# THE EFFECTS OF HYPERGLYCAEMIA AND EXERCISE ON GLUCOSE CONTROL AND METABOLISM: INSIGHTS FROM *IN VIVO* AND *IN VITRO* EXPERIMENTAL MODELS

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**GENERAL ABSTRACT**

Hyperglycaemia and exercise oppositely regulate human glucose metabolism, but whether these two opposite regulatory stimuli directly interact to influence resultant glucose metabolism is poorly understood. Therefore, the overarching aim of this thesis was to examine how hyperglycaemia and exercise/muscle contraction interact to regulate glucose metabolism to better understand how this interaction could be manipulated to improve human metabolic health. Chapter 4 demonstrates that prior *in vitro* skeletal muscle contraction blunts hyperglycaemia-induced insulin resistance in skeletal muscle cells. Chapter 5 reveals that this contraction-mediated protection coincides with increased expression of miR-194-1 and miR-1a-1 in skeletal muscle cells. Chapter 6 shows that prior hyperglycaemia blunts exercise-induced improvements in glycaemic control in healthy humans *in vivo*. Collectively, these results demonstrate that the timing of exercise/muscle contraction in relation to exposure to hyperglycaemia alters the resultant changes in glucose metabolism, with the first stimuli blunting the impact of the second in both cases. Overall, this thesis provides novel data that demonstrates that timing exercise to precede exposure to hyperglycaemia and/or targeting skeletal muscle miR-194-1 and miR-1a-1 expression may be effective approaches to modify the hyperglycaemia – exercise interaction and optimise human glycaemic control.

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## LIST OF CONFERENCE COMMUNICATIONS

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## AUTHORS DECLARATION

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

i. Dr Jonathan Barlow analysed conditioned media samples for IL-6 concentration within chapter 4.
ii. BGI Genomics Co., Ltd. (Hong Kong) completed RNA sequencing and bioinformatics of RNA samples isolated from skeletal muscle cells for chapter 5. This included screening for differentially expressed genes (DEGs) and assessing the clustering of DEGs.
iii. Dr Thomas Solomon, as primary supervisor, provided intellectual support throughout.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **2-DG** | 2-Deoxyglucose |
| **ACC** | Acetyl-CoA carboxylase |
| **ACCORD** | The Action to Control Cardiovascular Risk in Diabetes |
| **ADP** | Adenosine diphosphate |
| **AICAR** | 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside |
| **Akt** | Protein kinase B |
| **AMP** | Adenosine monophosphate |
| **AMPK** | AMP-activated protein kinase |
| **AS160** | Akt substrate 160 |
| **ATP** | Adenosine triphosphate |
| **BMI** | Body mass index |
| **Ca2+** | Calcium ion |
| **CaCl2** | Calcium chloride |
| **CAMKII** | Ca2+/calmodulin-dependent protein kinase II |
| **cAMP** | Cyclic AMP |
| **CGM** | Continuous glucose monitoring |
| **CM** | Conditioned media |
| **CON** | Control |
| **CRP** | C-reactive protein |
| **CS** | Citrate synthase |
| **DEG** | Differentially expressed gene |
| **DMEM** | Dulbecco’s Modified Eagles Medium |
| **DPBS** | Dulbecco's Phosphate-Buffered Saline |

| | |
|---|---|
| **EPS** | Electrical pulse stimulation |
| **FBS** | Fetal bovine serum |
| **FPKM** | Fragments per kilobase per million reads |
| **GIP** | Glucose-dependent insulinotropic polypeptide |
| **GLP-1** | Glucagon-like peptide-1 |
| **GLUT4** | Glucose transporter type 4 |
| **GO** | Gene Ontology |
| **H2O2** | Hydrogen peroxide |
| **HbA1c** | Glycated haemoglobin |
| **HCl** | Hydrogen chloride |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **HG** | Hyperglycaemia |
| **HGP** | Hepatic glucose production |
| **HOMA-IR** | Homeostasis model assessment-insulin resistance |
| **HRmax** | Maximal heart rate |
| **IFG** | Impaired fasting glucose |
| **IGT** | Impaired glucose tolerance |
| **IL-6** | Interleukin-6 |
| **KCl** | Potassium Chloride |
| **KEGG** | Kyoto Encyclopaedia of Genes and Genomes |
| **KH2PO4** | Potassium dihydrogen phosphate |
| **LDH** | Lactate dehydrogenase |
| **MAPK** | Mitogen-activated protein kinase |
| **MCP-1** | Monocyte chemotactic protein-1 |

| | |
|---|---|
| **MgSO4** | Magnesium sulfate |
| **miRNA** | Micro RNA |
| **mRNA** | Messenger RNA |
| **MVPA** | Moderate to vigorous physical activity |
| **Myh1** | Myosin heavy chain 1 |
| **Myh3** | Myosin heavy chain 3 |
| **Myog** | Myogenin |
| **NaCl** | Sodium chloride |
| **NADH** | Reduced nicotinamide adenine dinucleotide |
| **NADP** | Nicotinamide adenine dinucleotide phosphate |
| **NaHCO3** | Sodium bicarbonate |
| **NaNO3** | Nitrate |
| **NaOH** | Sodium hydroxide |
| **NG** | Normoglycaemia |
| **NICE-SUGAR** | Normoglycaemia in Intensive Care Evaluation and Survival Using Glucose Algorithm Regulation |
| **OGTT** | Oral glucose tolerance test |
| **PCr** | Phosphocreatine |
| **PDK4** | Pyruvate dehydrogenase kinase 4 |
| **PFI** | Palmitate, forskolin, and ionomycin |
| **PGC-1α** | Peroxisome proliferator-activated receptor-γ coactivator-1α |
| **PI3Kα** | Phosphoinositide 3-kinase alpha |
| **PKA** | Protein kinase A |
| **PLIN3** | Lipid-droplet protein perilipin 3 |

| | |
|---|---|
| **PLIN5** | Lipid-droplet protein perilipin 5 |
| **PPAR** | Peroxisome proliferator-activated receptor |
| **PPG** | Postprandial glucose |
| **qPCR** | Quantitative PCR |
| **Rac1** | Ras-related C3 botulinum toxin substrate 1 |
| **RIN** | RNA integrity number |
| **RNA** | Ribonucleic acid |
| **RNAseq** | RNA sequencing |
| **ROS** | Reactive oxygen species |
| **RPE** | Ratings of perceived exertion |
| **snoRNA** | Small nucleolar RNA |
| **T2DM** | Type 2 diabetes mellitus |
| **TBC1D1** | TBC1 Domain Family Member 1 |
| **TBC1D4** | TBC1 Domain Family Member 4 |
| **Tpm1** | Tropomyosin 1 |
| **Tnnt1** | Troponin T1 |
| **Tnnt3** | Troponin T3 |
| **$\dot{V}O_2max$** | Maximal oxygen consumption |
| **Wmax** | Maximum workload capacity |
| **ZMP** | 5-aminoimidazole-4-carboxamide-1-β-D-ribosyl monophosphate |

# CHAPTER 1

## 1 GENERAL INTRODUCTION

### 1.1 Type 2 diabetes is a global issue

Type 2 diabetes mellitus (T2DM) is a worldwide epidemic, with ~415 million adults estimated to have T2DM in 2019, increasing to ~520 million and ~630 million by 2030 and 2045, respectively (1). In the UK there are ~3 million confirmed diagnoses; equating to ~5% of the UK population (2). Additionally, 1 in 13 adults worldwide have impaired glucose tolerance (IGT) and are therefore at risk of progressing to overt T2DM (1).

Consequently, increasingly more people worldwide are at risk of developing diabetes-related complications. These include serious microvascular (e.g. retinopathy, neuropathy and nephropathy) and macrovascular (e.g. myocardial infarction and stroke) complications (3,4), as well as Alzheimer's disease (5,6) and some cancers (7). In 2019, more than 4 million deaths worldwide have been attributed to diabetes (1). Clearly, T2DM can have a significant detrimental impact on an individual's quality of life, and markedly increases the risk of mortality compared to non-diabetic individuals (8). Additionally, recent reports have suggested that T2DM accounts for ~10% of global healthcare expenditure, with the total cost associated with T2DM in the UK estimated to be ~£21.8 billion in 2011 (£8.8 billion direct costs; £13 billion indirect costs), and predicted to rise to ~£35.6 billion by 2036 (£15.1 billion direct costs; £20.5 billion indirect costs) (9).

Collectively, these facts and statistics clearly highlight that optimising effective preventative and therapeutic interventions to combat against the inexorable rise in the prevalence of this disease is a clinical necessity.

Diagnosis of individuals with diabetes, as well as classification of those at risk (i.e. impaired fasting glucose (IFG) and IGT), is completed primarily by assessing fasting and postprandial plasma glucose concentrations as well as glycated haemoglobin (HbA1c) concentration according to the diagnostic criteria summarised in Table 1.1.

**Table 1.1 Diagnostic criteria for diabetes**

| | Plasma glucose concentration (mmol/L) | | | |
|---|---|---|---|---|
| | Fasting | 2 h OGTT | Random | HbA1c (%) |
| Impaired fasting glucose | 6.1 – 6.9 | < 7.8 | | |
| Impaired glucose tolerance | < 7 | 7.8 – 11.1 | | |
| Diabetes | ≥ 7 | ≥ 11.1 | > 11.1 | ≥ 6.5 |

Fasting = ≥ 8 h no caloric intake. OGTT = Oral glucose tolerance test.

Many risk factors are associated with the deterioration of glycaemic control and the progression across the glucose tolerance continuum towards overt diabetes. Whilst certain genetic variants (10,11) and aging (12,13) confer increased risk of T2DM, many risk factors of T2DM are lifestyle-related. For example, positive energy balance (resulting from dietary caloric intake exceeding expenditure), obesity (14,15), physical inactivity (16), sedentary behaviour (17–19) and low physical fitness (20) all contribute to an increased risk of developing T2DM. Fortunately, many of these risk factors are modifiable through correct education and adoption of healthy lifestyles meaning T2DM is preventable in many cases. Likewise, whilst previously considered as a life-long condition, accumulating evidence supports the notion that remission of T2DM is possible (21).

In-depth characterisation of mechanisms underlying the deterioration of glucose homeostasis in individuals with T2DM compared to healthy individuals facilitates

identification of appropriate interventions to counteract the inexorable rise in prevalence and serious complications associated with T2DM. The following section summarises the important control mechanisms involved in healthy glucose homeostasis, followed by an overview of which mechanisms fail in the progression towards T2DM.

## 1.2 Glucose homeostasis

Glucose serves as the major fuel used by the human body, and the primary source for the brain (22). Significant reductions in blood glucose (i.e. hypoglycaemia) confer risk to brain function, whereas significant increases in blood glucose (i.e. hyperglycaemia) impair metabolic function in most tissues, with such impairments reaching toxic levels (termed glucotoxicity) in some tissues (e.g. pancreatic β-cells) (22,23). Humans experience numerous stimuli that challenge glucose homeostasis by either increasing (e.g. food or drink consumption) or decreasing (e.g. physical activity) blood glucose levels throughout daily life. Additionally, stress and infection as well as several other environmental stimuli (e.g. heat, cold and hypoxia) also challenge this homeostasis. Amazingly, tight regulatory mechanisms in healthy individuals ensure blood glucose quickly returns to healthy levels (fasting ~3.9 – 5.0 mmol/L), thus preventing prolonged exposure to either hypo- or hyperglycaemic insults (22). These tight regulatory mechanisms are summarised below.

### *1.2.1 Regulation of healthy glucose homeostasis*

For reasons detailed above, tight control mechanisms exist to ensure blood glucose concentrations remain within a normal range in healthy individuals. For example, a balance between glucose absorption from the intestine, production by the liver and uptake by peripheral tissues all contribute to governing this tight control (24).

In a postprandial state (i.e. following a meal) elevations in blood glucose are countered by an increased insulin secretion by the pancreatic β-cells (25,26). The resulting elevation in insulin levels serves to increase glucose uptake into insulin-sensitive peripheral tissues such as skeletal muscle, and inhibit hepatic glucose production (HGP; by inhibiting glycogenolysis and gluconeogenesis and stimulating glycogen synthesis) (24). Additionally, stimulation of enteroendocrine cells in the intestine following food ingestion induces secretion of incretin hormones, namely glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which act to maintain glucose homeostasis, such as via potentiation of glucose-induced pancreatic β-cell insulin secretion (27,28), as well as GLP-1 mediated glucose uptake by skeletal muscle (29). Collectively, these mechanisms ensure that blood glucose concentrations are reduced to re-establish normoglycaemia (25).

In a post-absorptive state (e.g. following an overnight fast), reductions in blood glucose concentration are countered by glucagon secretion from the pancreatic α cells, which acts to increase HGP (via increased glycogenolysis and gluconeogenesis) and thus raise blood glucose concentrations (30). In this way, normal glucose homeostasis is maintained thus ensuring only modest alterations in blood glucose levels in both states (31).

### *1.2.2 Pathophysiology of impaired glucose homeostasis in T2DM*

T2DM is characterised by chronic hyperglycaemia and large glycaemic fluctuations (32), manifestation of which indicates an impairment in the tight glucoregulatory control mechanisms detailed above (22,25,33). The initiating defect in the progression towards overt T2DM is considered to be the development of insulin resistance (i.e. a reduced response to insulin) in tissues typically considered to be insulin sensitive, such as skeletal

muscle, liver and adipose tissue (34). In healthy individuals, ~70% of an ingested glucose load is disposed of by peripheral, extra-splanchnic tissues during the postprandial period (35–38). In particular, skeletal muscle is the predominant site for insulin-stimulated glucose uptake following a meal (34), meaning a reduction in skeletal muscle insulin sensitivity is understandably a major contributor to the development of hyperglycaemia. Physical inactivity (39), obesity, endoplasmic reticulum stress, lipid accumulation, inflammation (40,41), mitochondrial dysfunction (42–44), and impaired microcirculation (45,46) have all been implicated in the pathogenesis of insulin resistance. Additionally, hepatic insulin resistance contributes to fasting hyperglycaemia by increasing basal glucose output (47), and to postprandial hyperglycaemia by impairing insulin-mediated suppression of HGP (37). Insulin resistance can occur many years before the onset of overt T2DM and is initially offset by an increase in insulin secretion by the pancreatic β-cells to maintain normoglycaemia (25). However, with time, pancreatic β-cells fail to secrete sufficient insulin to compensate for the peripheral insulin resistance and hyperglycaemia manifests (25).

Furthermore, as this dysregulation escalates, impaired secretion and/or action of incretin hormones (e.g. GIP and GLP-1) following a meal exacerbates the loss of pancreatic β-cell insulin secretory capacity (Deacon & Ahrén, 2011; Holst, Vilsbøll & Deacon, 2009), and hyperglycaemia *per se* impairs GLP-1 stimulated glucose uptake into skeletal muscle (29). If left untreated, this pathophysiological process deteriorates further, resulting in hallmarks of the diabetic phenotype, including persistent hyperglycaemia and marked glycaemic instability (32).

## 1.3 Complications associated with diabetic-like hyperglycaemia

The following two sections will outline the problems and complications that arise as a result of exposure to a) chronic hyperglycaemia and b) fluctuating hyperglycaemia.

### *1.3.1 Chronic hyperglycaemia*

The chronically elevated hyperglycaemia resulting from the aforementioned pathogenesis of T2DM has been implicated in the development of several microvascular and macrovascular complications (3,4), as well as exacerbating the rate of progression of the disease itself (25,31). These direct detrimental effects of hyperglycaemia, particularly when mediated by hyperglycaemia-induced apoptosis, have been referred to as glucotoxicity (25). These glucotoxic effects have been demonstrated experimentally, with exposure to hyperglycaemia reportedly inducing dysfunction in a range of cell types, including those involved in diabetes-related complications (e.g. cardiac muscle cells (48) and endothelial cells (49)).

Of particular interest to the current review, hyperglycaemia also impairs cell types central to the pathogenesis of the disease itself including skeletal muscle cells, pancreatic β-cells and hepatocytes. Specifically, treatment with high glucose has been shown to significantly impair insulin-stimulated glucose uptake in skeletal muscle cells (29,50–52) and adipocytes/tissue (53), along with reduced insulin-mediated suppression of HGP in hepatocytes *in vitro* (54). Additionally, hyperglycaemia further accelerates HGP by increasing the activity of glucose-6-phosphatase, thus allowing more glucose to escape from the liver (55). Furthermore, exposure to high glucose conditions also significantly impairs insulin secretory function of pancreatic β-cell lines (56,57) and islet models (58).

Such β-cell glucotoxicity is reportedly attributable to a complex interaction of several mechanisms, including oxidative stress (59), β-cell overstimulation, protein glycation and endoplasmic reticulum stress (60,61).

In line with the above *in vitro* evidence, glucotoxicity also induces dysfunction *in vivo*. For example, exposure to 24 h experimental hyperglycaemia (+ 5.4 mmol/L above basal) impairs pancreatic insulin secretory function and insulin sensitivity in healthy normal glucose tolerant individuals (62). Similarly, 72 h experimental hyperglycaemia induced hepatic insulin resistance as well as marked skeletal muscle insulin resistance in the nonoxidative (glycogen synthesis) pathways of glucose disposal in healthy individuals, attributable at least in part to hyperglycaemia-induced disruption of key enzymes involved in skeletal muscle glucose metabolism (e.g. glycogen synthase and pyruvate dehydrogenase) (63). Evidence that normalisation of hyperglycaemia in diabetes patients, such as through intensive insulin therapy, restores insulin secretory function and insulin sensitivity (64–68) further demonstrates the detrimental effects of hyperglycaemia *in vivo*.

Additionally, steady-state hyperglycaemia induces whole-body inflammation (69) and oxidative stress, as well as impairing endothelial function (flow-mediated dilation (70); factors that are key contributors to diabetes-related cardiovascular complications. Alongside these effects, hyperglycaemia can also increase serum osmolality, with such hyperosmolality implicated in the development of hyperglycaemia-related microvascular (e.g. neuropathy (71)) and macrovascular (e.g. stroke (72)) complications. Clearly, chronic hyperglycaemia, a hallmark of the T2DM phenotype, significantly contributes to the manifestation of diabetes-related complications, as well as exacerbates the loss of insulin

sensitivity and β-cell function, with the latter impairment ultimately determining the rate of disease progression (25).

### *1.3.2 Fluctuating hyperglycaemia*

As detailed previously, along with chronically elevated hyperglycaemia, large daily glycaemic variability (including periods of both hyper- and hypoglycaemia) is also a hallmark of the diabetic phenotype (32). Such glycaemic instability has been implicated, perhaps more so than steady-state hyperglycaemia (73), in the development of several hyperglycaemia-related complications, such as cardiovascular disease (74), as well as with all-cause and cardiovascular disease-related mortality (75). Consequently, interest in the potential exacerbating role that a fluctuating glucose profile may have upon hyperglycaemia-induced impairments has intensified over the last two decades.

The effects of fluctuating or oscillating hyperglycaemia have been investigated in several cell lines and animal models. For example, steady-state hyperglycaemia has been shown to induce apoptotic cell death in endothelial cells; effects that were exacerbated following exposure to fluctuating hyperglycaemia (49,76). These exacerbating effects of fluctuating hyperglycaemia in endothelial cells have been demonstrated using various models of fluctuating hyperglycaemia (77–79). Similarly, in pancreatic β-cells, fluctuating hyperglycaemia has been demonstrated to induce apoptosis compared to normoglycaemia (80), as well as impair insulin secretory function to a greater extent than steady-state hyperglycaemia in rat islets and pancreatic β-cells (INS-1) (81). In animals *in vivo*, fluctuating blood glucose levels in mice exacerbate atherogenesis (82). Mechanistically, fluctuating hyperglycaemia reportedly induces greater levels of cellular stress (e.g. oxidative stress) compared to steady-state hyperglycaemia in β-cells (81) and endothelial

cells (49,83,84). Therefore, the more deleterious effect of a fluctuating glucose profile may be partly attributable to a more marked increase in cellular stress induced by fluctuating compared to steady-state hyperglycaemia.

