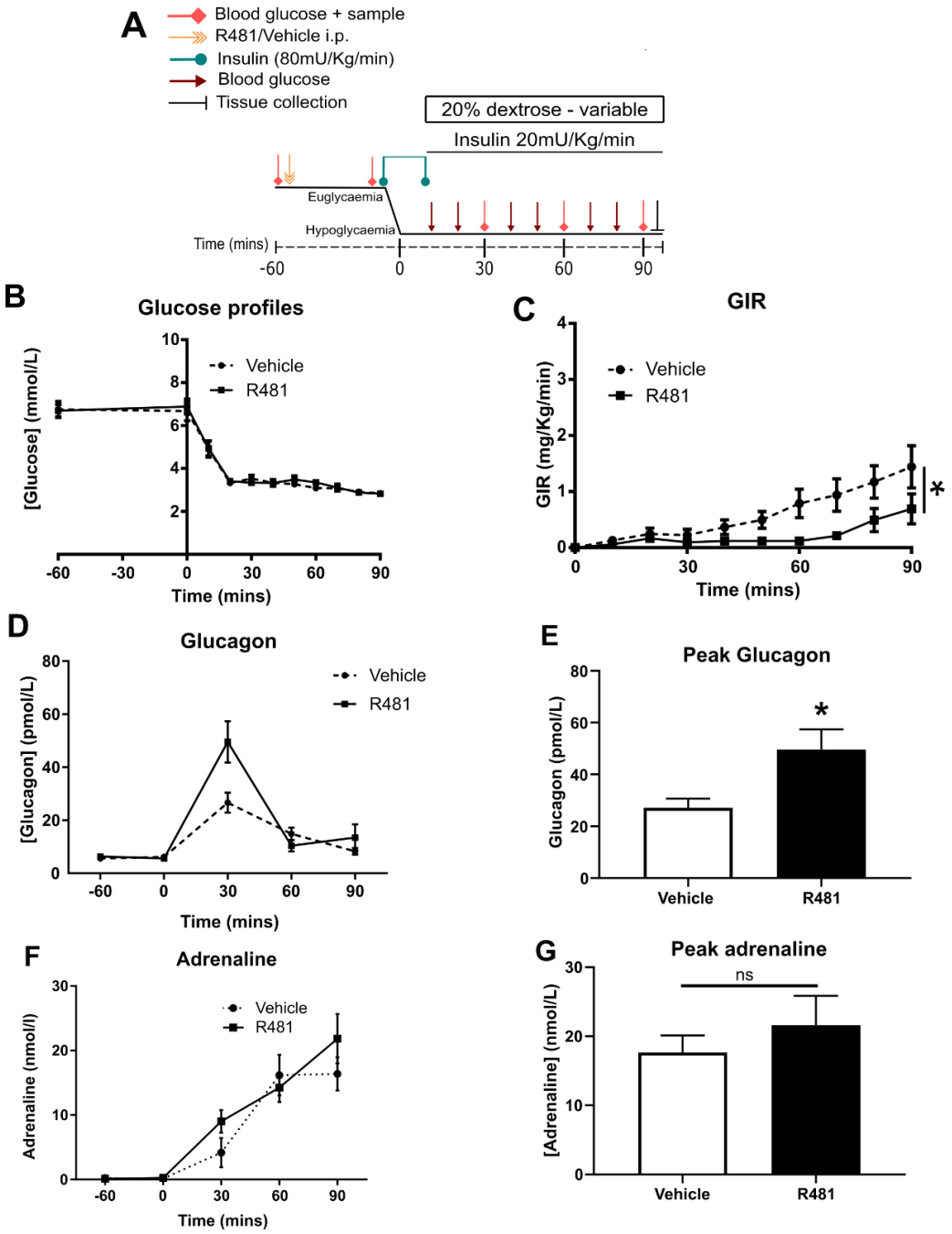



**Figure 5.6 R481 delays exogenous glucose requirements during hyperinsulinaemic-hypoglycaemic clamp by augmenting glucagon levels during hypoglycaemia**

Hyperinsulinaemic-euglycaemic clamps in healthy male Sprague-Dawley rats ~14 days following surgical implantation of carotid artery and jugular vein catheters. **A)** Study design. **B)** Blood glucose profiles during clamp measured from tail vein samples with glucometer (Vehicle n=10, R481 n=8). No overall drug effects $P>0.05_{(drug)}$.**C)** Glucose infusion rates (GIR; mg/Kg/min) during the clamp using a 20% dextrose solution. $*P_{(drug)}<0.05$, $***P_{(time)}<0.05$, $*P_{(interaction)}<0.05$; two-way ANOVA with repeated measures. **D)** Plasma glucagon profile with peak (t= 30) shown in **E**, measured by ELISA (Vehicle n=9; R491 n=9; *P<0.05, unpaired t-test). **F)** Plasma adrenaline profile with peak shown in **G**, measured by ELISA (Vehicle n=8; R481 n=8; ns, not significant).

# Discussion

Therapeutic approaches to manage glycaemia in diabetes primarily aim to reduce hyperglycaemia and risk of developing associated co-morbidities (American Diabetes Association, 2019b). Such strategies, namely long-term insulin treatment in T1D and advanced T2D, however, often lead to increased risk of hypoglycaemia (Fanelli *et al.*, 1994). One or recurrent hypoglycaemic episodes leads to defective CRR (Ma *et al.*, 2018), which further increases risk for hypoglycaemia eventually leading to impaired hypoglycaemia awareness (Gold *et al.,* 1994; Davis *et al.*, 1997; Geddes *et al.*, 2008). Strategies to manage hypoglycaemia and recover the CRR are scarce and a better understanding of the mechanisms regulating these processes is necessary to devise novel therapeutic approaches.

In this chapter, AMPK activator R481 was used to assess whether peripheral administration of a brain permeable AMPK activator could amplify the CRR to hypoglycaemia. Data from the collaborators that provided the compounds, at Rigel Pharmaceuticals (California, USA), not presented here, demonstrated that R481 penetrates the CNS, exhibiting a positive brain:plasma ratio and activates AMPK centrally for up to 4 hours following oral dosing in mice. Furthermore, their evidence suggests that R481 acts as an indirect metformin-like AMPK activator, increasing kinase activity by mild mitochondrial inhibition (ie. mitochondrial hormesis (Hawley *et al.*, 2010). The data presented here, performed in Exeter, demonstrated that R481 treatment activated the ANS to raise glycaemia, a response that was attenuated by pharmacological AMPK inhibition, without altering insulin secretion or peripheral insulin sensitivity; and amplified the CRR to hypoglycaemia by enhancing peak glucagon levels, in healthy rats.

The GT1-7 murine cell line was used as an *in vitro* model of GE neurone. These cells demonstrate AMPK-dependent glucose sensing capacity and are useful for examining responses in a single neuronal population (Beall *et al.*, 2012). In these cells, R481 significantly increased AMPK phosphorylation and activity (demonstrated by increase p-ACC) at low nanomolar concentrations and within 30 minutes of drug administration.

Treatment with R481, despite activating AMPK, did not compromise intracellular levels of ATP, even at concentrations 10 fold higher than that required to significantly increase AMPK phosphorylation. This suggests that mild energetic stress caused by the drug may increase AMP levels to activate AMPK, but the catalytic effect of the kinase and rapid nucleotide turnover maintain the total levels of ATP in the cell. The potent ability to activate AMPK as well as CNS-permeability demonstrated by R481 made this compound a useful tool to test the overarching hypothesis of this study.

The contribution of AMPK activation to the central regulation of feeding (Hardie *et al.,* 2012) generated the hypothesis that R481 would promote food intake *in vivo*. Contrary to the hypothesis, R481 treatment did not alter *ad libitum* feeding or feeding responses to hyperinsulinaemia or fasting. AMPK is essential for glucose/hypoglycaemia sensing by POMC and NPY/AgRP appetite regulatory neurones, and its activation is enhanced and reduced by orexigenic and anorexigenic stimuli, respectively (Kola, 2008). Hypothalamic AMPK activity is suppressed by peripheral leptin administration and enhanced by ghrelin administration, leading to decreased and increased food intake, respectively (Andersson *et al.*, 2004). Pharmacological activation of AMPK by injection of AICAR into the third ventricle or directly into the hypothalamic PVN promotes food intake (Andersson *et al.*, 2004). Conversely, direct leptin injections into the MBH decrease ARC and PVN AMPK activity, and adenoviral induction of dominant-negative AMPK into the MBH decreases food intake and body weight in rodents (Minokoshi *et al.*, 2004). Direct injection of R481 into an appetite regulatory brain region would, therefore, provide more concrete evidence of a role for the compound in food intake and until better tissue distribution of the drug is assessed, it is not possible to determine whether hypothalamic or hindbrain AMPK activity is enhanced by R481. It is possible that R481 treatment (particularly oral dosing) may not be sufficient to amplify hypothalamic AMPK activation induced by prolonged fasting (López *et al.*, 2008). Furthermore, R481 may activate AMPK without altering food intake, which would concur with findings demonstrating altered glucose metabolism downstream of hypothalamic AMPK activation without changes to food intake or body weight (Kinote *et al.*, 2012).