Fluctuating hyperglycaemia is also reportedly more deleterious than steady-state hyperglycaemia in humans *in vivo.* For example, glucose fluctuations, but not measures of sustained hyperglycaemia, strongly predict measures of oxidative stress in T2DM patients (85). In an experimental setting with healthy participants, the inflammatory response to experimental hyperglycaemia is reportedly exacerbated if the hyperglycaemia follows a fluctuating profile (69). Similarly, exposure to steady-state hyperglycaemia has been shown to induce whole-body oxidative stress and endothelial dysfunction in a healthy population; impairments which were reportedly exacerbated when the blood glucose levels fluctuate (70). It has also been suggested that glycaemic instability, particularly when accompanied by frequent hypoglycaemic episodes, could contribute to explain the higher mortality and comorbidity rates reported following intensive treatment compared to standard treatment of hyperglycaemia in T2DM (e.g. the ACCORD trial (86)) as well as critically ill patients (e.g. the NICE–SUGAR trial (87)). Since the endothelial dysfunction reported by (70) was ameliorated by an antioxidant (vitamin C), oxidative stress has been implicated as a mechanism for these *in vivo* glucotoxic effects (73). That said, the exact mechanism through which a fluctuating profile of hyperglycaemia induces significantly greater dysfunction than steady-state hyperglycaemia *in vivo* is not fully understood.

Clearly, the majority of the evidence regarding the effects of fluctuating hyperglycaemia relates to cell types (e.g. endothelial cells) and *in vivo* measures (oxidative stress, inflammation and endothelial function) that are integral to the pathogenesis of

hyperglycaemia related cardiovascular complications. Conversely, research into the role of glycaemic variability in the worsening of the disease progression currently remains limited to aforementioned *in vitro* work with pancreatic β-cells. Within this thesis, healthy participants will be exposed to experimental fluctuating hyperglycaemia to investigate whether this aspect of diabetic-like hyperglycaemia influences the otherwise beneficial glucoregulatory effects of exercise.

## 1.4 Physical activity and T2DM

Physical inactivity is a major risk factor for the development of T2DM (88). For this reason, it is perhaps unsurprising that vast amounts of evidence support the role of physical activity/exercise in the prevention and management of T2DM.

### *1.4.1 Overview of the preventative and therapeutic effects of exercise*

Epidemiological support for the role of physical activity in the prevention of metabolic diseases such as T2DM came from numerous large cohort studies which consistently concluded that greater physical activity is associated with a markedly reduced risk of T2DM (89,90). Furthermore, several large-scale randomized controlled trials, including the Diabetes Prevention Program (91) and the Finnish Diabetes Prevention Study (92), support conclusions that regular exercise as part of a healthy lifestyle (including improved diet) contributes to the attenuation of T2DM onset. Importantly, the Da Qing IGT study demonstrated that exercise alone reduced the risk of developing T2DM by 46% in at-risk individuals (93). The therapeutic effect of physical activity and exercise in individuals with T2DM has also been consistently demonstrated. For example, randomised controlled trials report reductions in hyperglycaemia following an exercise training intervention in T2DM

patients (94–96). Accordingly, exercise is clinically prescribed to prevent and treat T2DM (97).

### *1.4.2 Effects of aerobic exercise on whole-body glycaemic control*

Extensive research has consistently demonstrated the effectiveness of aerobic exercise training (e.g. moderate-intensity cycling or running) in improving the ability to regulate and maintain blood glucose levels within normal ranges (i.e. glycaemic control) in individuals at risk of and with T2DM, as well as in healthy individuals. It is important to note that resistance exercise also elicits many glucoregulatory effects (98), however for the remainder of this thesis the term 'exercise' will refer to aerobic exercise, unless otherwise stated.

Acute exercise improves several important measures of whole-body glucose metabolism, including lowering 24 h average glucose levels (99–102) and improving postprandial glucose control (100,102,103). Similarly, exercise training lowers HbA1c (95,104) and glycaemic variability (105,106). Such benefits result from coordinated exercise-induced changes in several exercise-responsive organs, including skeletal muscle, pancreas, liver and adipose tissue. For example, acute (107) and chronic (108–113) exercise training has been shown to significantly improve pancreatic β-cell insulin secretory function in those with and at risk of T2DM. Additionally, exercise improves adipose and hepatic insulin sensitivity, ameliorates control of HGP, reduces fat mass and suppresses inflammation, all of which contribute to exercise-induced glucose-lowering effects (114). Since many of the glucoregulatory effects of exercise originate from within contracting skeletal muscle, the following sections will address the contribution of these skeletal muscle-specific changes in more detail.

### *1.4.3 Central role of skeletal muscle-specific changes in exercise-induced improvements in whole-body glycaemic control*

Skeletal muscle glucose uptake is integral to the maintenance of healthy glucose homeostasis and the development of skeletal muscle insulin resistance is a key defect in the progression towards T2DM (34). Whilst skeletal muscle is susceptible to deleterious stimuli such as hyperglycaemia (29,50,62,63), skeletal muscle is also extremely responsive to exercise/muscle contraction. Such exercise-induced changes within contracting skeletal muscle are particularly important contributors to exercise-induced improvements in whole-body glycaemic control. For example, acute exercise/muscle contraction is a potent stimulus for acutely increased glucose uptake by the contracting muscle; an insulin-independent effect that is particularly important in reducing hyperglycaemia in otherwise insulin resistant individuals (115–118). This immediate upregulation of glucose uptake lasts for minutes to hours post-exercise, and is followed by increased skeletal muscle insulin sensitivity (117,119). Acute exercise improves microvascular recruitment/perfusion (120) as well as activates multiple important signalling pathways, including AMPK (121,122), TBC1D1 (121) and Rac1 (123), as well as induces GLUT4 translocation to the cell surface (124–126). All of these exercise-responsive effects, as well as improved capacity for substrate oxidation, contribute in varying degrees to the increased basal and insulin-stimulated glucose uptake in the hours – days following exercise (117,118,127).

The effects of a single exercise bout are transient (lasting up to ~2 days), meaning regular exercise is required for more prolonged improvements in skeletal muscle glucose metabolism (117). Indeed, repeated exercise bouts/muscle contraction induce persistent improvements in peripheral insulin sensitivity, including in those with (128–131) and at

risk of T2DM (132). Again, mechanisms responsible for these effects can be summarised as being attributable to enhanced delivery (e.g. via increased capillarization) and also greater capacity for disposal of delivered glucose (e.g. via intramyocellular changes) (117,119,133).

Furthermore, along with glucoregulatory changes occurring directly within the contracting skeletal muscle itself, muscle contraction also liberates factors such as cytokines and other peptides (classified as myokines) (134,135). These factors have been suggested to mediate inter-organ communication, including between skeletal muscle and pancreatic β-cells (136). Interestingly, recent evidence suggests that a myokine-mediated cross-talk effect between contracting skeletal muscle and distant organs may mediate the well-documented health-promoting effects of exercise, and this has recently been reviewed by several authors (134,137–140). Of particular interest here, it has been proposed that the contraction-induced release of myokines, such as interleukin (IL)-6, that occurs during exercise may mediate the exercise-induced adaptations in pancreatic β-cell function and health (141,142). That said, this skeletal muscle to β-cell cross-talk appears to be context-dependent, potentially modified by prevailing insulin resistance of skeletal muscle (136,143) and the inflammatory environment (141), therefore further research in this area is certainly warranted.

## 1.5 Scientific gaps to be addressed within this thesis

Extensive research efforts have highlighted that hyperglycaemia *per se* and exercise/muscle contraction *per se* oppositely regulate human glucose metabolism. That said, whether/how these two stimuli directly interact to determine resultant changes in glucose homeostasis remains poorly understood. In particular, whilst the general health

benefits of exercise are unequivocal, whether exercise/muscle contraction *per se* influences the deleterious effects of hyperglycaemia on glucose homeostasis is unknown. Additionally, hyperglycaemia has been associated with inter-individual variability in glycaemic response following exercise, but whether hyperglycaemia *per se* influences the glucoregulatory effects of exercise remains to be determined. The purpose of the next two sections is to summarise existing literature in this area and to contextualise specific scientific gaps that will be targeted during this thesis.

### *1.5.1 Does exercise/muscle contraction confer protection against hyperglycaemia-induced impairments in glucose metabolism?*

The aforementioned beneficial effects of exercise upon blood glucose control are well established in those with and at risk of T2DM. Additionally, as discussed further below, exercise/muscle contraction appears to confer protection against a range of insulin resistance-inducing stimuli (e.g. lipid and inflammation). However, whether the adaptations to chronic muscle contraction (as experienced during exercise training) *per se* confers direct protection specifically against hyperglycaemia-induced impairments in skeletal muscle glucose metabolism remains to be established.

Skeletal muscle of endurance-trained individuals possess superior oxidative capacity compared to untrained individuals (144). It has also been reported that endurance-trained individuals demonstrate a degree of protection against lipid-induced insulin resistance *in vivo* (145,146), possibly mediated by greater skeletal muscle sensitivity to fatty acid-induced uncoupling via adenine nucleotide translocator 1 (147) and/or greater myocellular glucose-6-phosphate levels for glycogen synthesis (146). Interestingly, even a single bout of exercise confers protective effects against lipid-induced insulin resistance (148).

Additionally, since skeletal muscle metabolic responses to exercise training *in vivo* are at least partially retained in cultured myotubes (149,150), studies have compared glucose metabolism in cells from exercise-trained/physically active donors to cells from untrained/sedentary individuals. Interestingly, evidence demonstrates that myocytes from exercise-trained/physically active individuals exhibit greater capacity for glucose uptake (151) and oxidation (152,153) than myocytes from untrained/sedentary individuals. Furthermore, human primary skeletal muscle cells from physically active individuals appear to be protected against ageing-associated insulin resistance (154) as well as lipid-induced insulin resistance (155) compared to myocytes from sedentary individuals.

Of direct relevance to experimental models used within this thesis, recent evidence has also demonstrated that electrical pulse stimulation (EPS)-induced skeletal muscle contraction *per se* confers protection against lipid-induced (156) and inflammation-induced (157) insulin resistance. However, from these studies, it is clear that the mechanisms that impart protection against insulin resistance-inducing stimuli are context/stimuli specific. For example, contraction-mediated protection against inflammation-induced insulin resistance is attributable to contraction-responsive suppression of inflammatory signalling pathways within human skeletal muscle cells (157). In contrast, contraction-mediated protection against lipid-induced insulin resistance was attributed to contraction-mediated regulation of both non-canonical (e.g. PI3Kα) and canonical activating pathways that were otherwise dysregulated by lipid exposure (156).

Overall, despite clear evidence demonstrating superior skeletal muscle metabolism and protection against insulin resistance-inducing stimuli (e.g. aging, lipid, inflammation) in physically active/trained or contracted muscle, whether chronic skeletal muscle contraction

(as experienced during exercise training) *per se* protects against hyperglycaemia-induced impairments in skeletal muscle glucose metabolism remains to be determined. Likewise, mechanisms through which muscle contraction may impart protection against hyperglycaemia specifically cannot be inferred from previous studies using lipid- or inflammation-based stimuli to induce skeletal muscle insulin resistance.

Understanding the molecular regulation of changes in skeletal muscle glucose metabolism in the context of contraction and hyperglycaemia may facilitate optimisation of strategies to prevent and manage hyperglycaemia-related conditions. Several rapidly evolving 'omics' approaches exist that enable a global, non-biased assessment of changes occurring at DNA (genomics), RNA (transcriptomics), protein (proteomics) or metabolite (metabolomics) level. These approaches are increasingly applied to investigate skeletal muscle biological networks that underpin the health benefits of exercise (158,159). Of these approaches, transcriptomics, i.e. the study of RNA within a cell or organism, has revealed that transcriptional changes within skeletal muscle are closely associated with impaired glucose metabolism in the context of insulin resistance and T2DM (160,161), as well as with improvements in glucose metabolism following exercise (162–164). Multiple tools now exist that facilitate cross-study comparisons (165,166).

Interestingly, in addition to coding RNA (e.g. mRNA), recent evidence links a set of non-coding RNA, particularly microRNA (miRNA), with different metabolic phenotypes in the context of type 2 diabetes, as well as exercise. Specifically, recent evidence highlights associations between skeletal muscle miRNA expression profiles with differences in skeletal muscle glucose metabolism and insulin sensitivity (160,167–173). Likewise, miRNAs are regulated by exercise and may contribute to the health promoting effects of

exercise (174,175), and of relevance to earlier discussion, may be implicated in the heterogeneity in exercise responsiveness (176). Consequently, targeting the skeletal muscle transcriptome has emerged as an appealing therapeutic option in the battle against hyperglycaemia-related conditions. Accordingly, a transcriptomic approach incorporating both coding (e.g. mRNA) and non-coding (e.g. miRNA) RNA will be applied within this thesis to characterise the underlying transcriptional changes associated with the hyperglycaemia – contraction interaction.

### *1.5.2 Does hyperglycaemia per se influence glycaemic responses to exercise, and therefore contribute to exercise response heterogeneity?*

While there is little doubt about the preventative and therapeutic effects of exercise in improving measures of glycaemic control, recent lines of evidence have identified the large inter-individual variability in the beneficial effects of exercise (177–183). These studies, and others (184), have also highlighted the potential for individuals to experience adverse glycaemic responses following an exercise intervention. In a clinical setting, such inter-individual variability makes it difficult to confidently prescribe a standard (or "one-size-fits-all") exercise regime to an individual with or at risk of T2DM. Additionally, lack of beneficial response despite complete adherence to an exercise regime could feasibly hinder individual motivation to continue, thus increasing the risk of exacerbating the development/progression of T2DM, as well as other conditions associated with physical inactivity and T2DM (e.g. cardiovascular disease). Therefore, identifying factors contributing to variability could have significant clinical relevance since it would contribute to the development of optimised and individualised exercise regimes for the

prevention and management of T2DM with the ultimate aim of ensuring an individual is a 'responder'.

The clinical benefit of identifying the causes of this exercise response heterogeneity is emphasised by the publication of several recent review articles focusing on this topic specifically within the context of T2DM (185–188). Within these, several potential contributory factors to this inter-individual variability have been identified, including exercise adherence, diet, drug treatment and genetic factors. Specifically, in studies where the exercise training is conducted in a free-living environment, diet and exercise adherence and characteristics (e.g. volume, intensity) may feasibly contribute such variability (185,188). Furthermore, an interaction between exercise and drug treatments commonly prescribed to T2DM patients also reportedly exists. For example, metformin may blunt the aforementioned well-documented benefits of exercise (189,190), or possibly vice-versa (191). Likewise, exercise-induced improvements in insulin secretion are greater in T2DM patients who are antidiabetic drug naïve (107). Similarly, statins reportedly blunt training-induced cardiorespiratory and skeletal muscle adaptations (192). Conversely, rosiglitazone may potentiate exercise benefits (193). Regarding genetic contributors to the variability, being a carrier of the PPARG $Pro^{12}Ala$ polymorphism is reportedly predictive of greater exercise training-induced improvements in glucose and insulin metabolism compared to non-carriers in sedentary non-diabetic (194,195) and type 2 diabetic individuals (196). That said, such polymorphisms may only explain <3% of the variance in response to exercise training (195).

Interestingly, even in well-controlled settings where diet, exercise adherence and drug treatments are fully accounted for, improvements in measures of glycaemic control are still

variable, therefore suggesting other factors must contribute to this variability (188). Recent evidence has implicated baseline/ambient hyperglycaemia in the variability of intervention efficacy, with some evidence suggesting that the greatest benefit of interventions to manage blood glucose levels, such as exercise training (179,180) and drug treatment (197), may be gained by those with poorer baseline glycaemia. Conversely, it has recently been demonstrated that exercise-induced improvements in skeletal insulin sensitivity (132,183), insulin secretory function (107,178,198,199) and resultant whole-body glucose control, including reduced fasting and postprandial hyperglycaemia (132), are blunted in those with higher pre-training hyperglycaemia.

Hyperglycaemia *per se* impairs insulin secretory function and peripheral insulin sensitivity *in vivo* (62). This is pertinent because poorer baseline β-cell function and skeletal muscle glucose metabolism both predict poorer exercise-induced improvements. Specifically, poorer baseline β-cell insulin secretory function is predictive of impaired exercise-induced improvements in insulin secretory function (108), with other evidence suggesting that a degree of pre-training insulin secretory function is a prerequisite for training-induced improvements (178). Likewise, impaired insulin sensitivity at baseline predicts poorer improvements in the same outcome following exercise training *in vivo* (132) and skeletal muscle contraction *in vitro* (200–202). Whilst the capacity for contraction-mediated GLUT4 translocation is preserved in hyperglycaemic individuals (203,204), activation of AMPK following exercise *in vivo* (205) and EPS-induced contraction *in vitro* is inhibited in muscle from hyperglycaemic (and insulin resistant) donors *vs*. healthy controls (202).

Similar blunted therapeutic effects have also been reported following bariatric surgery (206,207) and glucose-lowering medication (208) in those with long-duration and/or

poorly controlled diabetes pre-intervention. Therefore, collectively, such evidence suggests that hyperglycaemia is closely linked with blunted metabolic response to exercise. However, whether hyperglycaemia *per se* directly blunts the beneficial effect of exercise remains to be determined within an experimental design *in vivo* in humans.

## 1.6 Summary

Exercise represents a unique case in which a single intervention has remedial effects against a wide range of chronic diseases, including T2DM. Nevertheless, the full therapeutic potential of exercise is not always gained, meaning identifying ways to maximise such effects remains an important focus of ongoing research. The above review of the literature clearly identifies several questions that remain to be answered in the pursuit of this. In particular, whilst it is well known that exercise/muscle contraction improves whereas hyperglycaemia impairs glucose metabolism, several questions regarding the interaction of these two opposite regulatory stimuli remain unanswered (Fig. 1.1).

Firstly, whilst it is established that exercise/muscle contraction and hyperglycaemia exert opposite regulatory effects upon skeletal muscle insulin sensitivity, whether muscle contraction *per se* can protect against the deleterious effects induced by hyperglycaemia remains to be determined, nor are the transcriptional mediators underlying any interaction understood. Second, whilst recent evidence demonstrates an association between pre-training hyperglycaemia and the magnitude of training response, whether diabetic-like blood glucose profiles (steady-state and/or fluctuating hyperglycaemia) *per se* directly impairs the glucoregulatory benefits of acute exercise has not been determined experimentally.

By resolving the aforementioned gaps in existing knowledge, this thesis will provide insight into the role of the interaction between hyperglycaemia (of differing patterns) and exercise/muscle contraction in determining human metabolic health. Firstly, understanding whether skeletal muscle contraction *per se* can counteract deleterious effects of hyperglycaemia upon skeletal muscle glucose metabolism could facilitate optimisation of interventions to prevent the manifestation of hyperglycaemia-induced complications. In particular, characterising transcriptional mediators of this interaction may identify novel contraction-specific regulators of skeletal muscle glucose metabolism that could be targeted for this purpose. Secondly, insight into whether hyperglycaemia (of differing patterns) *per se* influences the metabolic response to acute exercise will have significant implications for understanding the exercise response heterogeneity in hyperglycaemia-related conditions, such as T2DM. Therefore, collectively the new information generated by this thesis will have clinical relevance in advancing the understanding of the hyperglycaemia – exercise interaction. Such knowledge will then facilitate future development of novel interventions that optimise the prevention and management of hyperglycaemia-related conditions.

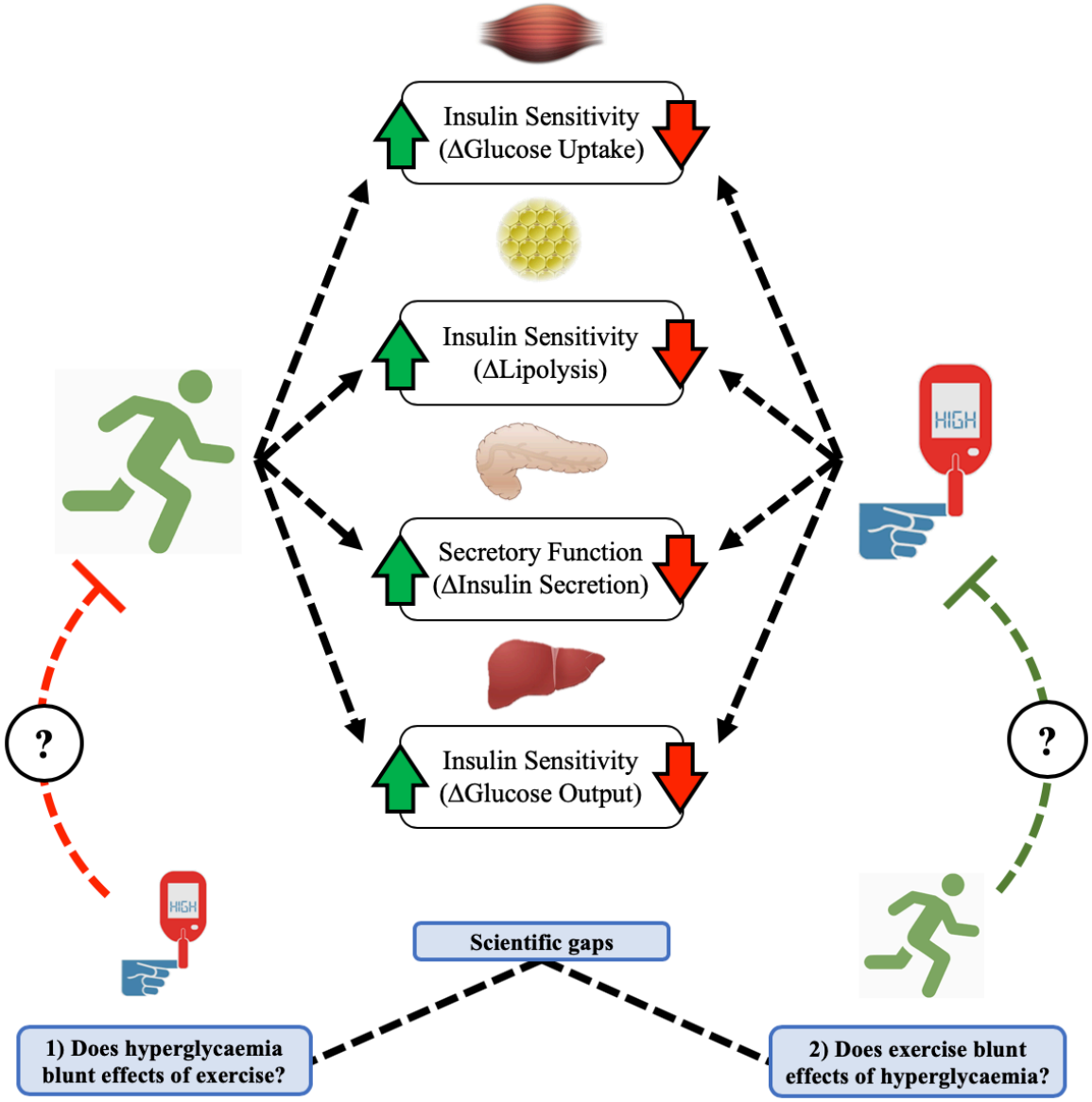



**Fig. 1.1 Exercise and hyperglycaemia exert opposite regulatory effects on multiple organs, but scientific gaps in existing knowledge remain**. Exercise improves insulin sensitivity of skeletal muscle, liver and adipose tissue, and enhances pancreatic β-cell insulin secretory function. In contrast, hyperglycaemia exerts direct opposite effects upon these vital glucoregulatory organs. That said, whether/how these two stimuli directly interact to determine resultant changes in glucose homeostasis remains poorly understood. In this thesis, scientific gap 1 will be addressed within an *in vivo* clinical study aiming to examine the effects of hyperglycaemia on exercise-induced changes in whole-body glucose control (which is a composite of the aforementioned multi-organ effects). Scientific gap 2 will be addressed using *in vitro* approaches, focusing specifically on skeletal muscle to examine the effects of muscle contraction *per se* on hyperglycaemia-induced changes in skeletal muscle glucose metabolism.

## 1.7 Specific aims of this thesis

The overarching aim of this thesis is to examine the interaction of hyperglycaemia and exercise/muscle contraction in regulating glucose metabolism to better understand how this interaction could be manipulated to improve metabolic health. Below is a summary of each chapter with their respective specific aims and hypotheses:

**Chapter 2:** Narrative review detailing the approaches used to examine the cell biology of exercise utilising *in vitro* experimental models.

**Chapter 3:** Overview of the method development process which led to key elements of the methodological approaches used within this thesis.

**Chapter 4:** Investigating the effect of skeletal muscle contraction *per se* on hyperglycaemia-induced impairments in skeletal muscle glucose metabolism. It was hypothesised that skeletal muscle contraction *per se* protects against hyperglycaemia-induced impairments in skeletal muscle glucose metabolism.

**Chapter 5**: Investigating the transcriptional changes associated with the interaction between contraction and hyperglycaemia. It was hypothesised that hyperglycaemia and skeletal muscle contraction induce distinct transcriptional profiles in skeletal muscle cells. In particular, it was hypothesised that hyperglycaemia without prior skeletal muscle contraction would induce a distinct transcriptional profile compared to hyperglycaemia with prior skeletal muscle contraction.

**Chapter 6:** Investigating the effect of hyperglycaemia *per se* on the glucoregulatory responses to a single exercise bout. It was hypothesised that hyperglycaemia *per se* impairs the glucoregulatory effects of acute exercise.

**Chapter 7**: Summary and contextualisation of research findings.

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# CHAPTER 2

## 2 *IN VITRO* EXPERIMENTAL MODELS FOR EXAMINING THE SKELETAL MUSCLE CELL BIOLOGY OF EXERCISE: THE POSSIBILITIES, CHALLENGES AND FUTURE DEVELOPMENTS

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## 2.1 Abstract

Exercise provides a cornerstone in the prevention and treatment of several chronic diseases. The use of *in vivo* exercise models alone cannot fully establish the skeletal muscle specific mechanisms involved in such health-promoting effects. As such, models that replicate exercise-like effects *in vitro* provide useful tools to allow investigations that are not otherwise possible *in vivo*. In this review, we provide an overview of experimental models currently used to induce exercise-like effects in skeletal muscle *in vitro*. In particular, the appropriateness of electrical pulse stimulation and several pharmacological compounds to resemble exercise, as well as important technical considerations are addressed. Each model covered herein provides a useful tool to investigate different aspects of exercise with a level of abstraction not possible *in vivo*. That said, none of these models are perfect under all circumstances, and the choice of model (and terminology) used should be informed by the specific research question whilst accounting for the several inherent limitations of each model. Further work is required to develop and optimise the current experimental models used, such as combination with complementary techniques during treatment, and thereby improve their overall utility and impact within muscle biology research.

## 2.2 Introduction

Vast evidence supports the role of physical activity and exercise in the prevention and treatment of many chronic diseases, such as cardiovascular disease and type 2 diabetes mellitus (T2DM) (1,2). Despite extensive research efforts, the exact underlying mechanisms responsible for these benefits remain to be determined. That said, skeletal muscle contraction *per se* likely contributes to some or many of the exercise-mediated health benefits, be that through direct effects within the contracting skeletal muscle cells (such as improved glucose uptake and insulin sensitivity (3,4)) or via cross-talk with other organs mediated by factors liberated from the skeletal muscle (e.g. myokines, metabolites, microRNAs, exosomes) (5,6). Determining the key mechanisms through which exercise ameliorates disease-linked pathogenesis and signal transduction dysfunction could optimise the beneficial role of exercise in health promotion, minimise instances of impaired exercise adaptation, and may facilitate the identification of novel exercise-responsive drug targets.

*In vivo* experiments with human volunteers provide the most integrated model with which to examine exercise-induced health-promoting responses. Skeletal muscle biopsies collected before and after acute exercise and/or exercise training programmes provide valuable observational insight into associations between *in vivo* health benefits and specific exercise-mediated changes in skeletal muscle gene expression, protein content and signal transduction pathways. For example, the glucoregulatory and insulin-sensitizing effects of exercise have been associated with changes in skeletal muscle expression and activity of several proteins, such as glucose transporter 4 (GLUT4) (3,7,8). However, to confirm the mechanistic importance of specific changes in skeletal muscle in the resultant exercise-

mediated health benefits, further experiments (such as gain and loss of function) are required. The unethical nature of such experiments in human participants, as well as feasibility and practicality considerations, preclude the ability to study individual molecular mechanisms of exercise in isolation, *in vivo* in humans. *In situ* contraction (i.e. contracting skeletal muscle whilst intact in whole-body animals), or *ex vivo* contraction (i.e. contracting whole muscle isolated from an animal) are intermediate approaches between *in vivo* and *in vitro* models that allow more invasive interrogation than *in vivo* human designs. That said, high breeding/housing costs, specialist animal handling expertise and facilities, as well as ethical considerations associated with these approaches mean they are not always possible. Additionally, *in situ* and *ex vivo* contraction inherently involves the tissues, nerves and vasculature surrounding the skeletal muscle, again precluding the ability to determine whether responses can be attributed to skeletal muscle cells *per se*. Accordingly, a prime challenge for researchers working to understand the role of exercise in the prevention and treatment of disease is to develop and optimise experimental models that resemble elements of exercise physiology in a more reductionist approach.

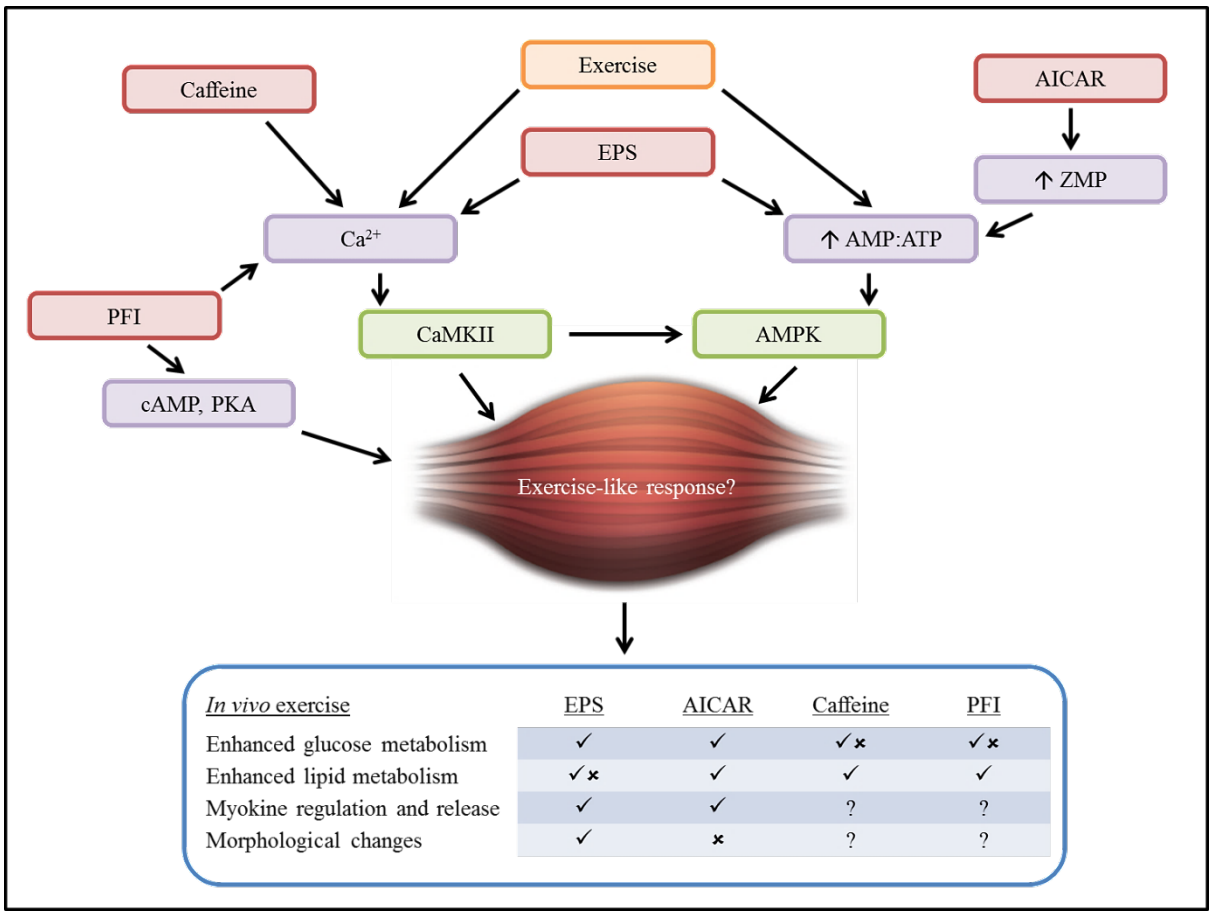
*In vitro* experimental models whereby skeletal muscle cells derived from immortalised cell lines (e.g. C2C12 and L6) or isolated from human or rodent skeletal muscle samples are exposed to various skeletal muscle-specific exercise-like treatments (shortened to 'exercise-like treatments' hereafter) such as electrical pulse stimulation (EPS) or pharmacological compounds have proved useful in this regard. These *in vitro* models can be more cost effective and flexible whilst enabling greater throughput and reduced ethical implications compared to aforementioned *in vivo, in situ* and *ex vivo* approaches. Such 'exercise in a dish' approaches allow investigations into the cellular effects of individual

aspects of exercise physiology, such as skeletal muscle contraction or activation of exercise-responsive signalling pathways, directly upon the skeletal muscle cells, as well as exercise-mediated cross-talk between different cell types. Accordingly, the purpose of this review is to provide the rationale, potential application and evaluation of several models that have been used to replicate and examine exercise-like effects *in vitro.*

### 2.3 Criteria of an *in vitro* skeletal muscle-specific exercise-like model

Models aiming to replicate skeletal muscle-specific aspects of exercise *in vitro* must be validated against the complex range of *in vivo* exercise-mediated changes. The exact mechanisms of exercise-induced responses are not fully understood, and will vary depending on the exercise stimulus (modality, intensity, duration) and whether such stimulus is performed acutely or as part of a training regime (9). That said, some aspects apply universally to skeletal muscle contraction, and these will be used for the purpose of evaluation of each exercise-like treatment discussed within this review. Specifically, exercise induces muscle contraction through increased intracellular calcium ion ($Ca^{2+}$) concentrations. Subsequent contractile activity during exercise elicits multiple intracellular perturbations within the contracting muscle, including decreased adenosine triphosphate (ATP) and phosphocreatine (PCr) content, increased adenosine monophosphate (AMP) to ATP ratio and increased free radical production, as well as other metabolic and mechanical stresses (10). Consequently, exercise *in vivo* induces a cascade of transcriptional (e.g. peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), GLUT4, enzyme and myokine mRNA expression), signalling (e.g. activation of AMP-activated protein kinase (AMPK) and $Ca^{2+}$ activated kinases), proteomic (e.g. increased GLUT4 expression), metabolic (e.g. enhanced glucose uptake and GLUT4 translocation (11,12))

and morphological (e.g. hypertrophy) changes within skeletal muscle (as reviewed previously (13)). Therefore, to be considered as an *in vitro* exercise-like treatment, an experimental model must demonstrate exercise-like effects with regards to the aforementioned transcriptional, signalling and metabolic changes observed *in vivo.* After such comparison, conclusions can be made regarding the extent to which a model can resemble exercise. In the following sections, we provide an outline of *in vitro* models that have been applied to skeletal muscle myotubes in conventional 2D cell culture models within the context of exercise, including a comparison of the effects of each *in vitro* exercise-like treatment to several exercise-induced responses common to multiple *in vivo* exercise stimuli. See Table 2.1 and Fig. 2.1 for summaries of this narrative. Please note that for reasons further discussed within the limitations section below we have not directly compared each model to specific types of exercise (e.g. endurance *vs.* resistance).




| *In vivo* exercise | EPS | AICAR | Caffeine | PFI |
|---|---|---|---|---|
| Enhanced glucose metabolism | ✓ | ✓ | ✓× | ✓× |
| Enhanced lipid metabolism | ✓× | ✓ | ✓ | ✓ |
| Myokine regulation and release | ✓ | ✓ | ? | ? |
| Morphological changes | ✓ | × | ? | ? |

**Fig. 2.1 Proposed experimental models to examine skeletal muscle-specific exercise-like effects in vitro.** Exercise in vivo (highlighted orange) potently activates a range of changes within skeletal muscle that contribute to the well-documented improvement of human health. Exercise-like treatments (highlighted red) elicit several intracellular perturbations (highlighted purple) and activate several exercise-responsive signalling pathways (highlighted green), and as discussed within the text, consequently induce different exercise-like functional and metabolic responses (highlighted blue) in skeletal muscle myotubes. References are provided within the text of the relevant section. ✓ indicates the same direction of change induced by exercise-like treatment compared to in vivo exercise; × indicates different direction of change induced by exercise-like treatment compared to in vivo exercise; ✓× indicates contradictory findings; ? indicates evidence lacking regarding the effect of exercise-like treatment.

**Table 2.1 A comparison between the in vivo effects of exercise and the in vitro effects of EPS and pharmacological compounds on skeletal muscle cells**

| *In vivo* exercise effects | EPS | AICAR | Caffeine | Forskolin alone | Ionomycin alone | PFI |
|---|---|---|---|---|---|---|
| **Intracellular perturbations** | | | | | | |
| ↑ $Ca^{2+}$ transients | ✓ | ? | ✓ | ? | ✓ | ? |
| ↓ ATP content | ✓ | × | ? | ? | × | ? |
| ↓ PCr content | ✓ | ? | ? | ? | × | ? |
| ↓ Glycogen content | ✓ | ? | ? | ? | ? | ? |
| ↑ Lactate production | ✓ | × | ? | ? | ? | × |
| **Gene expression** | | | | | | |
| ↑ PGC-1α | ✓ | ✓× | ✓ | ✓ | ✓ | ✓ |
| ↑ GLUT 4 | × | ? | ✓ | ? | ? | ? |
| ↑ IL-6 | ✓ | ✓ | ? | × | ✓ | ✓ |
| ↑ PDK4 | ✓ | × | ✓ | ? | × | ? |
| ↑ CS | ✓ | ? | ✓ | ? | ✓ | × |
| **Pathway activation** | | | | | | |
| ↑ AMPK | ✓ | ✓ | ✓× | × | ? | ✓ |
| ↑ CaMKII | ✓ | ? | ✓ | ? | ? | ? |
| ↑ MAPK | × | ✓× | ? | ? | ? | ? |
| ↑ ACC | ✓ | ✓ | ? | × | ? | ? |
| ↑ Akt | ✓ | × | ? | ? | ? | ? |
| ↑ AS160 | × | × | ? | ? | ✓ | ? |
| **Protein** | | | | | | |
| ↑ GLUT4 | × | ✓ | ✓ | ? | ? | ? |
| ↑ Akt | × | × | ? | ? | ? | ? |
| **Metabolism** | | | | | | |
| ↑ Glucose uptake and oxidation | ✓ | ✓ | ✓× | ? | ✓ | × |
| ↑ Fatty acid uptake and oxidation | ✓× | ✓ | ✓ | ? | ? | ✓ |
| ↑ Insulin responsiveness | ✓ | ✓ | × | ? | ? | ✓ |
| ↑ GLUT4 translocation | ✓× | ✓ | ? | ? | ✓ | ? |
| ↑ Mitochondrial content | ✓ | ✓ | ✓× | ? | ? | ✓ |
| ↑ Mitochondrial function | ? | × | ✓ | ? | ? | ? |
| **Myokine secretion** | | | | | | |
| ↑ IL-6 | ✓ | ✓ | ? | ? | ? | ? |
| **Morphology and structure** | | | | | | |
| ↑ Hypertrophy | ✓ | × | ? | ? | ? | ? |
| ↑ Sarcomere assembly | ✓ | ? | ? | ? | ? | ? |

↑ indicates values increase following *in vivo* exercise; ↓ indicates values decrease following *in vivo* exercise; ✓ indicates the same direction of change induced by exercise-like treatment compared to *in vivo* exercise; × indicates different direction of change

induced by exercise-like treatment compared to *in vivo* exercise; ✓✕ indicates contradictory findings; ? indicates evidence lacking regarding the effect of exercise-like treatment. References are provided within the text of the relevant section.