Pharmacological (and naturally occurring) AMPK activators have been developed as anti-hyperglycaemic agents for use in T2D, promoting glucose disposal primarily in SM (Merrill *et al.*, 1997; Kurth-Kraczek *et al.*, 1999). The non-permeable homologue of R481, R419, activates SM AMPK and enhances insulin sensitivity in DIO mice (Marcinko *et al.*, 2015). The effect of both R481 and R419 on glucose tolerance was assessed here by IPGTTs and data demonstrated that R419 did not alter peak glycaemia or glucose clearance, whereas, R481 significantly increased glucose excursion in response to glucose administration without altering glucose clearance. Pre-treatment with AMPK/Uncoordinated (Unc)-51-like kinase (ULK-1) inhibitor SBI-0206965 (Egan *et al.*, 2015) attenuated the transient increase in glycaemia and ANS blocker hexamethonium completely abolished R481-mediated peak glucose excursion, suggesting that R481 acts centrally to enhance glycaemia in a manner that is attenuated by AMPK inhibition. Kume and colleagues demonstrated that pharmacological activation of hypothalamic AMPK by intracerebroventricular (icv) AICAR injection suppresses first-phase GSIS via autonomic innervation of α-adrenergic pancreatic nerves, which is hypothesised to be a protective mechanism to promote glucose delivery to the brain during fasting (Kume *et al.*, 2016). The inhibition of R481-mediated peak glycaemic response by hexamethonium seen here suggests the involvement of the parasympathetic or sympathetic nervous systems in this response, which is in line with those observations. However, an acknowledged small caveat in this experiment is the lack of data on insulin levels during the GTT, which would help determine whether R481 alters insulin secretion. Furthermore, direct activation or suppression of hypothalamic AMPK activity differentially regulates HGP (Yang *et al.*, 2010; Lam *et al.*, 2011; Kinote *et al.*, 2012). Fructose-stimulated hypothalamic AMPK activation up-regulates HGP (Kinote *et al.*, 2012), whereas molecular and pharmacological inhibition of hypothalamic AMPK suppresses HGP (Yang *et al.*, 2010; Lam *et al.*, 2011). Future work with R481 would aim to assess blood glycaemia following pyruvate load, as a measure of glucose synthesis from pyruvate (gluconeogenesis) and assess levels of hepatic gluconeogenic enzymes, such as PEPCK, to determine whether R481 acts centrally to decrease glucose-induced inhibition of HGP (Kowalski *et al.*, 2017). This is supported by observations that pharmacological AMPK activation in the hypothalamus using AICAR or A-769662 increases

hepatic PEPCK expression and glucose synthesis during pyruvate tolerance test (Kinote *et al.*, 2012).

Pre-treatment with AMPK/ULK1 inhibitor SBI-0206965 attenuated the R481-mediated increase in peak glucose response during GTTs. SBI-0206965 was first reported, *in vitro* and *in vivo,* to act as a highly selective inhibitor of ULK1, a critical serine/threonine kinase for the initiation of autophagy (Egan *et al.*, 2015), whose activity is regulated by mTOR and AMPK (He and Klionsky, 2009). The compound has since been found to directly inhibit AMPK with 40 fold greater potency compared to conventionally used AMPK inhibitor Compound C (Dite *et al.*, 2018). During energetic stress, such as in hypoglycaemia, central activation of AMPK stimulates glucose production, raising plasma glycaemia (McCrimmon *et al.*, 2008), whereas during hyperglycaemia, peripheral AMPK activation is primarily glucose-lowering (Long and Zierath, 2006). Interestingly, during AA and growth factor deprivation, independently from autophagic events, ULK1 activation (whole-body) sustains glucose metabolic fluxes by directly phosphorylating glycolytic enzymes to enhance HK activity and promote glucose uptake, while suppressing gluconeogenic machinery (Li *et al.*, 2016). It would, therefore, be interesting to establish whether central activation of ULK1 plays a paradoxical action to its peripheral effect and further establish whether R481 activates ULK1 (dependently or independently of AMPK). SBI-0206965 (5 days treatment) has also demonstrated glucose clearing effects in mice fed a HFD (Yamamoto *et al.*, 2018), so a glucose-clearing effects of the compound cannot be ruled out as a possible explanation for the attenuated R481-mediated glucose response seen here. As R481 was administered peripherally, it is plausible that the drug activated AMPK in glucose-responsive tissues such as SM, to promote glucose disposal (Cokorinos *et al.*, 2017). Data from GTTs, however, suggests that, if R481 alters glycaemia by activating AMPK, its central effect supersedes its peripheral actions.

Hyperinsulinaemic-euglycaemic clamps were performed to assess the effect of R481 on insulin secretion and sensitivity, following the hypothesis that peripheral AMPK activation by the compound would enhance glucose disposal into SM. R481 did not alter GIR or the levels of C-peptide (used as a proxy for endogenous insulin secretion) before or during the euglycaemic clamp, suggesting that the

drug does not alter insulin secretion or sensitivity. Importantly, R481 was administered 60 minutes prior to the start of the clamp, so it is possible that R481 suppressed first-phase IS immediately after administration (supporting GTT data), but its effects begin waning by 60-90 minutes, when blood glucose returns to baseline levels during GTT and animals reach steady-state during clamp. It would be interesting to determine whether simultaneous administration of R481 and infusions at the start of the clamps would generate a different result. Nonetheless, AMPK has demonstrated both positive and negative regulatory effects on insulin secretion (Fu *et al., 20*13). Treatment of pancreatic β-cells and islets with increasing concentrations of AICAR increases insulin secretion (Akkan and Malaisse, 1994; Salt *et al.*, 1998; Salt *et al.*, 1998) with mild potentiation of insulin secretion seen during hyperglycaemia (Salt *et al.*, 1998; Wang *et al.*, 2005; Gleason *et al.*, 2007). Paradoxically, activation of AMPK and ACC with AICAR has demonstrated suppressive effects on GSIS (Zhang and Kim, 1995) and treatment with thiazolidinediones seen to activate AMPK while reducing insulin secretion (Wang *et al.*, 2007; Lamontagne *et al.*, 2009). This is supported by others demonstrating an inverse relationship between acute pharmacological AMPK activation and insulin secretion (Salt *et al.*, 1998; Leclerc and Rutter, 2004). Studies using longer-term pharmacological (Targonsky *et al.*, 2006; Cai *et al.*, 2007) and molecular (Salt *et al.*, 1998; Salt *et al.*, 1998; Da Silva Xavier *et al.*, 2000) AMPK activation have more consistently shown an inhibitory effect on insulin secretion. Importantly, decreased glucose availability (in β-cell model) leads to AMPK-dependent inhibition of insulin secretion (Lim *et al.*, 2009), which supports the notion that AMPK may suppress GSIS to prevent inappropriate hypoglycaemia. It is possible that this is not seen at euglycaemia, supporting the R481 effect seen here. Moreover, a single dose of the compound in lean rats, rather than chronic dosing in DIO mice, may explain the discrepancy between this study and evidence by Marcinko and colleagues using R419 (Marcinko *et al.*, 2015). Future studies would use glucose tracers prior to and during the clamp to determine rates of glucose appearance and disappearance from plasma to assess basal (fasting) HGP and glucose disposal into insulin-sensitive tissues (Zhang *et al.*, 2018). The glucose-raising effect of R481 may also be dose-dependent so it would be important to optimise the dose to prevent raised fasting glycaemia.

AMPK activation is important in hypothalamic glucose sensing, particularly during hypoglycaemia. The ability of R481 to amplify the CRR to hypoglycaemia was assessed here during hyperinsulinaemic-hypoglycaemic clamps, where the compound generated significantly lower GIRs, amplifying the CRR to hypoglycaemia by enhancing peak glucagon levels. R481-mediated hypothalamic AMPK activation was not assessed here, but these data are consistent with studies demonstrating amplified CRR by enhanced central AMPK activation. Direct AICAR-mediated activation of AMPK in the VMH has been shown to amplify the hormonal CRR to hypoglycaemia and increase HGP in healthy rats (McCrimmon *et al.*, 2006) and enhance the glucagon and adrenaline responses to hypoglycaemia in type 1 diabetic rats (Fan *et al.*, 2009). Conversely, downregulation of VMH AMPK suppresses glucagon (~60 %) and adrenaline (~40 %) responses to acute hypoglycaemia in healthy rats (McCrimmon *et al.*, 2008). VMH AMPK activation has also been shown to stimulate HGP in response to hypoglycaemia without altering counterregulatory hormone levels (McCrimmon *et al.*, 2004).

Here, R481 significantly increased glucagon without altering adrenaline levels, in response to hypoglycaemia. Release of glucagon and adrenaline are differentially regulated in response to decreasing plasma glucose (Sprague *et al.*, 2011). The mechanisms of glucagon release by α-cells remain poorly understood but studies in isolated α-cells, islets, rodents and humans suggest the involvement of paracrine mechanisms, such as inhibition of glucagon release by insulin (Cooperberg and Cryer, 2010) and somatostatin (Strowski *et al.*, 2000) as well as gut incretin-derived (Holst *et al.*, 2011) and neuronal mechanisms (Sprague *et al.*, 2011; Thorens, 2011). Sympathetic innervation of β2-adrenergic receptors also stimulates glucagon release (Gerich *et al.,* 1976; Fagerholm, Haaparanta and Scheinin, 2011) so it is possible that the ANS-dependent role of R481 alters glucagon release by α-cells. Peripheral glucose sensors, such as those in the hepatoportal vein may also regulate the ANS-mediated effects on glucagon secretion (Fujita *et al.*, 2007). Adrenaline release from the adrenal medulla is mediated by sympathoadrenal responses to a decrease in blood glucose and adrenaline acts in the liver to stimulate HGP (Exton, 1987) and on α-cells to further enhance glucagon release (Gustavson *et al.*, 2003).

It is plausible, given the wide variety of mechanisms regulating glucagon release, that R481 acts directly on α-cell or adjacent mechanisms to enhance glucagon secretion independently of significant changes to adrenaline. Indeed, direct pharmacological (metformin, A-769662) and molecular (adenoviral transduction) activation of AMPK in α-cells *in vitro* and ex-vivo mouse islets is sufficient to stimulate glucagon release at both high and low glucose concentrations, whereas inactivation of AMPK suppresses low glucose-induced glucagon secretion (Leclerc *et al.*, 2011). Furthermore, mice with α-cell specific AMPKα1 and LKB1 knockouts demonstrate enhanced glucose disposal during hyperinsulinaemic-hypoglycaemia clamps due to impaired glucagon release, suggesting the involvement of an LKB1-dependent signalling pathway involving, but not restricted to, AMPKα1 in glucagon secretion (Sun *et al.*, 2015). However, an R481-dependent effect on glucagon secretion is unlikely to mediate the transient glucose intolerance seen during GTTs, as glucagon levels were not elevated at the start of the hypoglycaemic clamps (60 minutes post-injection). R481 may activate an AMPK-ANS-HGP axis while concomitantly activating an ANS-α-cell axis or directly enhancing α-cell glucagon release. Further work is necessary to establish the effect of R481 treatment on markers of HGP, as the compound may attenuate the suppression of HGP during GTT (Alquier *et al.*, 2007), as well as direct effect of the compound on α-cell AMPK activation and glucagon release.

Importantly, the work presented here was conducted in healthy rodents with effective CRR and hypoglycaemia awareness. Acute glucoprivation by injection of 2-DG (icv) enhances activity of VMH and dorsomedial hypothalamus (DMH) AMPKα1 and α2 and leads to elevated glucagon and corticosterone levels, amplifying the CRR (Chun *et al.*, 1998; Marin-Spiotta *et al.,* 2004; Alquier *et al.*, 2007). However, recurrent glucoprivation with 2-DG (icv) impairs hormonal CRR, in a manner that is associated with blunted and delayed hypothalamic AMPK activation (Alquier *et al.*, 2007). Given that hyperglycaemia (Kim *et al.*, 2004; Minokoshi *et al.*, 2004) and recurrent hypoglycaemia/glucoprivation (Alquier *et al.*, 2007) attenuate hypothalamic AMPK activity and suppression of expression and activity of AMPK in the VMH suppresses the hormonal CRR to hypoglycaemia (McCrimmon *et al.*, 2008), it is plausible that AMPK activity is blunted in diabetes and that this may contribute to blunted CRR and awareness

of hypoglycaemia. AMPK activation may, therefore, be an important tool to recover impaired CRR. Future experiments using R481 would assess the effect of acute and chronic administration of the compound on glycaemia and CRR in diabetic models with impaired CRR and hypoglycaemia awareness.

**Summary and future work**

The data presented in this chapter demonstrate that peripheral administration of a brain permeable AMPK activator raises glycaemia in an ANS-mediated manner and amplifies the CRR to hypoglycaemia by enhancing glucagon levels. The glucose-raising effect of R481 was attenuated by AMPK inhibition and ANS blockade, suggesting that R481 acts centrally and providing proof-of-concept that pharmacological activation of central AMPK may represent an import therapeutic target for amplifying the defence against hypoglycaemia. It also highlighted that central AMPK activation likely supersedes peripheral activation to generate a whole-body net glucose-raising effect to protect the brain from glucoprivation (Kume *et al.*, 2016).

Data from Rigel Pharmaceuticals demonstrated whole-brain AMPK activation following oral administration of R481 in mice. However, no evidence exists of the regional specificity of the drug or difference in bioavailability between mice and rats, so a critical next step is to assess whether R481 is actively transported into specific brain regions or undergoes pan brain transport, and which ganglia it regulates. Furthermore, a better understanding of the tissue-specific effects of R481 on AMPK activation, particularly central versus peripheral effects, is required to establish whether the compound amplifies CRR by hypothalamic-hindbrain-to-periphery axis or by direct action on α-cells. Using R419 during glucose clamps would also aid in distinguishing the central from peripheral effects as the compounds exhibit structural homology but different brain:plasma ratios. Notably, *in vivo* regulation of AMPK activity is also complex as studies assessing the effect of central activation or inhibition of AMPK must consider distinct kinase isoforms, time-courses, doses and target regions (such as different hypothalamic nuclei) (Alquier *et al.*, 2007). Nonetheless, these data show promising effects to support the use of indirect peripherally administered (translatable approach) AMPK activators to treat diabetes.

# Chapter 6

# General conclusions

**Main findings and future considerations**

Energy balance is achieved by a well-tuned interplay between multiple organs that regulate energy intake and energy expenditure to maintain homeostasis. Energy imbalance represents a fundamental aetiological factor for the development of obesity, diabetes and a number of increasingly prevalent conditions aggravated by increasingly common human behaviours such as overeating and inactivity. Impaired glucose homeostasis is a predominant feature of diabetes and maintaining blood glucose is still a daily challenge for individuals with both T1D and T2D. More and better strategies are continually being developed to counteract these metabolic impairments from “nutraceuticals” to “exercise-pills” to better and more sensitive monitoring and diagnostic technologies. However, a better understanding of the mechanisms regulating these impairments is still required to devise better-targeted and effective therapeutic strategies. As such, the primary aim of the work presented in this thesis was to better understand and target the pathophysiology of impaired glucose homeostasis.

Impaired glucose clearance by skeletal muscle is the main driver of impaired glucose disposal in diabetes (primarily T2D). Appropriate nutrient balance in skeletal muscle is also critical for muscle function and for maintaining mass and strength. In Chapter 3, mechanistically focused *in vitro* experiments aimed to elucidate the mechanisms by which excessive lipid availability alters insulin and amino acid sensitivity in muscle cells. It was hypothesised that the purinergic signalling system, particularly extracellular ATP which has well-documented pro-inflammatory effects as a signalling molecule, would participate in the metabolic impairments and inflammatory response following treatment with high concentrations of fatty acids. Data in the chapter suggests that extracellular ATP derived from palmitate treatment does not mediate the loss of anabolic response, mitochondrial dysfunction or release of inflammatory cytokines brought about by treatment with the fatty acid. Instead, novel roles of extracellular ATP in the regulation of glucose metabolism were elucidated, showing that the nucleotide may have important insulin and exercise mimetic properties that may be therapeutically targetable. Findings summarised in Figure 6.1.

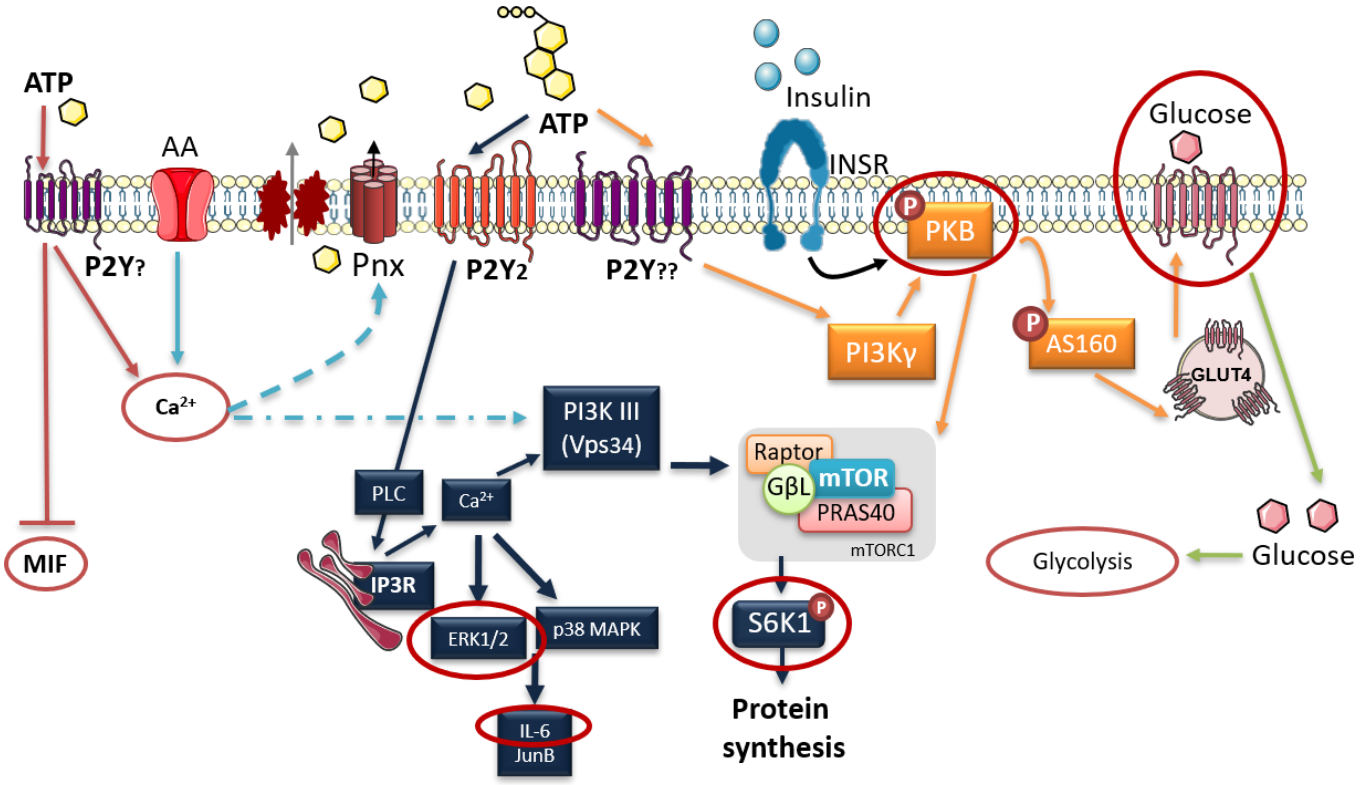



**Figure 6.1 Schematic summary of findings in Chapter 3**

Extracellular ATP-P2R-mediated regulation of glucose metabolism in skeletal muscle cells. Work by Ito *et al.,* (2018) (dark blue) demonstrated extracellular ATP-mediated phosphorylation of S6K1 (to stimulate hypertrophy) via activation of a P2Y2R/PI3K/PLC/IP3R pathway, through class III PI3K (Vps34) and not class I (PI3K/PKB) which primarily regulates insulin signalling. Work by Osorio-Fuentealba et al., (2013) (orange) demonstrated that ATP released from electrical stimulation of myotubes enhanced glucose uptake by increased mobilisation of GLUT4 transporters via a PI3Kγ-PKB-AS160. Work in this thesis (red circles) demonstrated that treatment with ATPγS enhanced the phosphorylation of ERK1/2 (positive control), S6K1 and PKB in a P2YR (but not P2X4R or P2X7R)-dependent manner and stimulated glucose uptake into C2C12 myotubes in an insulin-independent manner. In addition, treatment with ATPγS stimulated glycolysis in a P2YR-dependent manner. Furthermore, treatment with ATPγS resulted in increased release of IL-6 and suppression of MIF release. As expected, ATP treatment increased intracellular calcium, which was also observed following treatment with mixed essential amino acids (AA). AA treatment also enhanced extracellular ATP levels (dashed light blue lines; unknown mechanism) and enhanced S6K1 suggesting that the purinergic signalling system may, in part, modulate AA-dependent hypertrophy (feeding into a similar mechanisms to that demonstrated by Ito *et al.,* 2018). The P2R via which ATP exerts its glucose regulatory effects remains to be elucidated, as does the ability of ATP to stimulate glucose uptake in insulin resistant states (eg. lipid-induced insulin resistance).

The effects of increased lipid availability on muscle insulin and anabolic resistance may reside at the level of systemic impairments such as delivery of nutrients to skeletal muscle, and may be temporally regulated. As *in vitro* models are limited to assess these changes, the work in Chapter 3 was translated to a human study investigating the effect of elevated lipid availability in a disuse model of insulin resistance, in Chapter 4. Data in this chapter demonstrated, for the first time, that skeletal muscle glucose uptake is substantially impaired after only two days of immobilisation and that increasing lipid availability by overfeeding with saturated fats did not aggravate this effect. Novel evidence also demonstrated that postabsorptive and postprandial blood blow is not altered by immobilisation or affected further by diet, suggesting that nutrient delivery is preserved and alterations to nutrient balance occur due to impaired responses in muscle tissue.