### 2.4 Electrical pulse stimulation

*Background:* Skeletal muscle contraction that occurs during exercise induces multiple disturbances in intracellular homeostasis, and subsequently activates multiple contraction-mediated signalling pathways. Therefore, models that induce skeletal muscle contraction, and subsequent activation of contraction-mediated signalling pathways, provide perhaps the most obvious option when trying to resemble exercise *in vitro*. Electrical pulse stimulation (EPS) is an *in vitro* model of skeletal muscle contraction that has been used for this purpose. The application of EPS to skeletal muscle myotubes derived from cell lines (e.g. C2C12 and L6 cells) and cultured primary skeletal muscle cells has proved to be a major tool for studying contraction-induced adaptations, and a comprehensive overview has recently been published by Nikolić and colleagues (14).

*Applications:* While the use of EPS dates back to the 1970s, its specific use as an exercise-like treatment *in vitro* is a more recent development. In particular, EPS has been applied to study contraction-mediated changes in skeletal muscle signal transduction (15–25), metabolism (17,23,26–29), myokine regulation/release (19,30–32) and morphology (15,18,33). Additionally, contraction-mediated cross-talk between skeletal muscle cells and other cell types (e.g. pancreatic β-cells (34,35), hepatocytes (36), monocytes (37), endothelial cells (38)) and donor-specific adaptations (39–41) have been addressed using EPS.

*Comparison to in vivo exercise:* Many studies have now demonstrated that EPS induces visible contraction and elicits several exercise-like effects. Similar to *in vivo* exercise, EPS-induced responses depend upon the EPS stimulus (as discussed by Nikolić et al. (14)). For example, short-term, high-frequency stimulation has been proposed as a model of acute high-intensity exercise as it induces exercise-like upregulation of glucose uptake (27,28) and lactate production, decreased ATP and PCr content (27) and the activation of contraction-related signalling pathways (20). In contrast, chronic low-frequency stimulation has been used to resemble changes seen following exercise training given its exercise-like effects upon activation of exercise-mediated signal transduction (e.g. PGC-1α mRNA expression (19), AMPK (15,16,26), mitogen-activated protein kinase (MAPK), $Ca^{2+}$ transients (15,22)), stimulation of beneficial metabolic effects (e.g. enhanced glucose uptake and oxidation, and insulin sensitivity (26,27) and increased mitochondrial content (27)), myokine expression and release (19,26,30–32,42) and morphological changes (e.g. sarcomeric structure formation (15,26) and myotube hypertrophy (33)). Such EPS-induced sarcomeric structure formation reportedly relies upon calpain-mediated proteolysis (15). EPS has also been applied to better understand contraction-specific transcriptional responses in skeletal muscle myotubes (17,19,24,25,43). Additionally, EPS has been demonstrated to increase lactate production and reduce intracellular glycogen content (44) as well as protect against lipid-induced insulin resistance (45) and lipid accumulation (46).

However, some key changes seen following exercise *in vivo* have not been consistently reproduced *in vitro*. For example, GLUT4 mRNA and protein expression, and fatty acid oxidation are increased following exercise *in vivo*, but these effects have not been consistently demonstrated following EPS. Additionally, whilst C2C12 cells are responsive to EPS in regards to contractility, L6 myotubes (20) and primary human myotubes (14)

reportedly vary in their contractility depending on culture conditions, cell donor and cell differentiation meaning that the degree to which EPS induces exercise-like contraction can be variable. This variability in contractility may contribute to the heterogeneity of EPS-induced metabolic and transcriptional responses of C2C12 *vs*. L6 *vs.* human primary myotubes reported recently (17). For example, the magnitude of EPS-induced glucose uptake is significantly greater in C2C12 compared to human primary myotubes (17). Similarly, whilst GLUT4 translocation, a key process which contributes to the glucoregulatory effects of exercise *in vivo*, has been demonstrated in C2C12 myotubes (16,21,44), such effects have not yet been reported in human primary muscle cell-based EPS models. Collectively, the between-study discrepancies in results are likely, at least in part, attributable to variability in EPS parameters (as detailed previously (14)) and cell type used (17).

*Technical considerations:* While EPS provides a useful tool with which several key aspects of *in vivo* exercise can be induced *in vitro*, several key technical considerations must be acknowledged. Firstly, as discussed above, EPS-induced contraction activates multiple signalling pathways. Therefore, if the aim is to focus on a specific signalling pathway, alternative methods (such as pharmacological compounds discussed below) are required. Secondly, EPS can be applied using commercial (e.g. C-Pace IonOptix generators) or home-made systems. Commercially available systems are perhaps the more convenient option, but lack of tracking capabilities mean it is difficult to determine/confirm whether EPS parameters (e.g. frequency and voltage) are being consistently applied during the experiment. Conversely, while tracking is possible with home-made systems, such setups likely require expertise in electrical engineering (14).

Regarding protocol selection, whilst experimental data supports the use of two distinct protocols to resemble acute (i.e. short-term high-frequency stimulation) *vs.* chronic (i.e. long-term low-frequency stimulation) exercise-like stimulation (14,27), direct comparison of specific EPS protocols to different *in vivo* exercise programmes (e.g. aerobic *vs.* resistance *vs.* interval exercise) is more difficult. By altering the EPS parameters (e.g. duration, frequency, pulse trains *vs.* single pulses, etc.), attempts have been made to resemble these different types of exercise *in vitro*. Whilst different EPS models do evoke different responses that may align a particular EPS protocol with a particular type of exercise, any single EPS model can induce both similarities and differences to both endurance and resistance exercise (34). Therefore, it is perhaps best to consider EPS as a generalised skeletal muscle contraction model that can be applied acutely or chronically, rather than any specific type of exercise.

One consequence of the aforementioned attempts to resemble different types of exercise is the clear inconsistency in EPS parameters (duration, voltage, pulse duration and frequency) used in published studies. Inevitably, discrepancies in EPS-induced effects across studies are also evident. This is particularly true in studies performed on C2C12 and other rodent models, with protocols rarely used in more than 1-2 publications, resulting in 40+ different protocol combinations (as summarised by Nikolić et al. (14)). That said, some consistency can be seen with recent human EPS models with several studies setting their EPS parameters as follows: 1 Hz, 2ms, 11.5 V for 2-24 h (26,30,47–51). This protocol has also recently been applied to C2C12 cells (36).

As discussed by Nikolić and colleagues (14), correct care and cleaning of carbon electrodes after each use are vital to prevent chemicals leaching from the electrodes into

the media and inducing effects unrelated to the stimulus itself and/or compromising cell viability. This is particularly important when studying conditioned media mediated effects within cross-talk experiments. To do so, the following procedure can be used: (1) rinse electrodes with distilled water; (2) soak electrodes in distilled water for 2 days (refreshing water after 24 hrs); (3) disinfect electrodes with 70% ethanol; (4) position electrodes under UV lamp in tissue culture hood. Following these procedures after each use should prevent any issues developing as a result of incorrect care of the EPS electrodes. Nevertheless, measurement of lactate dehydrogenase (LDH) and/or creatine kinase activity in the supernatant should be completed to confirm that cell viability is not being compromised by incorrect electrode cleaning procedures and/or an unsuitable EPS protocol (16,26,27). Similarly, in all applications, the no EPS (control) condition should involve placement of electrodes as in the EPS condition, but without power. The addition of no cell/no EPS and no cell/EPS control conditions are worthwhile additions to exclude any non-specific effects that may result from factors leaching from the EPS electrodes rather than factors secreted by skeletal muscle myotubes (14).

### 2.5 Pharmacological compounds

Several compounds have been used *in vitro* to investigate the molecular regulation of exercise-induced skeletal muscle adaptations. Such compounds are primarily used based on their ability to activate specific exercise-like signalling or metabolic conditions that occur within exercising skeletal muscle. These compounds include 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; an AMPK activator), caffeine (a $Ca^{2+}$ ionophore), ionomycin (a $Ca^{2+}$ ionophore) and forskolin (a protein kinase A (PKA) activator), and on occasion a combination/cocktail of these. Below we provide the rationale

behind the use of each of these compounds, with the specific protocol parameters used in the cited studies summarised in Table 2.2.

### *2.5.1 AICAR*

*Background:* AMPK, a heterotrimeric protein complex, is a master regulator of energy metabolism in skeletal muscle (52). Exercise potently activates the AMPK pathway within skeletal muscle due to contraction-mediated increases in the intracellular AMP:ATP and adenosine diphosphate (ADP):ATP ratios (52,53). This exercise-induced activation of AMPK contributes to several exercise-mediated changes in skeletal muscle metabolism, such as enhanced substrate uptake and oxidation [47, 62]. Therefore, compounds that activate AMPK in skeletal muscle myotubes have been proposed as tools to study the importance of exercise-like activation of AMPK in the subsequent regulation of skeletal muscle metabolism. In particular, AICAR, which activates AMPK through its role as an AMP mimetic, has frequently been used for this purpose. Once transported into cells, AICAR is converted to 5-aminoimidazole-4-carboxamide-1-β-D-ribosyl monophosphate (ZMP; an AMP analogue), and subsequently results in AMPK activation (55).

*Applications:* Whilst AICAR is not always specifically used as an 'exercise-like treatment', AICAR has commonly been used as an AMPK activator within the context of exercise physiology. For example, AICAR applied to C2C12 and L6 myotubes to study signalling pathways (56–62), metabolism (63–65), myokine release (66) and protection against palmitate (67,68) that may be regulated by exercise-like AMPK activation. That said, studies have also used AICAR with the stated intention to induce 'exercise signals'(62), to serve as an 'exercise-mimetic precursor' (69,70) and to generate 'trained' cells (71) to study exercise-related signalling (69,72), myokine release (70) and cross-talk

(71). AICAR has also been applied to primary human myotubes to investigate donor-specific responses to AMPK activation, such as lean *vs.* obese (73), sedentary *vs.* obese *vs.* T2DM *vs.* athletes (74) and healthy *vs.* T2DM (75,76). For example, different responses between donors have been shown, with AICAR increasing AMPK activation in lean but not T2DM myotubes (75). Conversely, similar effects between donors have also been reported with regards to the AICAR-induced enhancement of glucose uptake (77).

*Comparison to in vivo exercise:* Whilst AICAR does not induce skeletal muscle contraction, AICAR administration to skeletal muscle myotubes induces several exercise-like effects *in vitro*. In C2C12 or L6 cells, AICAR has been shown to increase mRNA expression levels of PGC-1α (58,59,78) and interleukin (IL)-6 (79). Unsurprisingly, AICAR also robustly activates the AMPK-related pathway in C2C12 (57,61,65,70,71,79–82), L6 (69,72,83–85) and primary human myotubes (74,75), with only one study lacking this effect (85). Similarly, acetyl-CoA carboxylase (ACC) activation is a common finding across all cell types following AICAR treatment (C2C12, (64,80); L6, (83–85); human (86)). Other exercise-like changes reported following AICAR treatment include increased GLUT4 protein content (72,87), glucose uptake or oxidation (63,77,85,87,88), fatty acid uptake or oxidation (76,84,85,88), insulin responsiveness (68,73), GLUT4 translocation (83,87), mitochondrial content (74) and IL-6 secretion (70). However, in contrast to some of these above-described findings, other studies demonstrate a lack of effect of AICAR upon PGC-1α mRNA expression (70,89), pyruvate dehydrogenase kinase 4 (PDK4) mRNA expression (89,90), FNDC5 (gene that encodes for irisin) mRNA expression (70), as well as lack of effect upon protein kinase B (Akt) protein content (57) and activation (61,83), as well as Akt substrate 160 (AS160) activation (61,83). Findings regarding MAPK activation are currently inconsistent, with increased activation (79) and no effect

(64) reported. Additionally, during skeletal muscle contraction, intracellular ATP, PCr and glycogen levels decline. However, reports have demonstrated that ATP content remains unchanged following AICAR exposure in C2C12 (65) and L6 (83) myotubes, with no evidence of the effects upon PCr or glycogen content. Similarly, in contrast to *in vivo* exercise, AICAR treatment has been shown to have no effect upon myotube lactate production (64) and appears to inhibit hypertrophy (61) and mitochondrial function (65). The wide range of AICAR protocol parameters used (as detailed in Table 2.2), including duration (e.g. 2 mins up to 8 days), concentration (e.g. 0.05 mM up to 10 mM) and single *vs.* repeated/intermittent exposure, likely contribute to the aforementioned discrepant findings and therefore should be considered when interpreting results.

### *2.5.2 Caffeine*

*Background:* Skeletal muscle contraction results from sustained elevations in intracellular $Ca^{2+}$concentration, inducing several downstream effects including activation of the $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) pathway. $Ca^{2+}$ ionophores are compounds which increase intracellular $Ca^{2+}$ concentrations and thus have been used to study the effects of exercise-like CaMKII pathway activation in skeletal muscle myotubes. Caffeine triggers $Ca^{2+}$ release from the sarcoplasmic reticulum and increases intracellular $Ca^{2+}$ concentration in skeletal muscle (91) and is one compound that has been used for this purpose.

*Applications:* Caffeine treatment has been applied to skeletal muscle myotubes as a $Ca^{2+}$ ionophore, as well as with the specific intent to provide an 'exercise-like treatment' (62), mimic exercise activation (90,92) or exercise signals (72) and trigger exercise-like $Ca^{2+}$ changes (69). In this context, caffeine has been used to examine exercise-regulated changes

in signal transduction and metabolism in L6 (69,72,89,91,93) and C2C12 cells (62,94). To date, caffeine has been less readily applied to primary human myotubes than other compounds discussed in this review, and in the studies that have, healthy donor myotubes are primarily used (90,92).

*Comparison to in vivo exercise:* Caffeine has been shown to stimulate several exercise-like effects within skeletal muscle myotubes. Unsurprisingly, caffeine consistently induces $Ca^{2+}$ transients and thus can induce contraction (69,82,91,95). Caffeine has been shown to increase mRNA expression of markers of mitochondrial biogenesis and oxidative metabolism, such as PGC-1α (89,92,94), GLUT4 (92), PDK4 (89,90,92) and citrate synthase (CS) (89). Protein abundance of GLUT4 (72,94) and mitochondrial enzyme (91) proteins, as well as fatty acid uptake/oxidation (69) and mitochondrial function (94), have been shown to be increased following caffeine treatment, similar to exercise *in vivo.* Conversely, some inconsistent effects have also been reported. For example, AMPK activation has been shown to be increased (82,94) and unchanged (69) following caffeine treatment. Similarly, increased (94) and inhibited (95) glucose uptake or oxidation and increased (69,91) and unchanged (94) mitochondrial content have also been reported following caffeine treatment. Caffeine administration to primary human myotubes has been shown to induce several responses different to those seen following *in vivo* exercise, such as reduced FNDC5 expression (92) and insulin responsiveness (95), with the latter being a stark contrast to the insulin-sensitizing effect of exercise *in vivo.* Additionally, limited data exist regarding the effects of caffeine on other exercise-responsive signalling pathways, such as ACC, Akt, AS160 and MAPK activation, intracellular content of high energy phosphates (e.g. ATP and PCr) and aspects of skeletal muscle metabolism (e.g. GLUT4 translocation), morphology and myokine secretion. Similar to EPS and AICAR

models, variation in the exact protocols used within caffeine-based models (as detailed in Table 2.2) with regard to duration (e.g. 2 mins up to 48 hours), concentration (e.g. 0.05 mM up to 20 mM) and use of single *vs.* repeated/intermittent pulses likely contribute to inconsistent results between studies.

### *2.5.3 Palmitate, forskolin, and ionomycin (PFI)*

*Background:* Exercise enhances *in vivo* fat oxidation whilst stimulating both $Ca^{2+}$ and adrenergic (cyclic AMP (cAMP) and PKA) signalling pathways within skeletal muscle. As such, compounds that increase lipid oxidation in skeletal muscle *in vitro* and activate one or both of these pathways provide tools which can be used to delineate the role of the specific pathway in exercise-mediated changes within skeletal muscle. Palmitate (in low concentrations; increases lipid oxidative capacity of skeletal muscle myotubes without impairing insulin sensitivity (96)), forskolin (raises intracellular cAMP and activates PKA) and ionomycin (a $Ca^{2+}$ ionophore) have been used alone or in combination for this purpose.

*Applications:* Of the compounds discussed within this review, ionomycin/forskolin/PFI have the highest proportion of articles where it has been applied with the specific intention of providing an exercise-like treatment *in vitro*, with terminology used including 'exercise mimetic signalling compounds' (97), 'exercise mimetic stimulation' (98), '*in vitro* exercise model' (99) or 'exercise mimicking cocktail' (100). Ionomycin or forskolin alone have been applied to C2C12 or L6 cells to investigate skeletal muscle signalling (69,80,89), metabolism (69,101) or myokine release and expression (66). Primary applications of ionomycin or forskolin alone to primary human myotubes include investigations into intracellular signalling and myokine release or expression (92,97,102,103), with myotubes primarily established from healthy donors. These

compounds have also frequently been used in combination with palmitate as part of a lipolytic cocktail (palmitate, forskolin, ionomycin; or PFI). Within this context, PFI has almost solely been applied to primary human myotubes to investigate exercise-related signalling (74,100,104), metabolism (74,96) and myokine release/regulation (100). Additionally, most of these studies have worked to determine if donor specific responses exist, including sedentary *vs.* active *vs.* obese *vs.* T2DM (74), healthy *vs.* T2DM (100,104) and sedentary *vs.* active *vs.* T2DM (98) comparisons. For example, lipid-droplet protein perilipin 3 (PLIN3) content, which is associated with lipid metabolism *in vivo*, increases after both exercise and PFI treatment (105). Interestingly, the PFI-induced increase in PLIN3 protein was greatest in sedentary donor myotubes, with gradual increases in T2DM donor myotubes and minimal changes in active donor myotubes which almost exclusively favour the expression of another perilipin protein, PLIN5, following PFI treatment (98). Additionally, differential myokine expression has been reported following PFI treatment, with IL-8 increased *vs.* decreased in myotubes from sedentary *vs.* active donors following PFI, respectively (99).