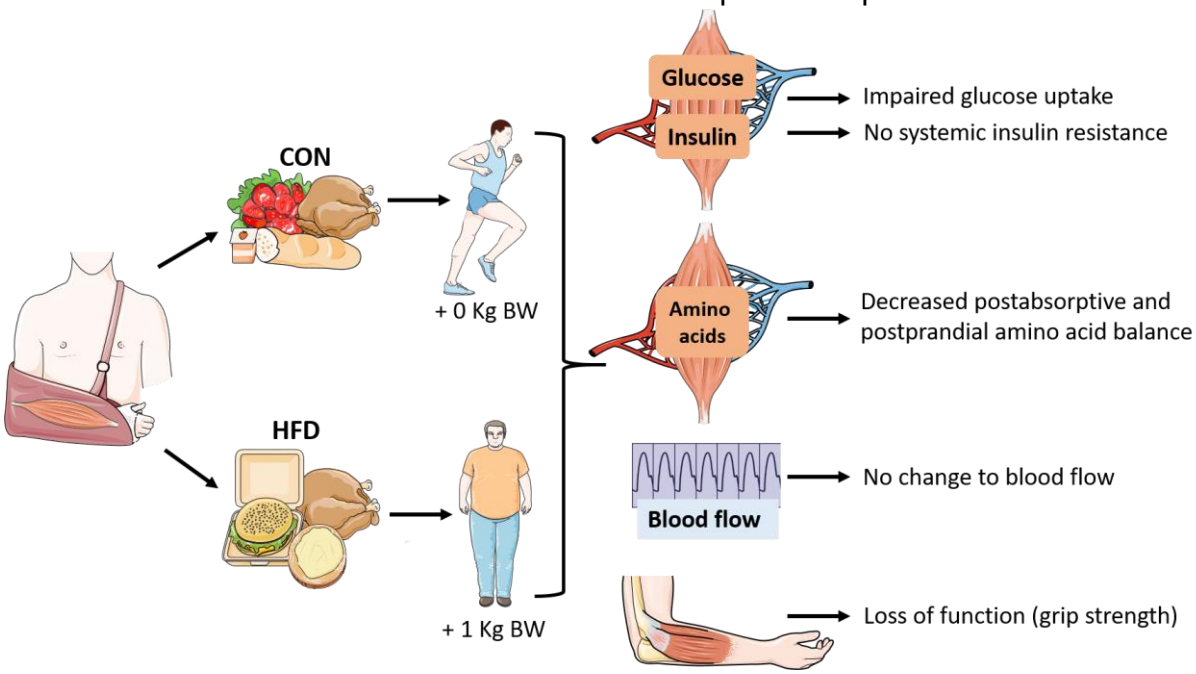



**Figure 6.2. Schematic summary of findings in Chapter 4**

Young healthy males were subjected to 7 days of forearm immobilisation under a controlled eucaloric diet (CON) or high-fat diet (HFD). Despite the increase in body mass (BW), the excess lipid availability in the HFD group did not exacerbate the effects of immobilisation on muscle substrate utilisation and function. Two days of immobilisation caused significant muscle insulin resistance (but not systemic) and this was not aggravated by an additional 5 days of disuse, suggesting that removal of contraction leads to a rapid decline in energy substrate demand by skeletal muscle (possibly due to intracellular negative feedback loops resulting in diminished substrate uptake). After 7 days, postprandial amino acid (leucine) balance was impaired, suggesting muscle anabolic resistance. Importantly, blood flow was not altered by immobilisation or diet, suggesting that impairments in nutrient balance do not occur as a result of diminished arterial delivery (despite possible microvascular impairments). It remains unclear whether insulin resistance causes anabolic resistance and whether impaired lipid metabolism contributes to their development.

Skeletal muscle energy metabolism is critical in diabetes pathophysiology, but glucose regulation occurs by more than one tissue/organ. Central regulation of glucose homeostasis is particularly important in the control of hypoglycaemia. Data in Chapter 5 addresses the pharmacological regulation of blood glucose, using AMPK as a target. AMPK activators are used widely in the treatment of T2D to lower glycaemia, primarily by acting on skeletal muscle and liver. However, emerging data suggest that central AMPK activation may be important to amplify the counterregulatory response to hypoglycaemia, to protect the brain from glucose deprivation. Data in Chapter 5 demonstrated that peripheral administration of a brain permeable AMPK activator (R481) regulates glycaemia by activation of the autonomic nervous system and amplifies the counterregulatory response to hypoglycaemia by increasing glucagon levels.

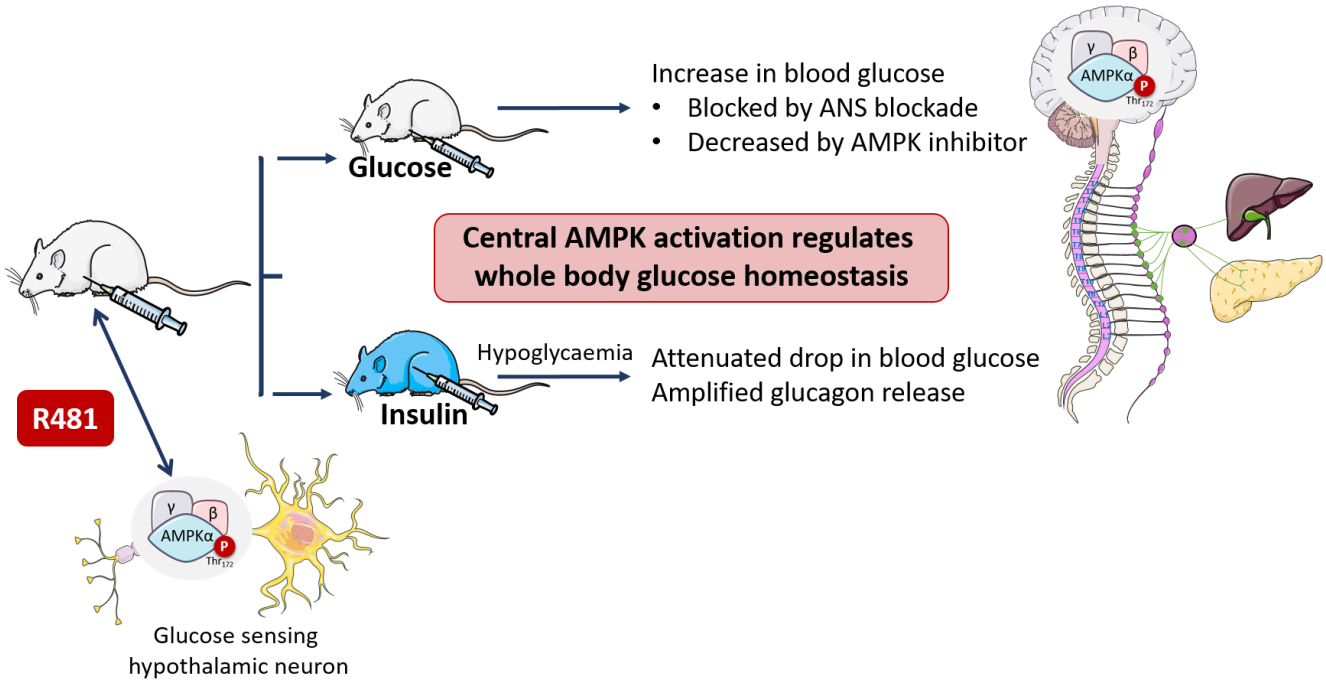



**Figure 6.3. Schematic summary of findings in Chapter 5**

Central AMPK activation in the regulation of whole body glucose homeostasis. *In vitro*, indirect AMPK activator R481 enhanced activation and activity of AMPK in glucose-sensing hypothalamic neurons. *In vivo*, peripheral administration of R481 (brain-permeable) enhanced glucose excursion in response to glucose administration in a manner that was blunted by blockade of the autonomic nervous system (ANS) with hexamethonium and attenuated by inhibition of AMPK with direct inhibitor SBI-0206965. In response to insulin treatment, R481 attenuated the insulin-induced drop in blood glucose and during a hyperinsulinaemic-hypoglycaemic clamp amplified the counterregulatory response to hypoglycaemia by enhancing glucagon release. Central AMPK activation may represent an important target for the recovery of impaired counterregulation in diabetes.

The different scientific approaches taken in these studies generated thought-provoking ideas and elucidated important processes, biomarkers and mechanisms that may be interconnected from an integrated physiology point of view. In this chapter, I would like to give an overview of concepts that were not discussed above and would represent interesting avenues to pursue following the work carried out here.

**Combating skeletal muscle metabolic impairments and atrophy in metabolic disease: ATP as a therapeutic tool**

Physical inactivity is a strong predictor of major metabolic diseases such as T2D (Booth *et al.*, 2002) and has been proposed as the 4$^{th}$ leading cause of death worldwide (Kohl *et al.*, 2012). Some situations result in inadvertent periods of inactivity, such as recovery from illness or injury and these are strongly associated with loss of muscle mass and metabolic health. Data in Chapter 4 demonstrated that two-days of immobilisation were sufficient to reduce muscle glucose uptake by 50 % in an insulin-stimulated state. After 7 days, muscle strength was 17 % decreased and glucose and amino acid balance were impaired. Although this local insulin and anabolic resistance develop more rapidly in experimental models of disuse compared to chronic physical inactivity (such as by sedentary lifestyles) these models enable mechanistic understanding and a platform to assess the effect of countermeasures to combat disuse-induced atrophy and metabolic impairments. Such countermeasures primarily focus on increasing/modifying physical activity and adjusting nutritional intake to match anabolic demand. Although effective, these are not always successful. Such measures have been studied extensively and have been expertly reviewed recently (Leenders and van Loon, 2011; Wall and van Loon, 2013; Dirks *et al.,* 2018; Hawley *et al.,* 2019) so will not be discussed here. Of interest, based on the work presented here, is the potential usefulness of molecules such as ATP (or small molecular ATP receptor agonists) to attenuate the loss of muscle mass and strength in metabolic disease.