*Comparison to in vivo exercise:* Forskolin alone demonstrates few responses similar to exercise *in vivo*. Whilst forskolin-induced upregulation of PGC-1α mRNA expression (92) is similar to exercise *in vivo*, negative effect upon IL-6 and FNDC5 mRNA expression (97) and a lack of effect upon AMPK and ACC activation (80) suggest that forskolin alone does not satisfactorily resemble enough aspects of *in vivo* exercise. In contrast, ionomycin application to skeletal muscle myotubes has been shown to increase PGC-1α (89,92), IL-6 (97,103) and CS (89) mRNA expression, $Ca^{2+}$ transients (69,103), AS160 activation (93), glucose uptake and oxidation (101) and GLUT4 translocation (93). That said, evidence from ionomycin only experiments have shown a lack of effect upon ATP and PCr levels

(69) and PDK4 mRNA expression levels (89). PFI has been primarily applied to primary human myotubes, and several exercise-like effects have been shown. Firstly, PFI treatment reportedly stimulates contraction of myotubes (96), as well as increases in PGC-1α (100) and IL-6 (99) mRNA expression, AMPK activation (74), insulin responsiveness (at the level of glycogen storage (96)), fatty acid uptake and oxidation (96) and mitochondrial content (74,96). However, unlike exercise, PFI has been shown to have no effect upon CS mRNA expression (96), glucose uptake (96) and lactate production (96), as well as inducing a reduction in FNDC5 mRNA expression (100). The apparent greater consistency of results between PFI-based studies (compared to relative inconsistency within the other models discussed) is likely attributable, at least in part, to the lower number of protocol variations used across studies (summarised in Table 2.2). That said, to date the effects of PFI upon several exercise-like responses, including GLUT4 mRNA expression, protein content and translocation, PDK4 mRNA expression, $Ca^{2+}$ transients, intracellular ATP, PCr and glycogen content as well as exercise-responsive signalling cascades (such as activation of ACC, Akt, AS160 and MAPK), have not been fully characterised.

### *2.5.4 Other pharmacological compounds*

Theoretically, any compound that can stimulate exercise-like responses at transcriptional, signalling and metabolic levels may be a viable tool to investigate exercise-related adaptations. A23187, a $Ca^{2+}$ ionophore also known as calcimycin, has been applied to C2C12 or L6 myotubes to increase intracellular $Ca^{2+}$ content (82,106–109), as well as with a stated intention to serve as an exercise-like treatment (110,111). A23187 has been shown to increase IL-6 mRNA expression (106), myosin heavy chain (MHC)-I expression (107), activate AMPK (82), increase hexokinase II expression (108), LDH activity (111) and

increase p38 phosphorylation (109). However, further studies establishing the effects of A23187 on primary human myotubes are required.

Improved glucose uptake and fatty acid oxidation have been reported in L6 myotubes following treatment with ex229 (an AMPK activator) (88). Similarly, AMPK activator, R419, reportedly increased glucose uptake and GLUT4 promotor activity in C2C12 myotubes (112). SRT170 provides another compound with which AMPK activation and mitochondrial biogenesis can be upregulated in C2C12 myotubes (113). Peroxisome proliferator-activated receptor (PPAR) β/δ levels increase in skeletal muscle during exercise and are central to exercise-mediated metabolic regulation. PPAR agonists, such as GW501516, have also been used to study the effects of exercise-like activation of PPAR pathways in primary human myotubes. GW501516 elicits AMPK and ACC activation, enhanced glucose uptake, fatty acid uptake and oxidation, as well as upregulation of lipid metabolism-related genes (e.g. PDK4) (114,115).

To induce exercise-like reactive oxygen species (ROS) production, hydrogen peroxide ($H_2O_2$) has been applied to C2C12 and L6 myotubes, demonstrating exercise-like effects with regards to increased mRNA expression of PGC-1α, CS and PDK4 (89) and genes involved in ribosome biogenesis (62). Finally, nitrate ($NaNO_3$) treatment of C2C12 myotubes has been shown to increase mRNA expression of PGC-1α, PPARδ, MYH7 and MYH2, as well as induce the release of suspected muscle-derived factors into conditioned media (e.g. irisin and beta-aminoisobutyric acid). Such observations have lead authors to conclude that $NaNO_3$ induces responses that mimic exercise (116).

All of the above-described approaches to induce exercise-like effects *in vitro* have their advantages. However, several science gaps urgently warrant exploration. Namely, the

effects of these different modalities on functional measures like glucose uptake, myokine secretion, and oxygen consumption should be determined in primary human myotubes. This would better characterise the response to each treatment and allow for a thorough comparison to corresponding *in vivo* measurements of the same functional outcomes.

**Table 2.2 A summary of the protocol characteristics of in vitro pharmacological models used to examine exercise-like skeletal muscle contraction and/or signalling**

| Model | Cell type | Purpose for application | Duration | Concentration | References |
|---|---|---|---|---|---|
| AICAR | C2C12 myotubes | Exercise-like treatment | 1 – 24 h | 0.5 mM | (70) |
| | | Exercise-like treatment | 6 h | 2 mM | (62) |
| | | Exercise-like treatment | 120 h | 0.25 mM | (71) |
| | | AMPK activator | 2 mins – 2 h | 0.5 mM | (57) |
| | | AMPK activator | 30 mins | 0.5 – 2 mM | (80) |
| | | AMPK activator | 30 mins – 2 h | 0.1 – 2 mM | (65) |
| | | AMPK activator | 1 – 24 h | 0.1 – 1 mM | (79) |
| | | AMPK activator | 1 – 24 h | 0.1 – 2 mM | (61) |
| | | AMPK activator | 3 h | 0.1 – 10 mM | (64) |
| | | AMPK activator | 3 h | 0.25 mM | (82) |
| | | AMPK activator | 3 – 18 h | 1 mM | (66) |
| | | AMPK activator | 3 – 24 h | 0.5 – 2 mM | (81) |
| | | AMPK activator | 3 – 36 h | 0.25 – 2 mM | (59) |
| | | AMPK activator | 6 – 24 h | 1 mM | (58) |
| | | AMPK activator | 16 h | 2 mM | (67,117) |
| | | AMPK activator | 18 h | 2 mM | (68) |
| | | AMPK activator | 24 h | 0.25 – 1 mM | (60) |
| | L6 myotubes | Exercise-like treatment | 15 – 60 mins | 2 mM | (89) |
| | | Exercise-like treatment | 5 h | 0.2 – 2 mM | (85) |
| | | Exercise-like treatment | 3 h/d for 5 d | 1 mM | (72) |
| | | AMPK activator | 40 mins | 0.01 – 0.75 mM | (63) |
| | | AMPK activator | 40 mins | 0.05 mM | (87) |
| | | AMPK activator | 40 mins | 1 mM | (56) |
| | | AMPK activator | 45 mins | 1 mM | (83) |
| | | AMPK activator | 2 h | 2 mM | (88) |
| | | AMPK activator | 16 h | 1 mM | (78) |

| | | | | | |
|---|---|---|---|---|---|
| | | AMPK activator | 1 – 5 h for 1 – 6 d | 1 mM | (84) |
| | | AMPK activator | 5 d | 1 mM | (69) |
| | Human myotubes | Exercise-like treatment | 20 mins | 2 mM | (77) |
| | | Exercise-like treatment | 5 h | 0.2 mM | (85) |
| | | Exercise-like treatment | 8 h – 5 d | 0.5 mM | (74) |
| | | Exercise-like treatment | 48 h | 1 mM | (90) |
| | | AMPK activator | 30 mins | 1 mM | (86) |
| | | AMPK activator | 1 – 16 h | 2 mM | (73) |
| | | AMPK activator | 1 – 24 h | 0.1 – 1 mM | (79) |
| | | AMPK activator | 6 h or 8 d | 0.1 – 2 mM | (76) |
| | | AMPK activator | 16 h | 0.5 mM | (75) |
| Caffeine | C2C12 myotubes | Exercise-like treatment | 6 h | 5 mM | (62) |
| | | $Ca^{2+}$ ionophore | 2 mins | 1 – 20 mM | (95) |
| | | $Ca^{2+}$ ionophore | 1 – 24 h | 0.05 – 0.1 mM | (94) |
| | | $Ca^{2+}$ ionophore | 3 h | 0.5 – 10 mM | (82) |
| | L6 myotubes | Exercise-like treatment | 1 – 3 h | 5 mM | (89) |
| | | Exercise-like treatment | 3 h/d for 5 d | 5 mM | (72) |
| | | Exercise-like treatment | 5 h/d for 5 d | 5 mM | (69,91) |
| | Human myotubes | Exercise-like treatment | 3 – 24 h | 2.5 – 20 mM | (92) |
| | | Exercise-like treatment | 48 h | 3 mM | (90) |
| Forskolin | C2C12 myotubes | Exercise-like treatment | 3 – 18 h | 1 μM | (66) |
| | | PKA activator | 30 mins | 20 μM | (80) |
| | Human myotubes | Exercise-like treatment | 3 – 24 h | 1 μM | (92) |
| | | Exercise-like treatment | 24 h | 4 μM | (97) |
| Ionomycin | C2C12 myotubes | Exercise-like treatment | 3 – 18 h | 1 μM | (66) |
| | | $Ca^{2+}$ ionophore | 30 mins | 1 μM | (101) |

| | | | | | |
|---|---|---|---|---|---|
| | L6 myotubes | Exercise-like treatment | 10 mins | 1 μM | (93) |
| | | Exercise-like treatment | 1 – 5 h | 1 μM | (89) |
| | | $Ca^{2+}$ ionophore | 5h/d for 5 d | 1 μM | (69) |
| | Human myotubes | Exercise-like treatment | 3 – 24 h | 1 μM | (92) |
| | | Exercise-like treatment | 6 h | 1 μM | (102) |
| | | Exercise-like treatment | 24 h | 0.5 μM | (97) |
| | | $Ca^{2+}$ ionophore | 3 – 12 h | 0.0005 - 0.002 μM | (103) |
| PFI | Human myotubes | Exercise-like treatment | 1 h/d for 3 d | P (30 μM), F (4 μM), I (0.5 μM) | (74,96,98–100,105) |
| | | Exercise-like treatment | 3 d | P (30 μM), F (2 μM), I (1.6 μM) | (104) |
| A23187 | C2C12 myotubes | Exercise-like treatment | 1 h | 1 μM | (110) |
| | | $Ca^{2+}$ ionophore | 3 h | 10 μM | (82) |
| | | $Ca^{2+}$ ionophore | 6 h | 0.4 μM | (106) |
| | | $Ca^{2+}$ ionophore | 9 – 24 h | 0.1 – 1 μM | (107) |
| | L6 myotubes | Exercise-like treatment | 30 mins – 5 h | 10 μM | (111) |
| | | $Ca^{2+}$ ionophore | 3 h | 1 μM | (109) |
| | | $Ca^{2+}$ ionophore | 6 h | 0.05 – 1 μM | (108) |
| ex229 | L6 myotubes | AMPK activator | 2 h | 0.5 – 1 μM | (88) |
| R419 | C2C12 myotubes | AMPK activator | 2 h | 0.2 μM | (112) |
| SRT1720 | C2C12 myotubes | SIRT1 activator | 1 – 24 h | 2.5 μM | (113) |
| GW501516 | Human myotubes | PPARδ activator | 1 – 18 h | 0.1 μM | (114) |
| | | PPARδ activator | 3 d | 0.0001 – 0.05 μM | (115) |
| $H_2O_2$ | C2C12 myotubes | Exercise-like treatment | 6 h | 1 mM | (62) |
| | L6 myotubes | Exercise-like treatment | 15 – 60 mins | 1 mM | (89) |
| $NaNO_3$ | C2C12 myotubes | Exercise-like treatment | 1 d | 25 – 500 μM | (116) |

## 2.6 Limitations and future developments applicable to all approaches

Each of the experimental models discussed has their own specific limitations. Additionally, there are several limitations that apply to most or all of the aforementioned models. These are discussed below along with suggestions for improving methodological clarity and improving the physiological nature of such models. See Fig. 2.2 for a summary of this discussion.

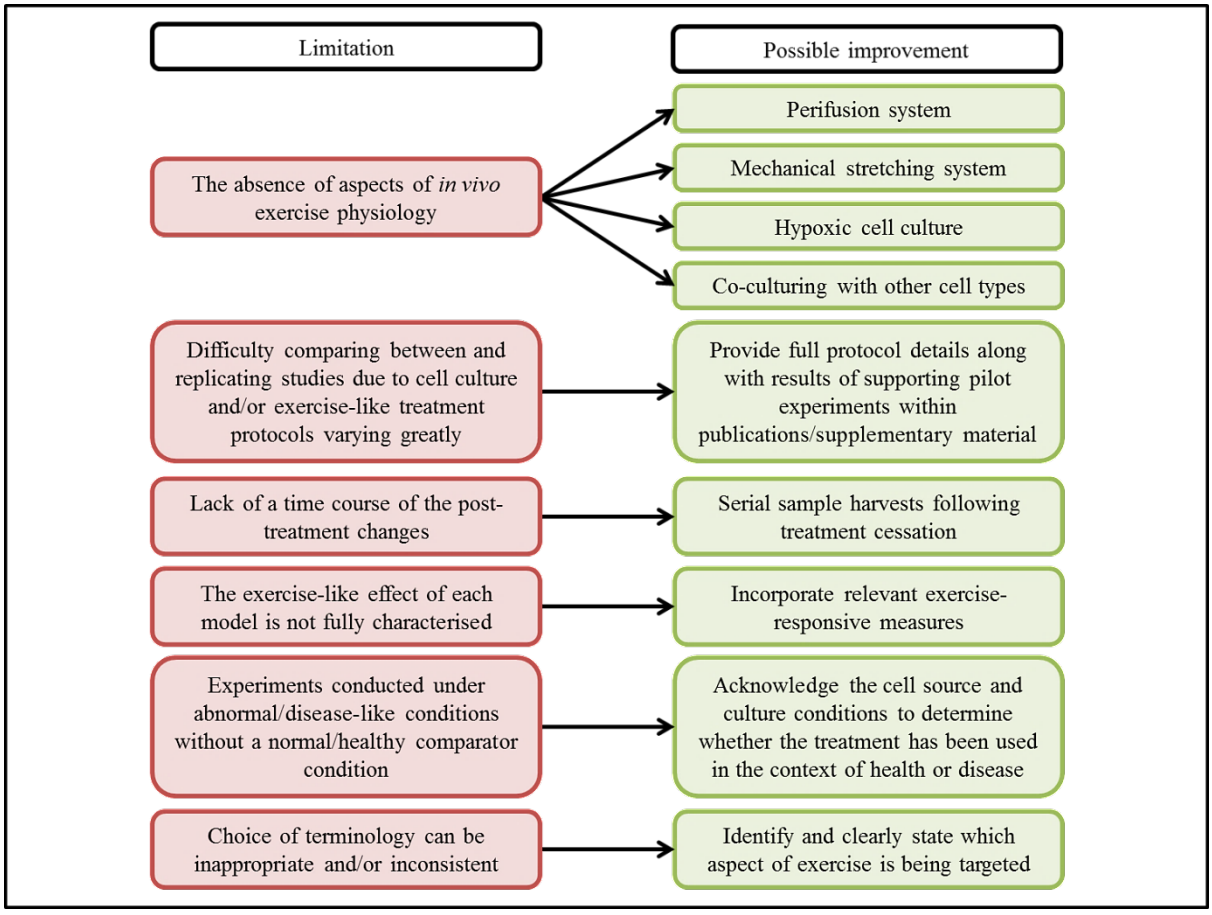



**Fig. 2.2 Summary of limitations and possible future improvements applicable to all in vitro skeletal muscle-specific exercise-like models.** Several limitations inherent to all models discussed have been identified from existing literature (highlighted red), and possible improvements have been suggested (highlighted green). See text for thorough discussion of each limitation/improvement.

### *2.6.1 The absence of aspects of in vivo exercise physiology*

A myriad of physiological changes occur during exercise *in vivo*, including increased blood flow and alterations in circulating substrates and molecules (e.g. hormones), microenvironment (e.g. local hypoxia, temperature) and autonomic nervous system (10). The absence of these exercise-mediated stimuli as well as a lack of cell-extracellular matrix interaction and mechanical load (101) should be acknowledged when making conclusions using data derived from these *in vitro* models. The lack of flow across skeletal muscle cells during culture/exercise-like treatment can result in a static accumulation of secreted factors, which is particularly relevant within cross-talk experiments where final concentrations of secreted factors can become supraphysiological. Additionally, substrate uptake is regulated by a balance of delivery, transport and metabolism (11), and during exercise *in vivo* the balance between carbohydrate and fatty acid oxidation shifts depending on exercise duration and intensity. However, the lack of flow across skeletal muscle cells (i.e. no change in substrate delivery) and the use of only one energy source in most studies during exercise-like treatment mean current models do not account for these important aspects of *in vivo* exercise physiology. Introduction of a perifusion system whereby culture media can be continuously circulated would prevent static accumulation of secreted factors, and allow alterations in the presence of exercise-responsive hormones and/or fuel source provision during treatment to be made. Inclusion of mechanical stretching to resemble exercise-like mechanical load may be a useful future addition because it has been shown to activate AMPK (118,119), Akt (119,120), CaMKII (101,119) and p70S6 kinase (118,121) related signalling pathways, and to increase glucose uptake (101), GLUT4 translocation (119) and myotube hypertrophy (120). It should be noted that mechanical stretching is not discussed fully in this review because it requires flexible and patterned

culture membranes within stretch chambers that are not applicable to conventional 2D culture, the focus of this review. Finally, it should be noted that adaptations to repeated exercise during a period of training result from transient activation followed by inactivation of exercise-related transcriptional and signalling responses within skeletal muscle (13). In contrast, exercise-like treatment models where the stimulus is often applied chronically over many hours or days may not accurately reflect the episodic nature of *in vivo* exercise and thus may explain some of the discordances in effects seen following *in vivo* exercise *vs.* exercise-like treatment *in vitro*. That said, efforts to resemble the episodic nature of *in vivo* exercise within exercise-like treatment models have been made, with EPS (43), AICAR (72,84), caffeine (69,72,91) and PFI (74,96,98–100,105) applied through daily pulses interspersed by periods of 'rest'. Overall, a combination of these exercise-like treatments with other techniques, such as hypoxic cell culture conditions (which has recently been combined with EPS (47)), mechanical stretching, co-culturing with other cell types and perifusion systems, could make each model more representative of *in vivo* exercise physiology.

### *2.6.2 Difficulty comparing between and replicating studies*

Differences in routine cell culture procedures and 'exercise-treatment' protocols used can make direct comparisons between and replication of studies difficult. For example, media constituents (e.g. serum and glucose concentration), days of differentiation, cell seeding densities, the presence of plate coating, media change frequency, cell passage and sample harvesting time could all feasibly influence how cells respond to each exercise-like treatment. In particular, many of these factors influence degree of cell differentiation, which in turn likely impacts consistency of these models. Equally, differences in

parameters used in EPS-based (duration, frequency, pulse duration; as detailed previously (14)) and compound-based (duration, concentration, single *vs.* repeated/intermittent pulses; as summarised in Table 2.2) experiments all likely contribute to different responses. Whilst such differences in protocols across studies is not a limitation of the models *per se*, these details are often omitted and/or vary greatly across studies, highlighting the need for caution before making direct comparisons across studies. That said, there is one exception: protocol parameter selection in the existing literature that has used PFI as a stimulus is admirably consistent. Additionally, the selection of the specific protocol parameters used in published work, such as concentration (compound-based methods), duration (all methods), frequency and voltage (EPS), have very likely been informed by dose-response and/or time course pilot experiments. The results of such pilot experiments, along with specific protocol information and video footage demonstrating myotube contractility, should be presented in publications or at the very least within the supplementary material. This would improve consistency and reduce systematic error in repeated work, and would facilitate fairer between-study comparisons and therefore improve the quality of data interpretations.

### *2.6.3 Lack of a time course of the post-treatment changes*

As determined by serial skeletal muscle biopsies following exercise cessation, the evidence demonstrates a time-dependent effect upon exercise-regulated signalling events within skeletal muscle *in vivo* (13,122–124). It is likely that time-dependent activation also likely occurs following cessation of exercise-like treatment *in vitro*. Indeed, serial sample harvests have demonstrated that PFI-induced changes in IL-6 mRNA expression (99), and EPS-induced GLUT4 recycling (16), IL-6 mRNA expression (42), PPARδ mRNA

expression (25) and myotube hypertrophy (33) exhibit significant time effects over the period following treatment cessation. However, most studies harvest samples at a single time point following treatment cessation, which can vary across studies, meaning the time-course for changes following each of these treatments is not well characterised. Therefore, time-course studies incorporating serial sample harvests following treatment cessation are required to ensure changes with different kinetics (e.g. rapid, transient or delayed) are not missed and thus allow full characterisation of responses to each model.