Recent evidence suggests that reintroduction of a level of muscle contraction by application of neuromuscular electrical stimulation (NMES) can enhance both postabsorptive and postprandial muscle protein synthesis rates and prevent/attenuate muscle loss during short periods of disuse (Dirks *et al.*, 2014; Dirks *et al.,* 2018). Interestingly, as discussed in Chapter 3, electrical stimulation of muscle fibres leads to increased ATP release (Buvinic *et al.*, 2009; Osorio-Fuentealba *et al.*, 2013) as does exercise (González-Alonso *et al.,* 2002; Mortensen *et al.*, 2011). To date, no evidence exists to suggest that the benefits of NMES in delaying impairments brought about by disuse are mediated by ATP. Data presented in Chapter 3, supported by work from others (Osorio-Fuentealba *et al.*, 2013; Ito *et al.,* 2018) suggests that extracellular ATP also promotes glucose uptake into skeletal muscle and enhances mTOR signalling pathway activity. It would, therefore, be interesting to establish whether ATP (P2 receptor agonists or ATPase inhibitors) could be used to mimic the exercise and insulin sensitising effects of NMES, during disuse.

ATP is readily available as an oral supplement and has already been shown to promote hypertrophy after resistance exercise (Wilson *et al.*, 2013) and lower blood pressure in hypertensive women after exercise (de Freitas *et al.*, 2018). As ATP is rapidly metabolised by the action of ecto-nucleotidases, it is challenging to assess whether ATP bioavailability increases in the muscle vicinity (after oral dosing) to elicit these effects. It would therefore be interesting to assess the effect of intravenous ATP infusion in a model of disuse such as forearm immobilisation to investigate alterations to strength and insulin/anabolic sensitivity. As ATP also acts as a potent vasodilator, this could have important implications in diabetes. In T1D, muscle blood flow, capillary density and mass are reduced (Rivard *et al.*, 1999), and in T2D microvascular function becomes impaired and exacerbates muscle loss (Groen *et al.*, 2014). However, physical activity can prevent or reverse microvascular impairments in T2D, so it would be interesting to establish whether ATP supplementation could mimic these effects.

ATP is an attractive candidate to modulate in these disease models. However, it is important to consider the caveats of such an intervention, particularly given the widespread expression of purinergic receptors across multiple tissues. An important first step would be to understand the time (length of treatment or

repeated supplementation) and concentration-dependent regulation of glucose metabolism across tissues other than muscle, such as adipose tissue (Burnstock, 2014; Burnstock and Gentile, 2018), liver (Burnstock *et al.,* 2014) or the gut (Burnstock, 2016). Elucidating a specific P2R-mediated effect would then enable more targeted therapeutic developments as P2R agonists/antagonists are already commercially available and continually being developed (Burnstock, 2017; da Silva Ferreira *et al., 201*9).

Furthermore, it is now possible to measure ATP (and other nucleotides/nucleosides) in whole blood in near real-time. Sarissa Biomedical (Coventry, UK) have developed enzyme-coated electrode biosensors (SMARTChip and SMARTCap (Dale *et al.*, 2019) that detect the levels of these markers in a small drop of whole-blood within ~5 minutes. An ATP SMARTChip was developed for the first time to be piloted in the studies conducted in this thesis. Following observations of increased eATP in response to palmitate treatment in Chapter 3, it was hypothesised that eATP levels would be elevated after ingestions of a high-fat drink (pilot study) and 7 days of high-fat overfeeding, which would have been measurable in the study conducted in Chapter 4. Unfortunately, the turnover of generation of this novel tool was extremely low, meaning that only a small proportion of electrodes were trialled. The pilot data generated from the acute high-fat drink ingestion experiment is demonstrated in Appendix D. Importantly, optimisation of this technique led to measurable levels of ATP in whole blood so the technique could be used in future to test hypotheses where the primary outcome measure is a change in blood nucleotide/nucleoside levels. This could be applied to an immobilisation model to investigate changes to venous ATP output from immobilised muscle, as loss of the anabolic effect of eATP may also contribute to the metabolic impairments observed during disuse.

**Immunometabolic regulation of glucose homeostasis: role for autophagy?**

Under stresses such as inflammation or nutrient starvation, autophagy is stimulated to break down proteins and organelles (often damaged) and lipid droplets (Singh *et al.*, 2009) to provide amino acids and precursors necessary for maintaining cellular and metabolic homeostasis (Singh and Cuervo, 2011).

Dysregulation of the physiological function of autophagy occurs in the pathophysiology of a number of conditions, including diabetes (Bhattacharya *et al.*, 2018). Under sufficient nutrient supply, mTOR directly phosphorylates ULK1 (S757) to inhibit its activation of autophagy whereas nutrient deprivation/starvation activates AMPK, which directly phosphorylates ULK1 (S317 and S777) (Egan *et al.*, 2011; Dunlop and Tee, 2013) (and inhibits mTOR (Kim *et al.*, 2011) to promote autophagy. ULK1 initiates autophagy by activation of the Class III PI3K complex (PI3KC3-C1), of which Vps34 is a critical component (Stjepanovic *et al.*, 2017).

Autophagy is regulated by a number of stimuli, pathways and pathophysiological processes mentioned in this thesis, but elucidating these mechanisms is challenging as they appear to be altered by nutrient/hormonal status. For example, regulation of autophagy by excess lipid availability, as modelled in Chapters 3 and 4, appears to be bi-phasic. This was demonstrated in the liver of mice fed a high-fat diet for 2 weeks, which enhanced autophagy, compared to mice on a high-fat diet for 10 weeks or exposed to an acute bolus of high lipid concentration, where autophagy was suppressed (Papáčková *et al.*, 2012). The latter may be a consequence of insulin resistance and hyperinsulinaemia, as this has been shown to blunt the expression of autophagy genes such as *vps34* (Liu *et al.*, 2009) or possible hyperactivation of mTOR, which is observed in hepatic tissue from HFD-fed mice (Korsheninnikova *et al.*, 2006; Wang *et al.*, 2010). In the brain, chronic HFD suppresses autophagy, and decreased hypothalamic autophagy appears to exacerbate diet-induced obesity (Meng and Cai, 2011). Data for the role of excess lipid availability on autophagy in skeletal muscle is less consistent (Campbell *et al.*, 2015), but reports suggest that alterations in mitophagy may play a role in the development of insulin resistance (Kim *et al.*, 2013), particularly given the role of lipid-induced mitochondrial dysfunction in this process (as seen in Chapter 3).

In Chapter 3, overnight treatment of muscle cells with palmitate also enhanced MIF release, whereas treatment with ATPγS decreased MIF release. MIF has been shown to induce autophagy in cardiomyocytes (Xu *et al.*, 2013, 2014), endothelial cells (Chen *et al.*, 2015) and in human hepatoma cell lines (Chuang *et al.*, 2012), but its roles in muscle and brain are understudied.

MIF secretion is enhanced during amino acid and serum starvation and its autophagy-inducing effects appear to be partly mediated by increased ROS generation (Chuang *et al.*, 2012). Although nutrient deficiency increases MIF, particularly under stresses such as inflammation and starvation, MIF plasma levels are elevated in humans with T2D (Toso *et al.*, 2008), impaired glucose tolerance (Kleemann and Bucala, 2010) and T1D (Hanifi-Moghaddam *et al.*, 2003). It is plausible that a decrease in energy availability leads to increased MIF release (Chuang *et al.*, 2012), as a signal of nutrient deficiency and that this is dysregulated in metabolic disease, where mitochondrial dysfunction leads to excessive ROS. It would be interesting to establish a relationship between amino acid availability, MIF and extracellular ATP and their roles in autophagy as, given the data presented here, it appears that amino acids regulate extracellular ATP and eATP regulates both mTOR signalling and MIF release. This could have important implication for skeletal muscle as although autophagy is critical for maintenance of muscle mass and strength (Masiero *et al.*, 2009), excess autophagy induces muscle atrophy (Sandri, 2013). Importantly, this relationship could also be relevant in the context of caloric/amino acid restriction and the metabolic and longevity benefits associated with these interventions. Indeed, methionine-restriction in rodents (known, like caloric restriction, to increase lifespan) leads to decreased glucose and insulin levels (Masoro *et al.*, 1992; Miller *et al.*, 2005) and increased hepatic MIF mRNA levels (Miller *et al.*, 2005). Furthermore, as discussed in Chapter 5, AMPK is an important inducer of autophagy and is differentially regulated by these nucleotides and cytokines.

Although AMPK activation is well-characterised as a potent stimulus to initiate autophagy, this regulation appears to change in response to glucose availability, similarly to its metabolic roles discussed in Chapter 5. For instance, silencing of AMPK can induce autophagy to protect β-cells from hyperglycaemia (Han *et al.*, 2010). Autophagy also mediates a number of processes that are altered by AMPK activation dependently or independent of activation of AMPK. For example, in hypothalamic AgRP and POMC neurones, genetic suppression of autophagy dysregulates their control of energy balance. Suppression of autophagy in AgRP neurones causes leanness (Kaushik *et al.*, 2011) and loss of autophagy in POMC neurones leads to increased adiposity, body weight and altered glucose

homeostasis (Coupé *et al.*, 2012) in mice. Pharmacological AMPK activators/inhibitors can also alter autophagy independently of AMPK. In cancer cells, Compound C induces protective autophagy by suppressing PKB/mTORC1 signalling (Vucicevic *et al.*, 2011) and AICAR suppresses autophagy by interfering with formation of the PI3KC3-C1 complex in fibroblasts (Viana *et al.*, 2008). Given the observations in Chapter 5, it would be interesting to better elucidate the effect of indirect and direct AMPK activators on autophagy, particularly during low glucose availability.

**Adenosine signalling in the pathophysiology of Diabetes: strategy to treat hypoglycaemia?**

Extracellular ATP has been discussed throughout this thesis as a pleiotropic signalling moiety that may be used as a tool to mimic insulin or exercise effects in skeletal muscle. This is primarily driven by the roles played by transiently increased levels of eATP on glucose metabolism, supported by work presented here. However, chronically elevated eATP likely leads to its well-characterised inflammatory effects in numerous tissues. In addition, an increase in eATP availability will also generate elevated levels of adenosine extracellularly, which may directly contribute to metabolic disease progression. Indeed, accumulating evidence highlights a role for extracellular adenosine signalling in the regulation of glucose homeostasis (Koupenova and Ravid, 2013) and the pathophysiology of T1D and T2D (Johnston-Cox *et al.*, 2012; Yip *et al.*, 2013; Csóka *et al.*, 2014).

Adenosine regulates glucose metabolism in tissues such as skeletal muscle, liver and adipose tissue by acting via P1 receptors $A_1R$, $A_{2A}R$, $A_{2B}R$ and $A_3R$ and has demonstrated both glucose-raising and lowering actions. For example, pharmacological stimulation of hepatic $A_{2B}Rs$ stimulates hepatic glucose production and glycogenolysis in primary rat hepatocytes (Harada *et al.*, 2001; Yasuda *et al.*, 2003) and in obese and diabetic mice (Figler *et al.*, 2011). Furthermore, inhibition of adenosine transporter ENT1 in hepatocytes leads to enhanced hepatic glucose production and suppresses the effect of AMPK activator AICAR on glucose production (Logie *et al.*, 2018). Adenosine (and analogues) also demonstrates glucose-raising effects by direct stimulation of glucagon secretion (in a concentration-dependent manner) from α-cells (Chapal *et al.*, 1985) and suppression of insulin secretion (via $A_1R$) in perfused rat

pancreas (Hillaire-Buys *et al.*, 1987). Paradoxical evidence, however, suggests that adenosine can enhance insulin secretion by activating $A_{2A}$Rs, and that $A_{2B}$R activity may suppress hepatic glucose production (Csóka *et al.*, 2014). Within the context of T2D, activation of $A_{2A}$Rs in brown adipose tissue appears to stimulate thermogenesis, which protects mice against diet-induced obesity (Gnad *et al.*, 2014) whilst activation of $A_{2B}$Rs in skeletal muscle is associated with the development of insulin resistance (Figler *et al.*, 2011). Furthermore, stimulation of $A_1$Rs in adipose tissue increases lipogenesis and reduces lipolysis, whereas $A_{2B}$R decrease adipogenesis and adipose tissue inflammation. In skeletal muscle, $A_1$Rs can increase or decrease glucose uptake (Vergauwen, Hespel and Richter, 1994) whereas $A_{2B}$R decreases glucose uptake (Antonioli *et al.*, 2015). These discrepancies often arise from differing experimental approaches, using pharmacology or genetic manipulation to agonise/antagonise these pathways; which highlights the need for clearer evidence for the roles played by the adenosine signalling system on glucose homeostasis. Nonetheless, a growing body of evidence suggests that adenosine signalling does modulate glucose homeostasis and a number of these functions may be therapeutically targetable. Of interest, is the understudied use of adenosine to modulate the counterregulatory response to hypoglycaemia.

Adenosine has a rapid half-life in plasma (< 10 s) as it is rapidly cleared and metabolised (Moser *et al.,* 1989; Marlinge *et al.*, 2017), meaning that adenosine raising compounds or P1R agonists may represent better therapeutic tools. Such compounds may already be used clinically but be repurposed for this use. For instance, rosuvastatin, a potent statin primarily used to lower plasma cholesterol, has been reported to increase HbA1c levels in individuals with and without diabetes (Ridker *et al.*, 2008; Ooba *et al.*, 2016). Interestingly, rosuvastatin has also demonstrated adenosine raising effects in humans (Meijer *et al.*, 2009) by inhibiting ENT1. Given the roles of adenosine receptor activation in liver and α-cells described above, it would be interesting to assess the effect of a drug such as rosuvastatin on hypoglycaemia counterregulation. Importantly, it would also be critical to establish whether this off-target effect is mediated via adenosine. To test such a hypothesis a series of *in vivo* experiments would be conducted in diabetic models using acute and chronic administration of the compound and

performing hyperinsulinaemic-hypoglycaemic clamps to assess changes to counterregulatory hormones and blood glucose.

The benefit of using a clinically-tested widely available compound is the rapid translatability to individuals with diabetes. As mentioned above, technologies such as the SMARTChip (Dale *et al.*, 2019) can be used to measure adenosine in near real-time in a clinical setting. It would be interesting to apply this to a study comparing daily glucose profiles (time in target blood glucose using CGMS) of individuals with T1D treated with rosuvastatin or placebo (chronically) to test a relationship between blood glucose and plasma adenosine levels.

**Conclusions**

This thesis highlights the complexity of the integrated physiology of glucose control. It is becoming increasingly apparent that activation of specific proteins/pathways may regulate glycaemia/metabolism differentially depending on energy status and target tissues. Here, extracellular ATP enhanced glucose uptake and utilisation in muscle cells suggesting that, in the whole-body, eATP may be blood glucose lowering. However, breakdown of ATP into adenosine leads to adenosine-mediated blood glucose-raising actions. Although these paradoxical mechanisms may occur synergistically and continuously, it remains important to identify the specific tissue impairments or metabolic status that dysregulates these processes to contribute to the pathophysiology of metabolic disease.

The same applies to lipid-induced insulin and anabolic resistance. It is clear from data presented here that lipid overflow in muscle cells causes insulin and anabolic resistance by impairing intracellular machinery. Although this likely occurs in humans, it appears from human data presented here that lipid-induced changes may be negligible if metabolic function is already impaired, such as during disuse. However, it is plausible that disuse dysregulates anabolic sensitivity by impairing lipid metabolism in the first place. Therefore, it is important to understand the crosstalk between these metabolic states and how they bring about impaired glucose (and in this case amino acid) balance.

During hypoglycaemia, central AMPK activation appears to protect the brain from glucose deprivation, but in peripheral tissues, AMPK activation is anti-hyperglycaemic. This represents a challenge pharmacologically as inadequate activation of AMPK peripherally during hypoglycaemia could aggravate this. R481-like compounds have therapeutic potential as they protect against hypoglycaemia without lowering blood glucose, suggesting that central effects of AMPK activators may supersede peripheral roles in response to a change in glucose availability.

Going forward, the understanding of disease pathophysiology or development of novel therapeutic strategies should re-enforce the importance of tissue-specific or metabolic status specific actions of the targets of interest.

# Chapter 7

# Appendices

## Appendix A

SPORT AND HEALTH SCIENCE
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**Project Title**
The effect of physical inactivity and overfeeding on on muscle amino acid uptake

**Principle Investigators:** Marlou Dirks, Dr Benjamin Wall, Dr Francis Stephens, Mandy Dunlop, Dr Jonathan Fulford, Dr Rob Andrews, Dr Sarah Jackman, Dr Lee Wylie, and Ana Cruz

**Information sheet for participants (Version 4: 13-04-2017)**

You are being invited to take part in this research study. Before you decide it is very important for you to understand why the research is being undertaken and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything which is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide not to take part, there will be no disadvantage to you of any kind and we thank you for considering our request.

**What is the purpose of this study?**
With ageing, we experience a progressive loss of muscle tissue, which affects strength and quality of life. Individuals with type-2 diabetes lose more muscle tissue with advancing age. The reason is likely linked to reduced physical activity and/or overeating, leading to an impaired ability of muscles to extract important nutrients from blood. This study aims to identify *how* physical inactivity and overeating affect the muscle's response to nutrition. We will measure how arm muscles of young people obtain nutrients from their blood after they have been made physically inactive and fed a normal or excessive energy diet for 7 days. As such, this study will inform future research to attenuate/prevent muscle loss in older individuals with and without type-2 diabetes, thereby supporting metabolic health and quality of life.

**What types of participants are needed?**
We are inviting healthy, young men aged 18-40 years to take part in this study. We are looking to recruit recreationally active individuals, if you are currently involved in an intensive endurance or resistance training programme you will also not be allowed to participate. After the screening procedure a decision will be taken if you are suitable for the study i.e. you do not violate any of the exclusion criteria. The exclusion criteria include:

- A body mass index (BMI) below 18 or above 30 kg/m$^2$

- Regular smoker
- Any diagnosed metabolic impairment (e.g. type 1 or 2 Diabetes).
- Any diagnosed cardiovascular disease or hypertension.
- Elevated blood pressure at the time of screening.
- Chronic use of any prescribed or over the counter pharmaceuticals.
- Regular use of nutritional supplements (e.g. creatine, protein supplementation)
- Metallic implants (including heart pacemaker, cochlear implants, medication pumps, surgical clips, plates or screws).
- A personal or family history of thrombosis, epilepsy, seizures or schizophrenia.
- Any previous motor disorders.

**What will participants be asked to do?**
The study will involve 5 visits to the laboratory. A simple summary of the study is that we want to assess what happens to muscle when it is not being used (contracted); this is known as 'muscle disuse'. To answer the question of what happens to a muscle when you do not use it we will make a variety of measurements before during and after the 7 days of forearm casting. During that week, you will be randomly allocated to energy matched standardized food, or high-fat overfeeding (providing 50% more energy than the energy-matched diet, predominantly from fat).

So firstly to immobilise the muscle we will use a cast (on one forearm) and a sling so you will not be able to use that arm for the entire 7 day period. You will always have to wear the cast, including when washing (a cover will be provided for the cast).

We will assess forearm muscle size of both arms 3 times over the study period and this will be done via a magnetic resonance imaging (MRI) scan. Each MRI scan will last approximately 20 minutes and will take place at the Medical School on St. Luke's campus. The scan will involve you lying on the bed of the MRI scanner facing upwards with your arms next to your body, and you will be rolled into the MRI scanner so both arms are inside the centre of the MRI scanner. The MRI scans will happen on the baseline visit (between 10 and 7 days before immobilization), on day 2 of immobilization and on day 7 (immediately following cast removal).

To assess the impact of inactivity and dietary intake on the uptake of nutrients from the diet, you will undergo a metabolic test on the same days as the MRI scans. For this test, you will be laying on a bed in the laboratory for a 4.5 h period. During this period you can watch movies or series on the television which is present. Before the test, we will have three cannulas inserted in your arms: one in each elbow and one on a hand. During the entire 4.5 h period, we will have an infusion of stable isotope amino acids running to quantify the amount of amino acids (the building blocks of proteins) that are taken up by the muscle. The other two cannulas will be used to take a total of 12 small blood samples throughout the test. On three occasions during the first and last visit a finger prick will also be performed to get a small blood sample. Additionally, we will use an ultrasound device on the inside of your elbow to measure blood flow at the same times as we take a blood sample. After 90 min, you will receive a drink containing a mixture of carbohydrates, protein and fat to assess the effect of food intake on the uptake of nutrients by the arm muscles. After another 3 hours, the test day will be finished.

Nowadays, inactivity is also accompanied by an increased energy intake, which could be contributing to the negative effects that are observed. In order to test this, we will provide you with a standardized diet during the 7 days of forearm immobilization. You will receive all your daily food, composed of three daily main meals and several daily snacks. You are requested to eat at certain set times, so the normal meal moments. During this week you are not allowed to consume other food/drink products than the

products you receive from us. Half of the participants will receive food products which are matched to their energy expenditure, and the other half of the participants will receive food products which are 50% higher in calories (predominantly from fat) than their normal diet.