### *2.6.4 The exercise-like effect of each model is not fully characterised*

As addressed in each section above and clearly demonstrated by the question marks in Table 2.1, several functional and molecular responses to *in vivo* exercise have not been similarly examined nor characterised in each *in vitro* model. For example, whilst EPS- and AICAR-based models are fairly well characterised, large gaps remain regarding the effects of the other pharmacological compounds on key exercise-responsive intracellular signalling and metabolic changes. Without such information, a full comparison of each *in vitro* model to *in vivo* exercise is not possible. Therefore, to fully characterise the extent to which each model resembles *in vivo* exercise, future studies should incorporate relevant exercise-responsive measures (referred to as positive controls in the next section) to address the gaps highlighted in Table 2.1.

### *2.6.5 Experiments conducted under abnormal/disease-like conditions without a normal/healthy comparator condition*

While cell lines offer consistent, robust and predictable cell growth patterns, excessive culture durations and handling make cell lines prone to deviate from normal function.

Additionally, donor characteristics (which are to an extent retained in cultured myotubes (125)), biopsy site (which can influence cell proliferation and differentiation rates (126) and fibre type heterogeneity/distribution) and reason for biopsy sample collection (including surgical biopsies, which can introduce confounding effects of non-specified chronic disease) could all influence how primary human myotubes respond to each treatment. Additionally, culture media glucose concentration prior to and during these exercise-like treatments varies from 5 to 25 mM. Since exposure to high glucose concentrations (i.e. 25 mM) can induce insulin resistance in skeletal muscle myotubes (127–129), studies that have cultured myotubes in hyperglycaemic conditions prior to and during the respective exercise-like treatment (for example, EPS-based (20,21,25,38,42,130), AICAR-based (58,70,71) and caffeine-based (82,94) models) may have been conducted under disease-inducing conditions. Therefore, acknowledging whether the effects of these exercise-like treatments have been investigated in the context of health (e.g. insulin responsive) or disease (e.g. insulin resistant) is important for increasing the accuracy of data interpretation and subsequent impact.

### *2.6.6 Choice of terminology*

As mentioned in each section, the choice of terminology used when describing each treatment varies greatly across studies, and may not always be fully appropriate. For example, terms such as ‘exercise mimetic’ or ‘exercise treatment’ inherently imply that the tool copies all aspects of exercise, which, as discussed throughout, is not the case. Equally, whilst altering the specific protocol parameters (EPS – duration, frequency, pulse duration; pharmacological compound – duration, concentration, single *vs.* repeated/intermittent pulses) can evoke different responses that may align a particular protocol with a particular

type of exercise, any one protocol can induce both similarities and differences to both endurance and resistance exercise. Moreover, an apparent focus of compound-based literature (particularly caffeine and PFI) upon endurance exercise-like responses, and a relative dearth of information addressing the potential effects upon resistance exercise-like responses preclude the ability to exclude the possibility that a single protocol does indeed evoke a hybrid of endurance-like and resistance-like effects *in vitro*. Therefore, whilst the notion of designing distinct 'endurance exercise-like' or 'resistance exercise-like' treatments is an appealing one, current evidence does not permit researchers to confidently state that *protocol A* fully resembles endurance exercise, and *protocol B* fully resembles resistance exercise. Such considerations strongly influenced our decision to not directly compare each experimental model to specific types of exercise, and clearly highlight the need for careful choice of terminology when referring to each treatment. For example, terminology such as *in vitro* skeletal muscle-specific exercise-like treatment (or shortened to 'exercise-like treatment' as throughout this review) may provide a more accurate and conservative description of this set of models in general. Better still, stating whether the model is being used to target skeletal muscle contraction or specific exercise-responsive signalling pathways would improve consistency between studies, as well as enhance the quality of data interpretation.

## 2.7 Model selection and practical considerations

*In vitro,* muscle-specific exercise-like treatments enable us to probe specific molecular mechanisms of exercise adaptations. This is not otherwise possible using human exercise studies alone. Each of these *in vitro* models induces several exercise-like changes in skeletal muscle cells. This includes the enhancement of glucose metabolism and activation

of several exercise-responsive signalling pathways. Nevertheless, each model has different advantages and careful consideration is required before selecting a single model.

### *2.7.1 Research aim*

Firstly, the specific research aim should form the basis of this decision. For example, if the aim is to investigate skeletal muscle contraction *per se* and all signalling pathways that contraction regulates, EPS may be preferable. In contrast, if the aim is to target a single, specific exercise-responsive signalling pathway (e.g. AMPK, cAMP/PKA, CaMKII, etc.) to investigate downstream targets/metabolic outcomes, then utilising the appropriate pharmacological agonist would likely be more appropriate. The main target for each application is summarised in Fig. 2.1. That said, as discussed above, these compounds often activate multiple pathways and have additional off-target effects. This must be considered when designing protocols and interpreting results.

### *2.7.2 Equipment available*

EPS requires specialised equipment that can be expensive and difficult to maintain, whereas the use of pharmacological agonist compounds simply requires the addition of such compounds to cell culture media. Therefore, a cost-benefit analysis of logistics, equipment, and finances available must also be considered.

### *2.7.3 Controls*

When investigating the effects of each of these exercise-like treatments it is important to include measurements that can be used to confirm the treatment has induced some exercise-like effect. For example, assessment of muscle contraction, glucose metabolism,

exercise-related signalling and myokine secretion provide appropriate positive controls to determine the extent to which a model is able to resemble aspects of *in vivo* exercise. In cross-talk experiments, additional controls whereby the exercise-like treatment is applied to media without cells are also important. Implementation of such controls in EPS-based models is recommended by Nikolić et al. (14) and is discussed above. Within compound-based experiments, such controls would involve incubating the target cell in fresh (i.e. non-conditioned) compound-containing media to investigate whether the compound alone impacts the target cell. Such controls have been implemented previously (71), and are vital to exclude the possibility that observed effects result from the compound that remains in the skeletal muscle cell conditioned media rather than factors secreted from skeletal muscle cells.

### 2.8 Future applications

As discussed throughout, each model has been used to answer different exercise-related questions. Because many research questions remain, *in vitro* models will likely form a large component of future research in this field. For example, application of these models, where possible, to primary human skeletal muscle myotubes as a more physiologically and genetically relevant model of human health and disease (125) would enhance the impact of findings gained. In particular, the evidence demonstrates the preservation of metabolic phenotype in myotubes established from different donors (e.g. healthy, obese, diabetic (125)), as well as following acute/chronic exercise *in vivo* (e.g. improved lipid metabolism and glucose metabolism) (131,132) and surgery (41). Therefore, application of these *in vitro* exercise-like treatments to myotubes derived from human donors of different disease states and/or exercise statuses may prove most useful in the identification of new

molecular targets to prevent/treat disease. Similarly, application of these models to myotubes isolated from individuals showing different adaptations to the same exercise stimulus (i.e. responders *vs.* non-responders), will advance our understanding of inter-individual variability in the response to exercise. Such an approach would contribute to reducing the occurrence of a non-response to exercise.

## 2.9 Conclusion

The application of EPS or various pharmacological compounds to skeletal muscle myotubes provide distinct experimental models able to induce some exercise-like effects *in vitro* and therefore provide valuable high-throughput tools to study the skeletal muscle cell biology of exercise-mediated health benefits. By targeting muscle contraction (e.g. using EPS) or exercise-related signalling pathways (e.g. AMPK or CaMKII using pharmacological compounds), specific aspects of muscle function and metabolism can be isolated using *in vitro* approaches. Future optimisation to enhance the physiological nature of these current *in vitro* models will further enhance their impact within muscle biology research and thus contribute to addressing key challenges in this field, such as maximising the therapeutic potential of exercise and facilitating the identification of novel exercise-responsive drug targets.

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# CHAPTER 3

## 3 METHOD DEVELOPMENT

The methodological approaches and techniques used within the experimental chapters in this thesis are detailed in full within the respective chapter and therefore not repeated here. The final study design and methods used required extensive preliminary work to ensure the approach used was best suited to addressing the study aims. The purpose of this chapter is to outline the processes and decisions which led to selection of key elements of these methodological approaches, as well as to outline performance (CV%) of assay-based analytical measures used throughout this thesis.

### 3.1 Development of models to assess the interaction of hyperglycaemia and exercise *in vitro*

The overarching aim of chapter 4 was to assess whether skeletal muscle contraction *per se* protects against hyperglycaemia-induced insulin resistance. This was followed up within chapter 5, the overarching aim of which was to characterise transcriptional changes in response to these experimental conditions. To address these, establishment of the following was required: 1) appropriate outcome to assess insulin sensitivity *in vitro*, 2) an insulin sensitive skeletal muscle cell model to serve as 'healthy' control condition, 3) hyperglycaemic conditions that can be applied short-term to induce skeletal muscle cell insulin resistance and 4) an *in vitro* skeletal muscle contraction model that induces 'exercise-like' effects in skeletal muscle cells.

### *3.1.1 Rationale for assessing glucose uptake to measure insulin sensitivity in vitro*

Insulin stimulates a complex cascade of signalling events involving the insulin receptor substrates (IRS) and several downstream kinases such as Akt that ultimately potently increases skeletal muscle glucose uptake, glucose oxidation and glycogen synthesis (1–3). Theoretically, analysing the effect of insulin on any part of this process could provide a measure of *in vitro* skeletal muscle insulin sensitivity. However, whilst phosphorylation of proteins such as IRS1 and Akt play key roles in insulin signal transduction, a change (increase or decrease) in insulin-stimulated phosphorylation of Akt or IRS1 does not always coincide with a linear change in insulin-stimulated glucose uptake (3–5). Therefore, studies concluding reduced insulin-stimulated phosphorylation of a single protein alone is sufficient to confirm insulin resistance, for example, should be interpreted with caution. Instead, metabolic end-points are considered 'gold-standard' measures of insulin sensitivity and are required to confirm insulin resistance (6,7), therefore justifying the use of glucose uptake to assess insulin sensitivity in chapters 4 and 5. Nevertheless, the assessment of relative phosphorylation of these key signalling proteins could have provided valuable insight into possible mechanisms underpinning the observed metabolic responses, but unfortunately this was not possible within this thesis.

### *3.1.2 Process of establishing an insulin sensitive skeletal muscle cell model*

Skeletal muscle cell culture allows researchers to investigate how skeletal muscle may respond to a variety of treatments or stimuli with a level of abstraction not otherwise possible *in vivo*. In chapter 4 and 5, C2C12 cells were used as a model of skeletal muscle. Whilst C2C12 cells are an immortalised cell line derived from murine skeletal muscle, key processes such as myogenesis and differentiation resemble those processes seen in human

primary muscle cells *in vitro*. Therefore, C2C12 cells provide a convenient and robust model which is regularly used to investigate muscle physiology in the context of both exercise and diabetes. However, they are highly glycolytic cells, therefore historically cultured in culture media containing high glucose (25 mmol/L) concentrations. Doing so allows researchers to reduce their workload, for example by avoiding the need for daily media changes and potentially expediting proliferation and differentiation. However, culturing skeletal muscle cells in high glucose induces insulin resistance (8–10).

Furthermore, skeletal muscle exposed to 25 mmol/L glucose is not representative of normal physiology in a healthy individual, and insulin resistant muscle is not representative of healthy muscle. Unfortunately, many studies utilising C2C12 continue to investigate the effect of their treatments in cells cultured in high glucose (and insulin resistance inducing) conditions, yet often fail to acknowledge the effect that skeletal muscle insulin resistance may exert on how the muscle responds to their experimental treatment. This should always be considered when interpreting study findings.

The above consideration was particularly important when planning and conducting experiments reported in chapters 4 and 5. Specifically, the aim within these chapters was to examine the effect of conditions of hyperglycaemia and/or skeletal muscle contraction on glucose metabolism in 'healthy, insulin sensitive' skeletal muscle cells. Therefore, continuing to culture C2C12 cells in high glucose, and therefore insulin resistant conditions, would have been inappropriate and ultimately significantly altered the research question that was being addressed. For example, routinely culturing hyperglycaemic (25 mmol/L) muscle cells, followed by investigating effects of short-term experimental treatment of a) 5 mmol/L *vs*. 25 mmol/L glucose or b) rest *vs*. contraction upon insulin

sensitivity would address the questions of a) ‘Does reducing glucose concentration reverse insulin resistance in hyperglycaemic muscle cells?’ or b) ‘Does contraction reverse insulin resistance in hyperglycaemic muscle cells?’. Both questions are interesting and could have important implications. However, this approach would not address my overarching research question of ‘Does skeletal muscle contraction *per se* protect against hyperglycaemia-induced insulin resistance?’. This question requires application of treatments to insulin sensitive cells.

Accordingly, C2C12 cells were routinely cultured in normal (5 mmol/L) glucose concentrations, which successfully established an insulin sensitive model of skeletal muscle. This was confirmed by preliminary experiments comparing insulin-stimulated glucose uptake in cells cultured in normo- *vs*. hyperglycaemic conditions throughout the whole culturing process (Fig. 3.1). Visual inspection confirmed that cell viability, growth rates and capacity to differentiate were unaffected by switching to normal (5 mmol/L) glucose culture conditions. That said, frequency of media changes during differentiation was increased from every 48 h in 25 mmol/L to every 24 h in 5 mmol/L conditions to maintain comparable rates and degree of differentiation (Fig. 3.1). This approach was adopted and used for all subsequent C2C12 experiments, including during routine culture of cells in chapters 4 and 5. Since the degree of cell differentiation is a particularly important determinant of myotube contractility, careful visual inspection of each well of all culture plates was completed to confirm consistent differentiation prior to all experiments. Whilst myotube number or diameter were not quantified, RNAseq data collected during chapter 5 confirm that key transcriptional markers of myotube differentiation and maturation were highly expressed in myotubes produced using the specific culture protocols used throughout this thesis. Specifically, expression of myogenin (*Myog*),

myosin heavy chain 1 (*Myh1*), myosin heavy chain 3 (*Myh3*), tropomyosin 1 (*Tpm1*), troponin T1 (*Tnnt1*) and troponin T3 (*Tnnt3*) were ranked amongst the top 0.7% of the ~20,000 genes assessed.

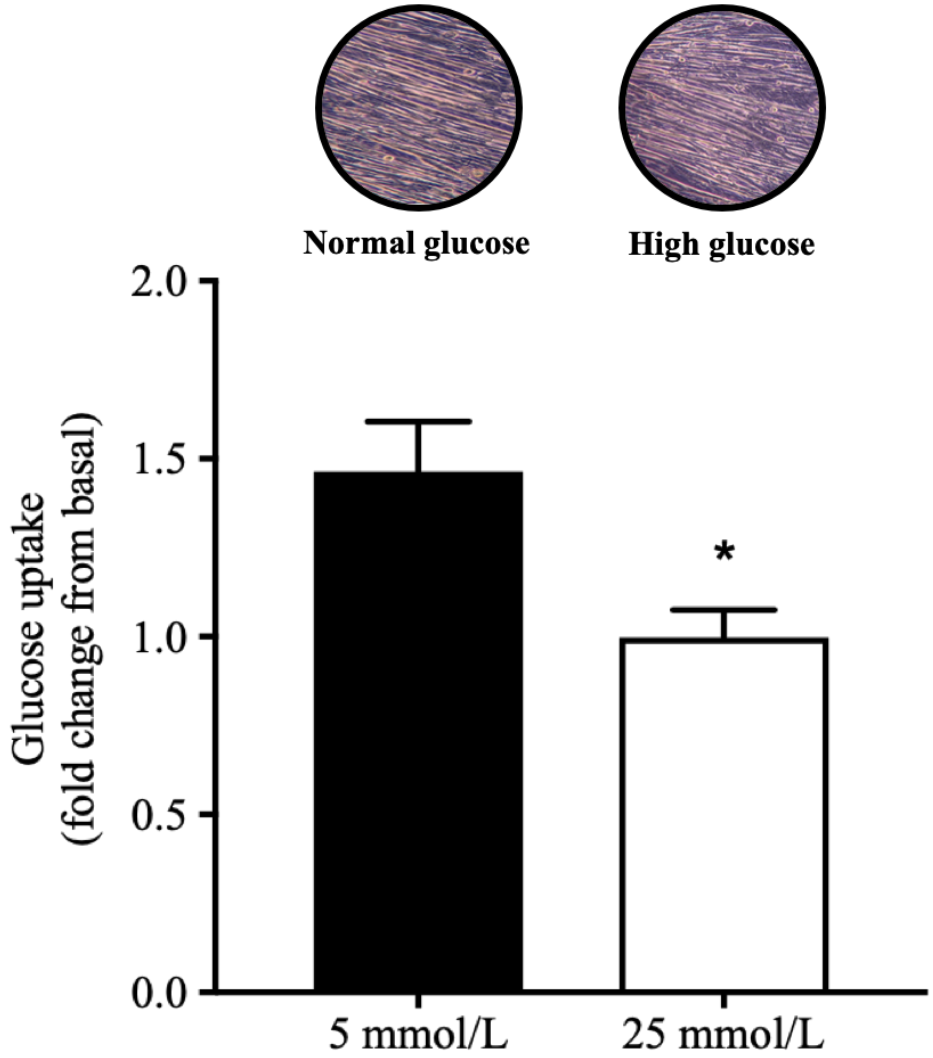



**Fig. 3.1 Switching routine cell culture from hyperglycaemic to normoglycaemic conditions produces an insulin sensitive model of skeletal muscle myotubes.** Basal and insulin-stimulated glucose uptake were assessed in C2C12 myotubes cultured in media containing either 5 mmol/L glucose or 25 mmol/L glucose throughout all routine culture procedures (i.e. storage → thawing → proliferation → differentiation). Microscope images demonstrate similar degree of myotube differentiation between conditions at the time that insulin-stimulated glucose uptake was assessed. Data are mean ± SEM and is based on preliminary experiments ($n = 5$; *$P<0.05$ *vs*. 5 mmol/L control).

### *3.1.3 Process of establishing insulin resistance inducing treatments*

As detailed above, a key research question of the current study was to examine whether hyperglycaemia *per se* induced skeletal muscle insulin resistance. Once an insulin sensitive skeletal muscle cell model had been established (as discussed above), the next task was to establish acute experimental treatments that can induce insulin resistance *in vitro*. Accordingly, a series of dose-response experiments were conducted, revealing conditions which induced skeletal muscle insulin resistance compared to control conditions (i.e. 5 mmol/L glucose). In line with the information provided above, 24 h exposure to 25 mmol/L glucose significantly impaired insulin-stimulated glucose uptake *vs*. 5 mmol/L control conditions ($P<0.05$), with statistical significance not reached for remaining concentrations ($P>0.05$). Results from this experiment are presented in Fig. 3.2.

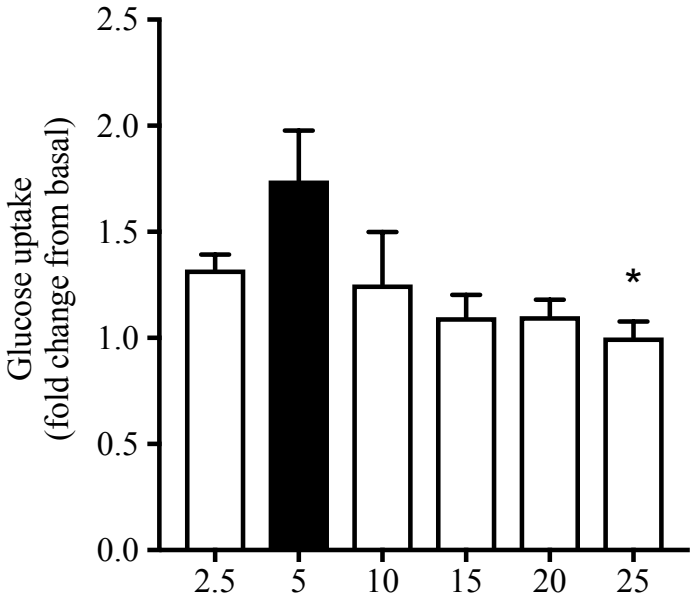



**Fig. 3.2 The effect of increasing glucose concentration on skeletal muscle glucose uptake.** C2C12 cells were cultured in normal glucose (5 mmol/L) culture conditions as detailed above. On day 6 of differentiation, myotubes were exposed to 2.5, 5, 10, 15, 20 and 25 mmol/L glucose for 24 h. Basal and insulin-stimulated glucose uptake were assessed. Data are mean ± SEM and is based on preliminary experiments ($n = 5$; *$P<0.05$ *vs*. 5 mmol/L control).

### *3.1.4 Process of establishing an in vitro skeletal muscle contraction model*

Multiple experimental models exist that enable different aspects of exercise to be isolated in skeletal muscle cells using *in vitro* approaches. Skeletal muscle contraction can be induced using electrical pulse stimulation (EPS), and specific exercise-related signalling pathways can be activated using pharmacological compounds such as 5-aminoimidazole-4-carboxamide-1-β-D- ribofuranoside (AICAR; an AMPK activator), caffeine (a $Ca^{2+}$ ionophore), ionomycin (a $Ca^{2+}$ ionophore) and forskolin (a protein kinase A (PKA) activator), and on occasion a combination/cocktail of these.

Given the importance of selecting the most appropriate model, a thorough narrative review of existing literature utilising these models was conducted (11) and is provided in chapter 2. This information was used to select the most appropriate model and protocol to address my experimental aims. Firstly, induction of contraction and subsequent activation of all associated signalling pathways was integral to correctly addressing the study aims, therefore the pharmacological compounds which focus on specific pathways were excluded. Instead, EPS was selected as the most appropriate model. Regarding the most appropriate EPS protocol, induction of effects comparable to those seen with chronic exercise, as opposed to acute exercise, was desired to generate a 'trained' cell model. Additionally, retaining the possibility of transferring the same EPS protocol to both C2C12 and human primary myotubes was also preferred. Based on these criteria, the following EPS protocol was applied: 24 h, 11.5 V, 1 Hz, 2 ms pulses. This protocol had previously been shown to induce exercise-like effects in both C2C12 (12) and human primary myotubes (13–18).

Glucose uptake and IL-6 secretion into conditioned media are frequently used as positive markers that EPS is resembling important aspects of *in vivo* exercise, and assessment of lactate dehydrogenase (LDH) accumulation into conditioned media is also required to confirm EPS protocols are not damaging cells during exposure (19). The specific EPS model selected met all of these criteria, therefore, confirming that this EPS model induces 'exercise-like' effects in the insulin sensitive C2C12 model discussed above. Results of these characterisation experiments are provided in full in chapter 4.

## 3.2 Development of models to assess the interaction of hyperglycaemia and exercise *in vivo*

The overarching aim of chapter 6 was to assess whether pre-exercise hyperglycaemia influenced exercise-induced changes in glycaemic control. Type 2 diabetes is characterised by chronically elevated blood glucose levels, as well as significant glycaemic instability. Since both aspects of diabetic-like hyperglycaemia induce differing degrees dysfunction when induced experimentally in healthy individuals (20,21), an additional aim was to assess whether the profile of hyperglycaemia (i.e. steady-state *vs*. fluctuating) influenced exercise-induced changes in glycaemic control. To address these distinct patterns of diabetes-like hyperglycaemia, establishment of the following distinct infusion models was required: 1) steady-state experimental hyperglycaemia and 2) fluctuating experimental hyperglycaemia.

### *3.2.1 Process of establishing steady-state hyperglycaemia*

To establish diabetic-like steady-state hyperglycaemia, the initial protocol involved a variable-rate intravenous glucose infusion to clamp participant blood glucose at 10

mmol/L. Within this protocol, blood glucose concentration is measured every 5 mins and infusion rate is adjusted accordingly based on calculations from a computer-based algorithm. For successful application of this procedure, it is vital that measured blood glucose values are a) very close to the target value (i.e. 10 mmol/L) and b) stable (i.e. low CV%). Meeting these criteria confirm attainment of successful clamp conditions. However, during preliminary trials these vital criteria were not met, meaning satisfactory clamp conditions could not be established. Specifically, glucose CV% was strikingly higher than is acceptable for this procedure (mean ± SEM: 12.6 ± 2.1%), as can be seen at both an individual (Fig. 3.3A) and group level (Fig. 3.3B). In particular, visual inspection of glucose profiles, especially towards the latter stages, demonstrates the relative similarity between steady-state and fluctuating hyperglycaemia profiles, albeit at different absolute concentrations (Fig. 3.3B).

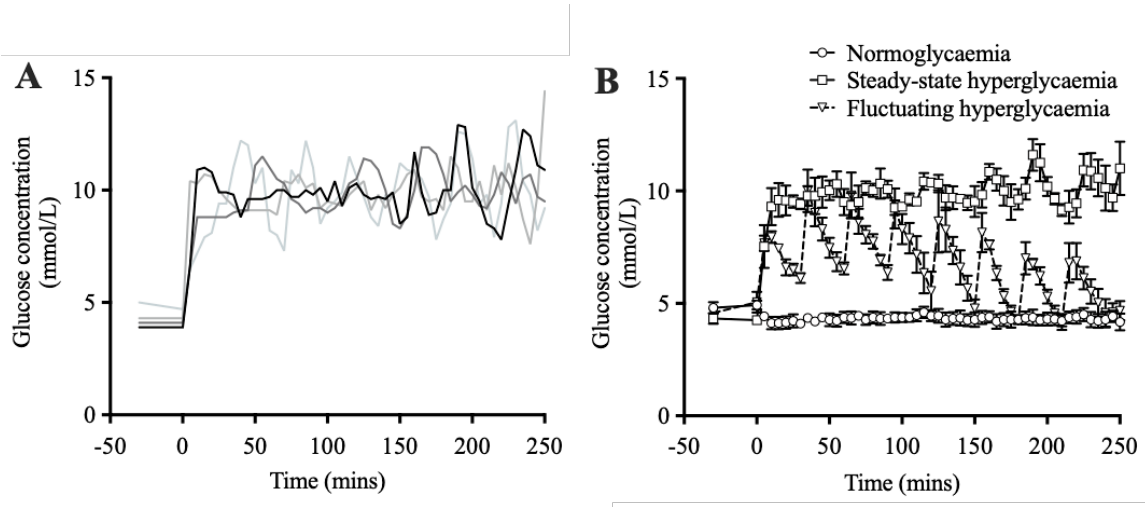



**Fig. 3.3 Blood glucose profiles during three glycaemic interventions.** Healthy participants underwent normoglycaemic, steady-state hyperglycaemic and fluctuating hyperglycaemic experimental conditions. Data presented are from preliminary trials for four participants. A) Glucose profiles for participants during the steady-state hyperglycaemia trial only, during which attempts were made to clamp glucose concentrations at 10 mmol/L via variable-rate intravenous glucose infusion. Each line represents a single participant. B) Mean glucose profile during three glycaemic interventions. Data are mean ± SEM.

Various possible causes of this high CV% were identified, of which inaccurate measurements of blood glucose (determined using HemoCue Glucose 201+, Radiometer, Copenhagen, Denmark) was deemed the most likely cause. Since the infusion rate is adjusted based on these blood glucose measurements, any inaccuracies inevitably introduce downstream errors. Three reference solutions provided by the manufacturer of known glucose concentrations were tested in triplicate on three separate occasions to determine mean error from known concentration (%) and mean measurement CV% (Table 3.1). For example, a mean CV% of the reference of closest concentration to the intended clamp goal (i.e. reference 2, 10.5 mmol/L) represents a variability of 0.42 mmol/L, which is unlikely to be conducive for achieving stable hyperglycaemic clamp conditions.

**Table 3.1 Mean measurement error and CV for HemoCue Glucose 201+ analyser**

| Reference solution | Glucose concentration (mmol/L) | Mean error (%) | Mean CV (%) |
|---|---|---|---|
| 1 | 4.9 | 1.8% | 4.6% |
| 2 | 10.5 | 1.2% | 4.0% |
| 3 | 15 | 1.0% | 3.6% |

An additional disadvantage of using the above clamp protocol is that total glucose infused during the clamp can only be accurately determined once the trial is complete. Therefore, directly matching total glucose infused between steady-state hyperglycaemia *vs*. fluctuating hyperglycaemia would only be possible by predetermining trial order to ensure steady-state always preceded fluctuating, thus precluding trial order randomisation and introducing risk of bias. Consequently, based on not having access to an alternative glucose analyser and the desire to retain randomised trial orders, the decision to alter the infusion protocol was made. Specifically, instead of clamping participant blood glucose at 10 mmol/L, 1.2 g/kg glucose was infused intravenously and distributed at a constant rate across a 3.5 h infusion period (equivalent to 5.71 mg/kg/min). Further preliminary work

demonstrated that this approach successfully elevated blood glucose several mmol/L above basal. This model was subsequently used to induce steady-state hyperglycaemia during the study detailed in chapter 6, full details and results of which are provided in the respective chapter.

### *3.2.2 Process of establishing a fluctuating glycaemic profile*

Establishing diabetic-like fluctuating hyperglycaemia was considerably more straightforward, with far fewer challenges encountered compared to establishing the steady-state hyperglycaemia condition. Briefly, 1.2 g/kg glucose was infused intravenously and distributed via 8 equal boluses every 30 mins across a 3.5 h infusion period (equivalent to 0.15 g/kg per bolus infused over 3.5 mins at a rate of 42.86 mg/kg/min) interspersed with 26.5 mins periods of no infusion. Results from the preliminary trials using this protocol are presented in (Fig. 3.3B). A notable advantage of amending the steady-state hyperglycaemia protocol is that matching total glucose infused across both steady-state and fluctuating hyperglycaemia infusion periods (i.e. 1.2 g/kg in both protocols) whilst retaining randomisation of trial orders was now possible. This model was subsequently used to induced fluctuating hyperglycaemia during the study detailed in chapter 6, full details and results of which are provided in the respective chapter.

## 3.3 Assay performance

Intra-assay and, where appropriate, inter-assay precision of assay-based analysis conducted throughout experimental chapters (4, 5 and 6) within this thesis are detailed in Table 3.2**.** Intra-assay precision is calculated as CV% between replicates within one plate, with the average intra-assay CV% of all measurements of the same outcome provided below. Inter-

assay CV% is calculated as CV% between replicates across multiple plates, with the average inter-assay CV% of all measurements of the same outcome provided below. Analysis of LDH, IL-6 and lactate in conditioned media, as well as qPCR derived gene expression was conducted once all samples had been collected and required only one plate/assay run per outcome meaning inter-assay CV% is not calculated. Similarly, inter-assay calculations for 2-DG concentration were not possible since samples only remain viable for analysis for 7 days after collection (22), meaning retaining a single sample for analysis on all plates was not possible.

**Table 3.2 Intra-assay and inter-assay CV for assay-based analysis**

| Outcome | Method | Chapter | Intra-assay CV (%) | Inter-assay CV (%) |
|---|---|---|---|---|
| LDH | Enzymatic assay | 4 | 5.5 % | - |
| IL-6 | Immunoassay | 4 | 6.3 % | - |
| Lactate | Enzymatic assay | 4 | 1.8 % | - |
| 2-DG | Enzymatic assay | 4 | 2.0 % | - |
| Protein | Bradford assay | 4 | 3.7 % | - |
| | | | | |
| mmu-miR-1a-1 | qPCR | 5 | 0.5 % | - |
| mmu-miR-23a | qPCR | 5 | 0.5 % | - |
| mmu-miR-7-1 | qPCR | 5 | 0.3 % | - |
| mmu-miR-486 | qPCR | 5 | 0.5 % | - |
| mmu-miR-27a | qPCR | 5 | 0.5 % | - |
| mmu-miR-378a | qPCR | 5 | 0.5 % | - |
| mmu-miR-99b | qPCR | 5 | 0.5 % | - |
| mmu-miR-215 | qPCR | 5 | 0.8 % | - |
| mmu-miR-133a-1 | qPCR | 5 | 0.8 % | - |
| mmu-miR-1249 | qPCR | 5 | 0.5 % | - |
| mmu-miR-194-1 | qPCR | 5 | 0.5 % | - |
| mmu-miR-125a | qPCR | 5 | 0.5 % | - |
| mmu-miR-155hg | qPCR | 5 | 0.4 % | - |
| mmu-miR-186 | qPCR | 5 | 0.3 % | - |
| mmu-miR-17hg | qPCR | 5 | 0.4 % | - |
| mmu-miR-22hg | qPCR | 5 | 0.3 % | - |
| ath-miR-159a | qPCR | 5 | 0.5 % | - |
| | | | | |
| Plasma insulin | ELISA | 6 | 3.9 % | 5.0% |
| Plasma IL-6 | ELISA | 6 | 6.2 % | 6.3% |
| Plasma CRP | ELISA | 6 | 4.2 % | 8.3% |

Full reagent and procedure details are provided in the relevant experimental chapter.

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# CHAPTER 4

# 4 SKELETAL MUSCLE CONTRACTION PROTECTS AGAINST HYPERGLYCAEMIA-INDUCED INSULIN RESISTANCE

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## 4.1 Abstract

**Background and aims:** Hyperglycaemia *per se* can impair skeletal muscle insulin sensitivity, but whether prior contractile activity of skeletal muscle directly protects against these hyperglycaemia-induced impairments remains to be determined.

**Methods:** Insulin-stimulated glucose uptake was measured in C2C12 myotubes exposed to normoglycaemic (5 mmol/L) *vs.* hyperglycaemic (25 mmol/L) conditions (0, 6, 18, 24 h) with and without prior contractile activity (electrical pulse stimulation; EPS; 24 h, 11.5 V, 1 Hz, 2 ms pulses).

**Results:** Similar to exercise *in vivo*, the *in vitro* contraction model induced visible myotube contraction, increased interleukin (IL)-6 accumulation in myotube conditioned media and upregulated glucose uptake ($P<0.05$). In non-EPS treated cells, 18 and 24 h of hyperglycaemia exposure significantly impaired insulin-stimulated glucose uptake *vs*. control ($P<0.05$). Prior skeletal muscle contractile activity ameliorated this hyperglycaemia-induced insulin resistance ($P>0.05$ *vs*. control).

**Conclusion:** Results demonstrate that the models of *in vitro* contraction and insulin resistance induce responses comparable to effects seen *in vivo* at the levels of IL-6 secretion, glucose uptake and insulin sensitivity. Using this model, findings demonstrate that skeletal muscle contraction *per se* prevents hyperglycaemia-induced insulin resistance in skeletal muscle cells. In chapter 5, this model will be used to further investigate the transcriptional changes associated with this contraction-mediated protective effect.

## 4.2 Introduction

Maintenance of normal glucose homeostasis relies on an intricately balanced and dynamic interaction between pancreatic β-cell insulin secretion and the sensitivity of target tissues (skeletal muscle, liver, fat) to the resultant circulating insulin (1). Whilst β-cell failure is ultimately requisite for the development of overt type 2 diabetes mellitus (T2DM), insulin resistance is considered as the first metabolic defect in the pathogenesis of T2DM (2). In particular, skeletal muscle represents a major site of glucose uptake, and insulin-mediated glucose uptake into skeletal muscle serves as a vital glucoregulatory mechanism, particularly important in the postprandial state (3). Failing to address this impairment in muscle insulin action contributes to the development of hyperglycaemia, which in turn contributes in a self-perpetuating manner, to the progression towards overt T2DM (1) and manifestation of hyperglycaemia-related complications (4–6). Clearly, optimising interventions to ameliorate insulin sensitivity is a vital step towards achieving healthy glycaemic control.

Hyperglycaemia and exercise oppositely regulate skeletal muscle insulin sensitivity. Specifically, hyperglycaemia *per se* impairs insulin-stimulated glucose uptake within human *in vivo* experimental models (7,8), as well as skeletal muscle cells in culture (9–12). Conversely, a single exercise bout enhances glucose uptake during and immediately following exercise; benefits which occur independently of insulin thus making exercise an vital nonpharmacological intervention to reduce hyperglycaemia in insulin-resistant individuals such as T2DM (13). Additionally, transient improvements in insulin sensitivity are gained over the following ~2 days (14). Whilst the insulin sensitising effect of each exercise bout reportedly lessens as muscle becomes trained (15), contraction-mediated

glucose uptake per exercise bout (16), as well as basal/rested insulin sensitivity (17) is enhanced in trained *vs*. untrained muscle. Importantly, exercise also enhances insulin sensitivity in those presenting with insulin resistance (18,19), further highlighting why exercise remains one of the most potent interventions to improve skeletal muscle glucose metabolism. That said, whether, and how, exercise directly protects against hyperglycaemia remains to be determined.

Dissecting the regulatory pathways through which exercise and hyperglycaemia oppositely regulate skeletal muscle insulin sensitivity could optimise the beneficial role of exercise, including minimising instances of impaired response, and may facilitate identification of new molecular targets to prevent/treat insulin resistance and associated metabolic abnormalities. Therefore, the aim of this chapter was to develop an experimental model that would allow us to evaluate the responses to contraction and hyperglycaemia *in vitro,* both alone and in combination.

Several experimental models have been proposed to investigate skeletal muscle-specific mechanisms of exercise *in vitro* with a level of abstraction not possible from *in vivo* studies (20). Of these, electrical pulse stimulation (EPS) offers the most appropriate *in vitro* model for researchers to specifically isolate the effect of skeletal muscle contraction *per se* upon cellular metabolism (20,21), but whether EPS-induced contraction directly prevents deleterious effects of hyperglycaemia is unknown. Accordingly, the purpose of the current study was to establish and characterise models of *in vitro* skeletal muscle contraction (i.e. EPS) and hyperglycaemia-induced insulin resistance. The overall aim was to combine these models to investigate whether skeletal muscle contraction *per se* protects against hyperglycaemia-induced impairments in glucose metabolism. It was hypothesised that

skeletal muscle contraction *per se* protects against hyperglycaemia-induced impairments in skeletal muscle glucose metabolism.

## 4.3 Research Design and Methods

### *4.3.1 Cell culture*

Mouse skeletal muscle C2C12 myoblasts (CRL-1772) were maintained in growth medium consisting of: Dulbeccos's Modified Eagles Medium (DMEM: GIBCO #11966025) containing 5 mmol/L glucose, 10% (v/v) fetal bovine serum (FBS), 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L GlutaMAX. For experiments, cells were seeded into 6-well plates at a density of 2 x $10^5$ cells/well in the above-described growth medium. At ~90% confluence, medium was changed to differentiation medium consisting of: DMEM containing 5 mmol/L glucose, 2% (v/v) horse serum, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L GlutaMAX. Differentiation medium was replaced daily for 5 days until myotubes had formed, as confirmed via visual inspection using an inverted light microscope. Cell passages between 11 and 15 were used for experimentation.

Experimental treatment consisted of exposing C2C12 myotubes to 24 h of contractile activity (electrical pulse stimulation; EPS) or rest (no EPS), followed by 0, 6, 18 or 24 h of normo- (NG; 5 mmol/L glucose) or hyperglycaemic (HG; 25 mmol/L glucose) conditions. The four experimental conditions are identified as follows: No EPS followed by normoglycaemia (CON); EPS followed by normoglycaemia (EPS); No EPS followed by hyperglycaemia (HG); EPS followed by hyperglycaemia (EPS HG). The experimental design is demonstrated in Fig. 4.1, with a full description of experimental treatment and analysis provided below.