Having an adequate supply of a molecule called nitric oxide is very important for maintaining a healthy body. However, it is possible that the consumption of a high fat diet could lower its availability by reducing the amount of a particular type of oral bacteria that are responsible for producing it. To test if this is the case, we will also ask you to provide an oral swab sample, and a saliva sample, on the same days as the MRI visits. The oral swab samples simply involve the researcher brushing your tongue for 3-5 seconds with a small, soft brush.

Lastly you will be asked to record your diet (both food and drink) intake to get an idea of your habitual dietary intake. To do this we need you to write down everything you eat and drink with a description of the items e.g. how much (weight or volume) and brand names. You will be given recording sheets to do this during the screening visit and will be asked to record your diet for a total of 3 days, to be completed prior to the baseline visit.

A detailed overview of the visits for this study is provided below:

| Visit number | Visit description | Task completed during visit |
|---|---|---|
| 1 | Screening | • Informed consent gained (if not already previously gained).<br>• Explanation of study by experimenter and shown equipment and tests.<br>• Height and weight measurement.<br>• Body composition measurement e.g. % body fat and muscle via BODPOD.<br>• Shown how to fill out diet diary. You will do this for 3 days prior to the baseline visit (visit 2).<br>• Health screening form completed.<br>• Oral health questionnaire completed. |
| 2 | Baseline (between day -10 and -7) | • Arrive at the lab at 8:15, while being fasted.<br>• MRI scan of both arms arm to assess muscle size.<br>• Take place on the bed, where you will be rested for 4.5 h. You can watch movies during this time.<br>• Insertion of three venous cannulas: two in the elbow veins and one on the hand. From these cannulas, a total of 12 blood samples will be taken during this day. Finger pricks will also be conducted alongside three of the samples. Together with every blood sample, blood flow measurements are performed using an ultrasound device.<br>• Start infusion of the stable isotope infusion.<br>• After 15-30 min, you will be asked to provide a saliva and oral swab sample.<br>• After 90 min, you will be given a test drink. |

| | | |
|---|---|---|
| | | • 3 hours after consuming the drink, the test is finished.<br>• Grip strength will be assessed. |
| 3 | Immobilisation<br><br>(day 0) | • Arrive at the lab at 7:30; you do not need to be fasted.<br>• Forearm cast is applied.<br>• You will be given a sling to put your arm in.<br>• You will receive weighed and packaged food for the first 3 days (day 0, 1, and 2). |
| 4 | Experimental<br><br>(day 2) | • Arrive at the lab at 8:15, while being fasted<br>• MRI scan of the immobilised arm to assess muscle size<br>• Take place on the bed, where you will be rested for 4.5 h. You can watch movies during this time.<br>• Insertion of two venous cannulas: one in the elbow vein and one on the contralateral hand. From these cannulas, a total of 12 blood samples will be taken during this day. Together with every blood sample, blood flow measurements are performed using an ultrasound device.<br>• You will be asked to provide a saliva and oral swab sample.<br>• After baseline measurements, you will be given a test drink.<br>• 3 hours after consuming the drink, the test is finished.<br>• You will receive weighed and packaged food for the last 4 days (day 3, 4, 5, 6). |
| 5 | Experimental<br><br>(day 7) | • Arrive at the lab at 8:00, while being fasted.<br>• The cast will be removed.<br>• MRI scan of the (previously immobilised) arm to assess muscle size.<br>• Take place on the bed, where you will be rested for 4.5 h. You can watch movies during this time.<br>• Insertion of three venous cannulas: two in the elbow veins and one on the hand. From these cannulas, a total of 12 blood samples will be taken during this day. Finger pricks will also be conducted alongside three of the samples. Together with every blood sample, blood flow measurements are performed using an ultrasound device.<br>• Start infusion of the stable isotope infusion.<br>• After 15-30 min, you will be asked to provide a saliva and oral swab sample.<br>• After 90 min, you will be given a test drink.<br>• 3 hours after consuming the drink, the test is finished.<br>• Grip strength will be assessed. |

**Are there any risks associated with taking part?**
The risks involved in this study are minimal. Insertion of the cannulas for blood sampling may cause some temporary discomfort. However, these techniques are regularly used

in physiology testing. The investigators are trained and experienced in all aspects of these procedures to ensure they are completed safely. Every effort will be made to minimise the above risks by thorough instruction and observation of symptoms during testing sessions.
Please be aware that any concerns that you have about any of the procedures will be addressed in full by the researcher prior to you giving your consent to participate.

**Can I change my mind?**
You can stop being in the project at any time without giving a reason and without any disadvantage to yourself of any kind.

**What data or information will be collected, and how will it be used?**
The samples will be code labelled without showing the participant's name and stored securely. Samples will be disposed of immediately once the analysis has been completed. Your anonymity will be protected in the case of publication of our findings. Any data collected will be used to establish response profiles across the group of subjects involved and these cumulative scores may be available for public inspection in research journals and/or seminars and conferences. In addition, individual response profiles indicative of the typical response may also be presented. However, in all cases, anonymity will be strictly preserved. Therefore, while results of this project may be available for public inspection; any data so displayed will in no way be linked to any specific individual. Upon completion, the data will be securely stored in such a way that only the researchers involved in this investigation will be able to gain access to it. At this point, you are most welcome to request a copy of the results of the project should you wish and we will be available to explain and interpret your specific data and how it compares to the results of the group as a whole.

**What if participants have any questions?**
If you have any questions about our project, either now or in the future, please feel free to contact Marlou Dirks or Mandy Dunlop:

| | | |
|---|---|---|
| Marlou Dirks | 01392 725496 | m.dirks@exeter.ac.uk |
| Mandy Dunlop | 01392 724715 | m.v.dunlop@exeter.ac.uk |
| Ana Cruz | | a.cruz@exeter.ac.uk |

This project has been approved by the Ethics Committee of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter.

**Appendix B**

**Medical Questionnaire**

This questionnaire belongs to the study 'The effect of physical inactivity and overfeeding on muscle amino acid uptake'. Please fill out this questionnaire as completely as possible. All information in this questionnaire is considered confidential.

| General questions | | |
|---|---|---|
| Name and initials: | | |
| Address: | | |
| Postal code and city: | | |
| Date of birth: | | |
| Gender: | ☐ Male | ☐ Female |
| Ethnicity | | |
| Phone number: | Home: | |
| | Work: | |
| | Mobile: | |
| E-mail address: | | |
| Occupation / Study: | | |
| Weight: | kg | |
| Height: | m | |
| Are you currently in another study? | | ☐ Yes ☐ No |
| If yes, which study and with which researcher? | | |
| Have you participated in studies in the past? | | ☐ Yes ☐ No |
| If yes, with which researcher? | | |

| General health | |
|---|---|
| Do you feel healthy at the moment? | ☐ Yes ☐ No |
| If no, what are the complaints? | |
| Are you currently being treated by a doctor, dentist, paramedic or alternative healer? | ☐ Yes ☐ No |
| If yes, by whom? | |
| For what reason? | |
| Have you been ill in the past 12 months? | ☐ Yes ☐ No |
| If yes, how many days? | |
| When? | |
| What were the complaints? | |
| Do you have a sensitivity or allergy? | ☐ Yes ☐ No |
| If yes, for what?<br>☐Food<br>☐ Medicine<br>☐ Penicillin (antibiotics)<br>☐Iodine or brown bandaids<br>☐ Other: | |
| When did you first notice? | |
| Do you have complaints about your **back, legs, knees or shoulders**? | ☐ Yes ☐ No |
| If yes, where?<br>☐Back<br>☐ Legs<br>☐ Knees<br>☐Shoulders | |
| What is the nature of these complaints? | |
| Have you donated blood in the past 3 months? | ☐ Yes ☐ No |
| If yes, when | |
| Medical history | |
| Have you ever had problems with your kidneys or urinary tract? (for example inflammation, kidney stones, blood loss) | ☐Yes ☐ No |
| If yes, what kind of problem(s)? | |
| When? | |
| How often? | |
| How was/is it treated? | |
| Have you ever undergone **surgery**? | ☐ Yes ☐ No |
| If yes, on what? | |
| When? | |
| Why? | |
| Do you have **diabetes**? | ☐Yes ☐ No |

| | |
|---|---|
| If yes, since when? | |
| How is/was it treated? | |
| Have you or anyone in your family ever had thrombosis? | ☐Yes ☐ No |
| If yes, who and when? | |
| What was the cause and how was/is it treated? | |

| Drugs | |
|---|---|
| Are you **currently** using any sleeping aids or drugs? (including aspirin or paracetamol) **If yes, which?** | ☐ Yes ☐ No |
| Drug: - | |
| Why? - | |
| How often? | |
| Since when? - | |
| Drug: - | |
| Why? - | |
| How often? - | |
| Since when? - | |
| Have you used any sleeping aids or drugs in the **past year**? (including aspirin or paracetamol) **If yes, which?** | ☐ Yes ☐ No |
| Drug | |
| Why? - | |
| How often? | |
| Since when? - | |
| Drug: - | |
| Why? | |
| How often? - | |
| Since when? | |

| Nutrition | |
|---|---|
| Has your **body weight** the past 3 months: | |
| **Increased** with more than 3 kg? | ☐ Yes ☐ No |
| If yes, how many kg? | kg |
| Reason: | |
| **Decreased** with more than 3 kg? | ☐ Yes ☐ No |

| | |
|---|---|
| If yes, how many kg? | kg |
| Reason: | |
| Are you on a (medically prescribed) diet? (For example, cholesterol lowering diet, weight loss diet or macrobiotic diet.) | ☐ Yes ☐ No |
| If yes, what kind of diet? | |
| Why are you on this diet? | |
| Are you a **vegetarian**? | ☐ Yes ☐ No |
| Do you normally eat breakfast? | ☐ Yes ☐ No |
| Are you using dietary supplements? | ☐ Yes ☐ No |

| If yes, which? | Which brand? | How often? | Why? |
|---|---|---|---|
| ☐ Vitamins | | | |
| ☐ Mineral | | | |
| ☐ Fiber | | | |
| ☐ Other: | | | |

On average, how many times do you eat the following foods (please circle)

Meat, meat products and meat dishes

| | | | |
|---|---|---|---|
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week |

Fish and fish products

| | | | |
|---|---|---|---|
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week |

Eggs and dairy products (milk, yoghurt, cheese)

| | | | |
|---|---|---|---|
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week |

Beans, peas and lentils

| | | | |
|---|---|---|---|
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week |

Nuts and whole grains (e.g. bulgur, quinoa, amaranth, oats)

| | | | |
|---|---|---|---|
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week |

| | | | | |
|---|---|---|---|---|
| Tofu, soya meat, TVP, soya products | | | | |
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week | |
| Protein enriched foods (e.g. protein shakes/bars etc.) | | | | |
| Never or < 1x per month | 1-3x per month | Once a week | >2-4 x per week | |

| | | |
|---|---|---|
| Recreational drugs / alcohol | | |
| Do you drink alcoholic beverages? | ☐ Yes | ☐ No |
| If yes, what kind? | | |
| How many?<br>☐ 1-7 glasses per week<br>☐ 8-14 glasses per week<br>☐ 15-21 glasses per week<br>☐ more than 21 glasses per week | | |
| Do you smoke tobacco? | ☐ Yes | ☐ No |
| If yes, how often?<br>☐ less than once per day<br>☐ 1-5 times per day<br>☐ 6-10 times per day<br>☐ more than 10 times per day | | |
| If no, have you smoked in the past? | ☐ Yes | ☐ No |
| Do you use nicotine patches or nicotine gum? | ☐ Yes | ☐ No |
| If yes, what kind? | | |
| How often? | | |
| Do you use recreational drugs? | ☐ Yes | ☐ No |
| If yes, what kind? | | |
| How often? | | |
| Physical activity | | |
| Generally how active would you say you are? (please circle) | | |
| Very Slightly Average Quite active Very active | | |
| Approximately how long do you spend sitting per day? (please circle) | | |
| < 3 hours 3-6 hours 6-9 hours 9-12 hours >12 hours | | |
| Do you exercise regularly? (fitness, sports) | ☐ | ☐ No |
| If yes, what kind of sport(s)? | | |
| How many hours per week? | | |

| **Do you have anything else you want to report about your health?** | | |
|---|---|---|
| | | |
| **Completed as true and correct,** | | |
| **Place** | **Date** | **Signature** |
| | | |

## Appendix C

Score:…….. PAL:…..

### INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

**PART 1: JOB-RELATED PHYSICAL ACTIVITY**

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐ Yes

☐ No *Skip to PART 2: TRANSPORTATION*

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

☐ No vigorous job-related physical activity *Skip to question 4*

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?

_____ hours per day

_____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.

_____ days per week

☐ No moderate job-related physical activity *Skip to question 6*

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ hours per day

_____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

_____ days per week

☐ No job-related walking *Skip to PART 2: TRANSPORTATION*

7. How much time did you usually spend on one of those days walking as part of your work?

_____ hours per day

_____ minutes per day

**PART 2: TRANSPORTATION PHYSICAL ACTIVITY**

These questions are about how you travelled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

_____ days per week

☐ No traveling in a motor vehicle *Skip to question 10*

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

_____ hours per day

_____ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

_____ days per week

☐ No bicycling from place to place *Skip to question 12*

11. How much time did you usually spend on one of those days to bicycle from place to place?

_____ hours per day

_____ minutes per day

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?

_____ days per week

☐ No walking from place to place *Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY*

13. How much time did you usually spend on one of those days walking from place to place?

_____ hours per day

_____ minutes per day

***PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shovelling snow, or digging in the garden or yard?

_____ days per week

☐ No vigorous activity in garden or yard *Skip to question 16*

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?

_____ days per week

☐ No moderate activity in garden or yard *Skip to question 18*

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

_____ days per week

☐ No moderate activity inside home *Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY*

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

_____ hours per day

_____ minutes per day

***PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY***

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

_____ days per week

☐ No walking in leisure time *Skip to question 22*

21. How much time did you usually spend on one of those days walking in your leisure time?

_____ hours per day

_____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

_____ days per week

☐ No vigorous activity in leisure time *Skip to question 24*

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?

_____ hours per day

_____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

_____ days per week

☐ No moderate activity in leisure time *Skip to PART 5: TIME SPENT SITTING*

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

_____ hours per day

_____ minutes per day

***PART 5: TIME SPENT SITTING***

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

_____ hours per day

_____ minutes per day

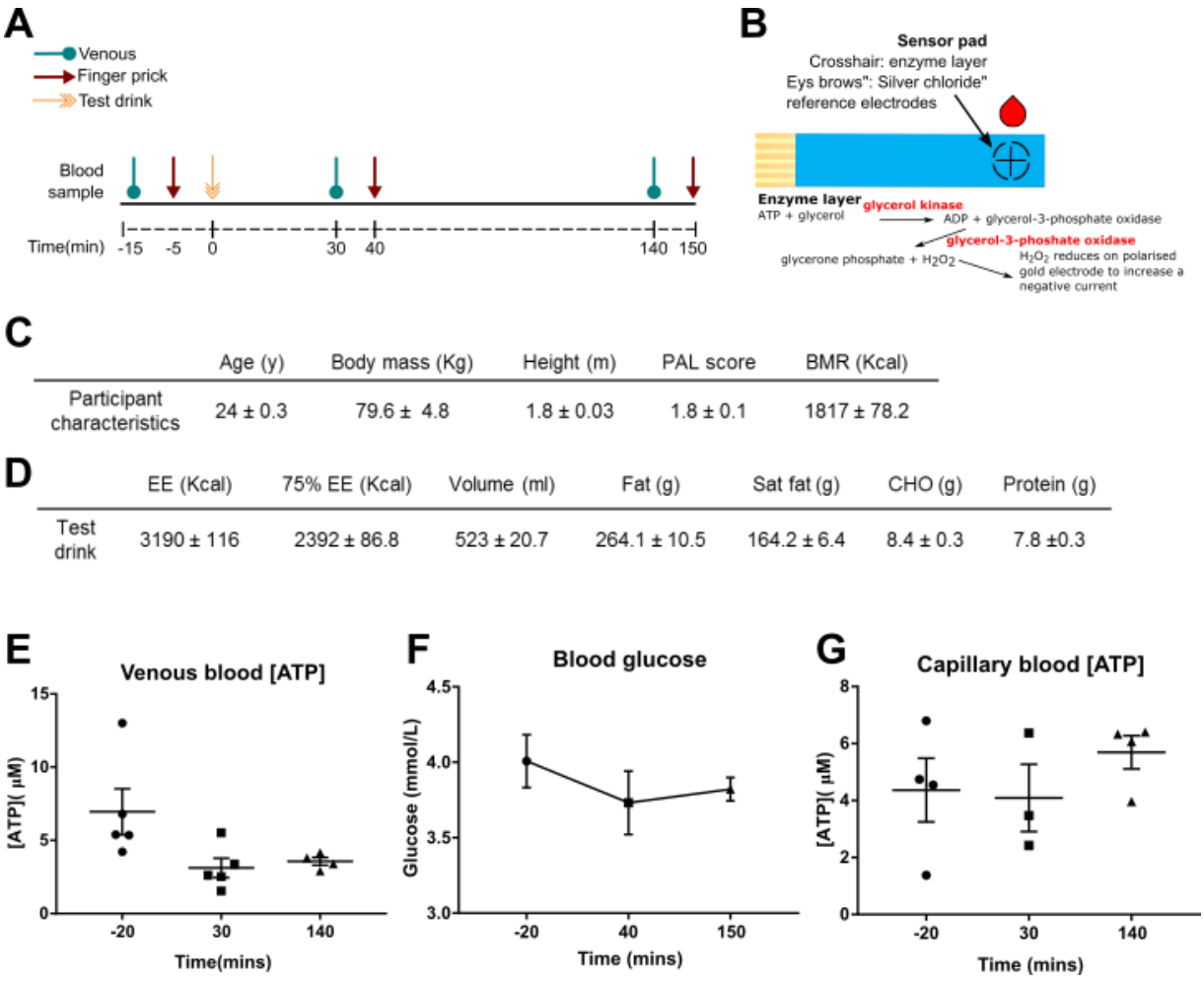
27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

_____ hours per day

_____ minutes per day

**This is the end of the questionnaire, thank you for participating.**

## Appendix D




C

| | Age (y) | Body mass (Kg) | Height (m) | PAL score | BMR (Kcal) |
|---|---|---|---|---|---|
| Participant characteristics | 24 ± 0.3 | 79.6 ± 4.8 | 1.8 ± 0.03 | 1.8 ± 0.1 | 1817 ± 78.2 |

D

| | EE (Kcal) | 75% EE (Kcal) | Volume (ml) | Fat (g) | Sat fat (g) | CHO (g) | Protein (g) |
|---|---|---|---|---|---|---|---|
| Test drink | 3190 ± 116 | 2392 ± 86.8 | 523 ± 20.7 | 264.1 ± 10.5 | 164.2 ± 6.4 | 8.4 ± 0.3 | 7.8 ±0.3 |




**Pilot experiment using ATP SMARTChips to assess changes to whole-blood ATP following high-fat drink**

**A)** Study schematic. Participants came into the lab fasted for ~8 hours and asked to lie in a semi-supine position. A venous cannula was introduced in an antecubital vein and saline infusion used to maintain it patent. Following an acclimatisation period (~30 minutes) a venous blood sample was taken to measure fasting blood ATP (SMARTChip)(-20 min) and glucose (using YSI system) immediately. As soon as ATP measurement were complete (after calibration and reading), a capillary blood sample was taken from the hand via a finger prick (with lancet). Blood ATP was immediately measured. 20 minutes after the first sample, participants were given a high-fat test drink **(C)** and sampling method repeated. Two more samples were taken 140-150 minutes following drink ingestion. **B)** Schematic representation of the ATP SMARTChip. Chips are calibrated in a potentiostat by polarising at +50mV. ATP calibrant (10 µM) is used and generates a certain net current. A sample is then measured and generates a second net current which is used to calculate a "calibration factor". Calibration factor x sample current = concentration **C)** Baseline characteristics of participants (n=5). **D)** Drink composition. Target caloric composition was 75 % of total daily estimated energy (EE) intake (using BMR and PAL) comprised of fat, primarily saturated fat (double cream). **E)** Venous blood ATP concentration measured using SMARTChip. **F)** Blood glucose measured from venous samples following ATP measurement. **G)** Capillary blood ATP concentration measured using SMARTChip.

# Chapter 8

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