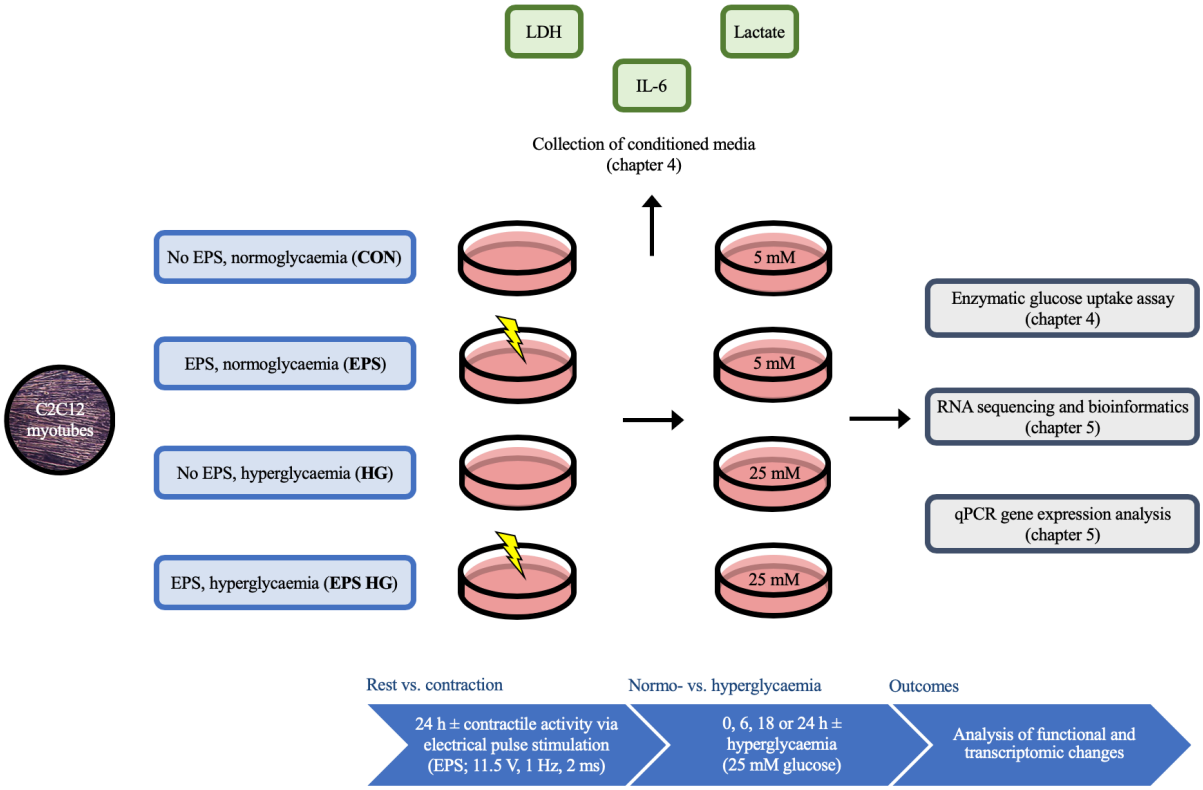



**Fig. 4.1 Schematic of experimental design.** C2C12 myotubes were exposed to 24 h of EPS *vs*. no EPS followed by 0, 6, 18 or 24 h normo- *vs*. hyperglycaemic conditions, after which functional (glucose uptake; this chapter) or transcriptional (RNA sequencing and qPCR; chapter 5) changes were assessed.

### *4.3.2 Electrical pulse stimulation*

On day 6 of differentiation, myotubes were washed into fresh differentiation medium, and exposed to 24 h rest (no EPS) or contraction (EPS; 11.5 V, 1 Hz, 2 ms pulses) using a C-Dish combined with a commercially available pulse generator (C-Pace EP, IonOptix, Westwood, CA, USA). In EPS treatment, C-Dish carbon electrodes were submerged into medium and power was applied. Muscle contraction was confirmed and documented via visual inspection and video recordings using an inverted light microscope. In rest (no EPS) treatment, C-Dish carbon electrodes were also placed into medium but without power as a sham control as recommended previously (21). After 24 h, ± EPS treatment was stopped

and the myotube-conditioned medium was collected and centrifuged, with the supernatant fraction filter sterilised using 0.45 μM Millipore syringe filters and stored at -80ºC until analysis of IL-6, lactate and LDH concentration.

### *4.3.3 Normo- vs. hyperglycaemia exposure*

Immediately following conditioned-medium collection, myotubes were washed into differentiation media containing either 5 mmol/L glucose (NG) or 25 mmol/L glucose (HG) and cultured for an additional 0, 6, 18 or 24 h. Finally, functional (basal and insulin-stimulated glucose uptake) responses to these conditions of rest *vs*. contraction and/or normo- *vs*. hyperglycaemia were evaluated.

### *4.3.4 IL-6 secretion*

Interleukin (IL) – 6 concentration in conditioned media from EPS and non-EPS treated myotubes was measured by homogenous time-resolved fluorescence (#63ADK043PEB, Cisbio Bioassays, Codolet, France) using the manufacturer's instructions.

### *4.3.5 Cytotoxicity assay*

Accumulation of lactate dehydrogenase (LDH) into conditioned medium is recognised as a marker of cell cytotoxicity. Assessment of LDH release into conditioned media from EPS and non-EPS treated myotubes is required to exclude the possibility that the EPS protocol caused cellular damage (21) and was achieved in the current study using a method described previously (22,23) with slight modification. Briefly, conditioned medium from ± EPS treated cells, as well as positive control (cell lysate collected by incubating C2C12 myotubes on ice in 1% Triton X-100 for 10 min) and negative control (fresh NG

differentiation media not exposed to cells), were transferred to a 96-well assay plate. Next, an assay medium containing 2 mmol/L sodium pyruvate and 200 μmol/L NADH were added to samples. Consumption or production of NADH were monitored at 37°C by assessing changes in absorbance at 340 nm using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany).

### *4.3.6 Lactate assay*

Lactate concentration in conditioned media from EPS and non-EPS treated myotubes was analysed using an autoanalyser (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) using a commercially available kit (Randox Laboratories, County Antrim, UK) following the manufacturer's instructions.

### *4.3.7 Glucose uptake*

Glucose uptake in differentiated C2C12 myotubes following the above treatments was measured using a non-radiolabelled 2-Deoxyglucose (2-DG) enzymatic assay as previously described (24). Specifically, following the above treatments, C2C12 myotubes were washed twice with DPBS and incubated in serum-free DMEM for 2 h at 37°C, 5% $CO_2$. Next, cells were washed twice with DPBS and then incubated for 30 mins at 37°C, 5% $CO_2$ in Krebs-Ringer Bicarbonate (KRB) buffer containing 115 mmol/L NaCl, 4.7 mmol/L KCl, 10 mmol/L HEPES, 1.2 mmol/L $MgSO_4$, 1.28 mmol/L $CaCl_2$, 1.2 mmol/L $KH_2PO_4$, 24 mmol/L $NaHCO_3$, 0.1% BSA, and either 0 nmol/L or 100 nmol/L insulin (Sigma-Aldrich I9278) to assess basal- and insulin-stimulated glucose uptake, respectively. Subsequently, cells were washed twice with DPBS and then incubated for 20 mins at 37°C, 5% $CO_2$ in KRB containing 1 mmol/L 2-DG. Next, cells were washed twice with DPBS

and lysed via agitated incubation in the presence of 0.1 N NaOH for 10 mins at 65 °C and a further 50 mins at 85 °C. Lysates were solubilised with 0.1 N HCl and then incubated, whilst shaking, for 10 mins at room temperature in 200 mmol/L triethanolamine, pH 8.

Lysates were exposed to one freeze-thaw cycle as recommended by (25) to ensure full degradation of any enzymes remaining following lysis that would otherwise interfere with the analysis. Finally, lysates were transferred to an assay plate containing assay medium with 50 mmol/L triethanolamine (pH 8), 50 mmol/L KCl, 15 U/mL glucose-6-phosphate dehydrogenase, 0.2 U/mL diaphorase, 0.1 mmol/L NADP, 0.02% (*w*/*v*) BSA, and 2 μmol/L resazurin (Invitrogen, Carlsbad, CA, USA). Selection of this specific assay solution composition is based on its superiority when assessing 2-DG uptake in cultured cells (26). This reaction mixture was incubated at 37 °C for 60 mins to allow 2-DG dependent reduction of resazurin to the fluorescent resorufin ($\lambda_{ex/em}$ = 540/590 nm). Resultant fluorescent was measured using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany). Glucose uptake was normalised to protein concentration as determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Glucose uptake results are derived from five independent experiments, each in triplicate.

### *4.3.8 Statistical analysis*

Paired t-tests or one-way ANOVA were used to compare the effect of EPS *vs*. CON on glucose uptake, IL-6, LDH and lactate (Fig. 4.2). Data from independent experiments displayed in Fig. 4.3 and Fig. 4.4 are presented normalised to appropriate time-matched internal (no EPS and/or no insulin stimulation) CON condition within each experiment, with differences detected using ANOVA followed by Bonferroni correction for multiple

comparisons. All analyses were performed in GraphPad Prism 7.0 (La Jolla, CA) with a value of $P<0.05$ considered to be statistically significant. Data are presented as mean ± SEM, with replicate numbers indicated in figure legends.

## 4.4 Results

### *4.4.1 Characterisation of the electrical pulse stimulation model (EPS vs. CON)*

EPS evoked contraction of skeletal muscle myotubes (based on visual inspection) without exerting damaging effects on myotubes, shown by the lack of difference in LDH release between EPS *vs*. CON ($P>0.05$, Fig. 4.2C). EPS-induced contractile activity of C2C12 myotubes significantly upregulated glucose uptake immediately following EPS cessation ($P<0.05$, Fig. 4.2A), and increased the accumulation of IL-6 in conditioned medium ($P<0.05$, Fig. 4.2B) *vs*. time-matched CON. No significant differences existed between ± EPS conditions with regards to media lactate concentration ($P>0.05$, Fig. 4.2D).

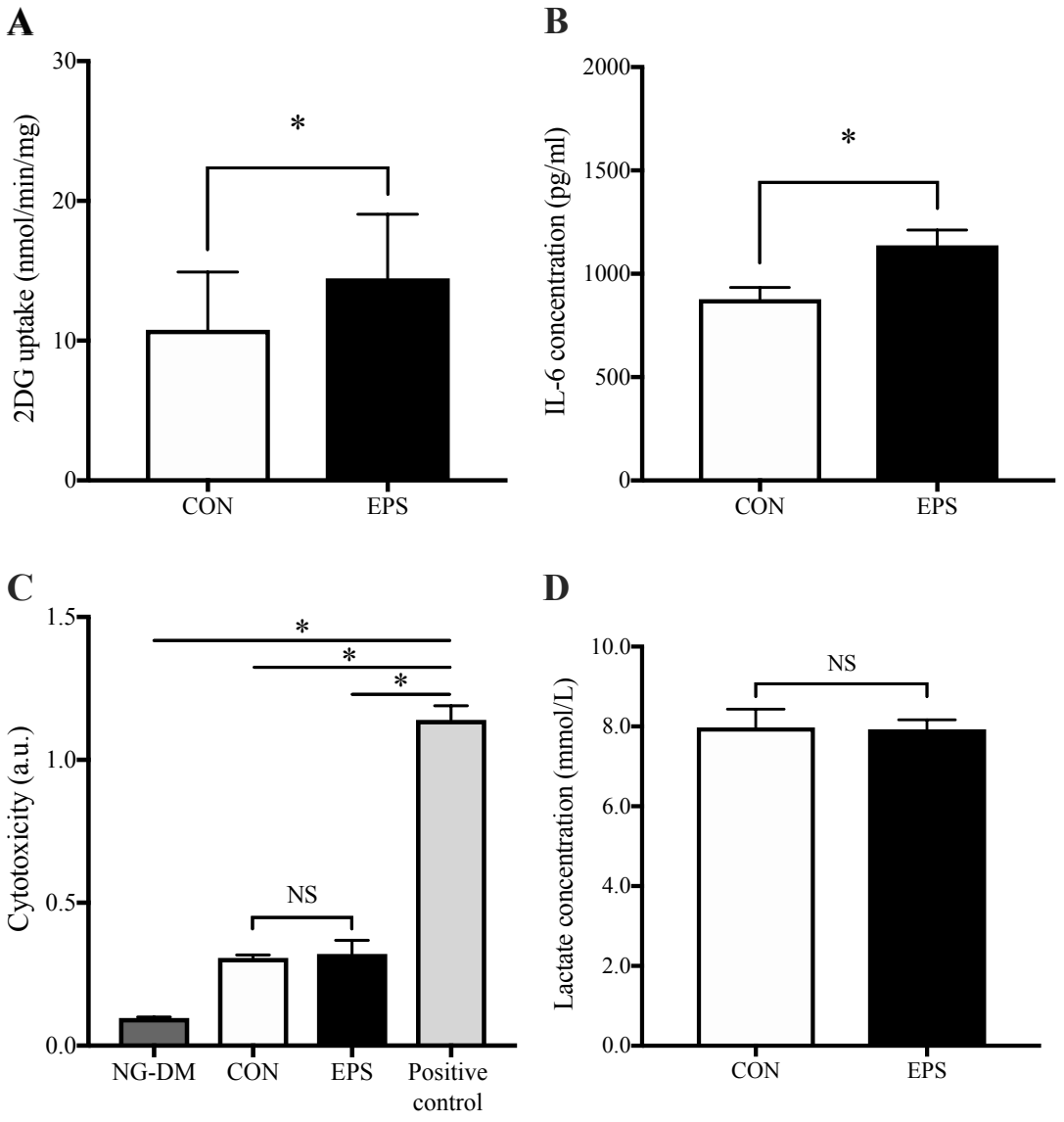



**Fig. 4.2 The effects of EPS on skeletal muscle glucose uptake, IL-6 secretion, cell membrane damage and lactate accumulation.** A) Glucose uptake in skeletal muscle cells treated with and without EPS ($n$ = 5; *$P$<0.05 *vs*. CON basal). B) IL-6 accumulation in conditioned media from myotubes treated with and without EPS ($n$ = 9; *$P$<0.05 *vs*. CON). C) Cytotoxicity as determined by LDH accumulation in conditioned media from myotubes treated with and without EPS ($n$ = 14; N.S, no significant difference *vs*. CON). D) Lactate accumulation in conditioned media from myotubes treated with and without EPS ($n$ = 16; N.S, no significant difference *vs*. CON). Data are from samples collected immediately following ± EPS cessation, and are mean ± SEM.

The magnitude of change in basal and insulin-stimulated glucose uptake follow a distinct pattern following exercise *in vivo*. The time-course profiles of contraction-mediated (i.e.

basal (non-insulin) glucose uptake in EPS relative to time-matched CON) and insulin-stimulated (i.e. insulin-stimulated glucose uptake in EPS relative to time-matched CON) glucose uptake are displayed below (Fig. 4.3). Notably, EPS significantly increased contraction-mediated glucose uptake only when assessed immediately following EPS cessation (0 h) *vs*. time-matched CON ($P<0.05$), whereas EPS significantly increased insulin-stimulated glucose uptake only when assessed 24 h post EPS cessation ($P<0.05$).

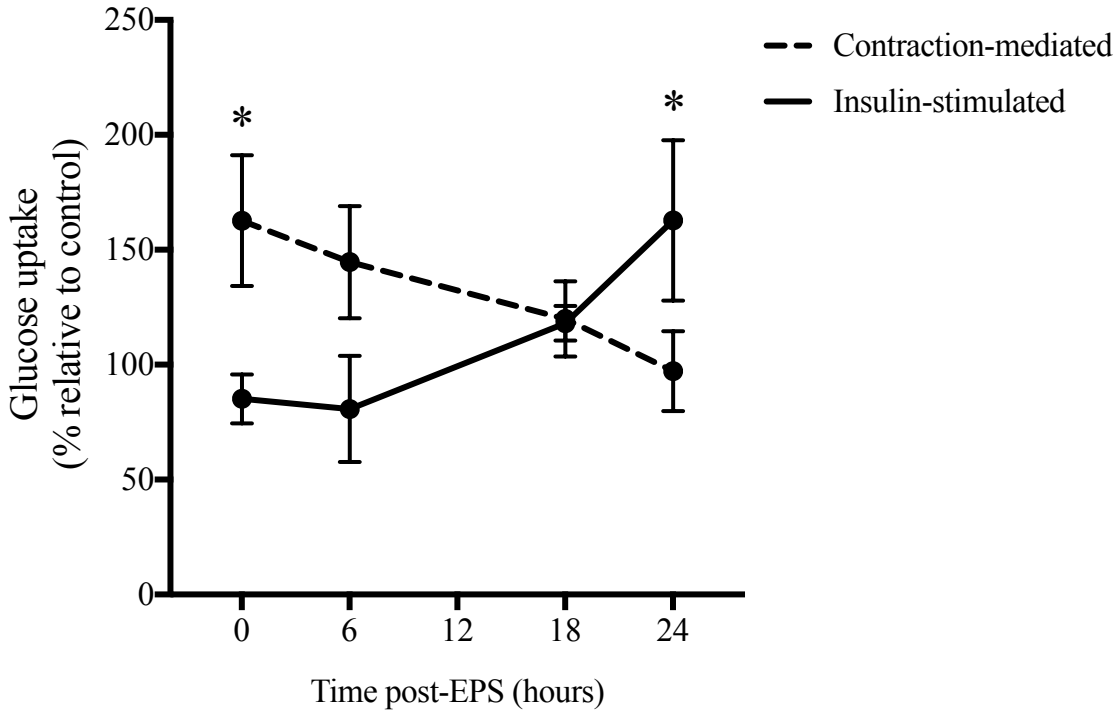



**Fig. 4.3 Time course of changes in glucose uptake in EPS *vs*. non-EPS treated myotubes.** EPS-induced changes in contraction-mediated (dashed line) and insulin-stimulated (solid line) glucose uptake were determined at 0, 6, 18 and 24 h following ± EPS cessation. For 6, 18 and 24 h post-EPS time points, treated cells were incubated in fresh normoglycaemic conditions immediately following ± EPS until subsequent assessment of glucose uptake. All values are presented relative to relevant time-matched CON as described above and are mean ± SEM ($n = 5$; *$P<0.05$ *vs*. time-matched CON).

### *4.4.2 Characterisation of insulin resistance model (HG vs. CON)*

To assess whether hyperglycaemia *per se* elicits changes in skeletal muscle cell glucose metabolism, basal and insulin-stimulated glucose uptake was assessed after 6, 18 and 24 h normoglycaemia (CON) *vs*. hyperglycaemia (HG) treatments. In CON (i.e. non-EPS, normoglycaemia treated cells), insulin significantly increased glucose uptake *vs*. basal levels ($P<0.05$). In 'rested' myotubes, where cells did not receive EPS treatment, insulin-stimulated glucose uptake was preserved following 6 h hyperglycaemia *vs*. CON ($P>0.05$), but significantly impaired at the level of glucose uptake by 18 and 24 h hyperglycaemia *vs*. CON (Fig. 4.4, $P<0.05$).

### *4.4.3 EPS protects C2C12 myotubes from hyperglycaemia-induced insulin resistance (EPS HG vs. CON)*

In contrast, in myotubes exposed to EPS-induced contractile activity no duration of hyperglycaemia induced any significant changes in insulin-stimulated glucose uptake *vs*. time-matched CON (Fig. 4.4, $P>0.05$). Combined, prior skeletal muscle contractile activity attenuated hyperglycaemia-induced impairment in insulin-stimulated glucose uptake.

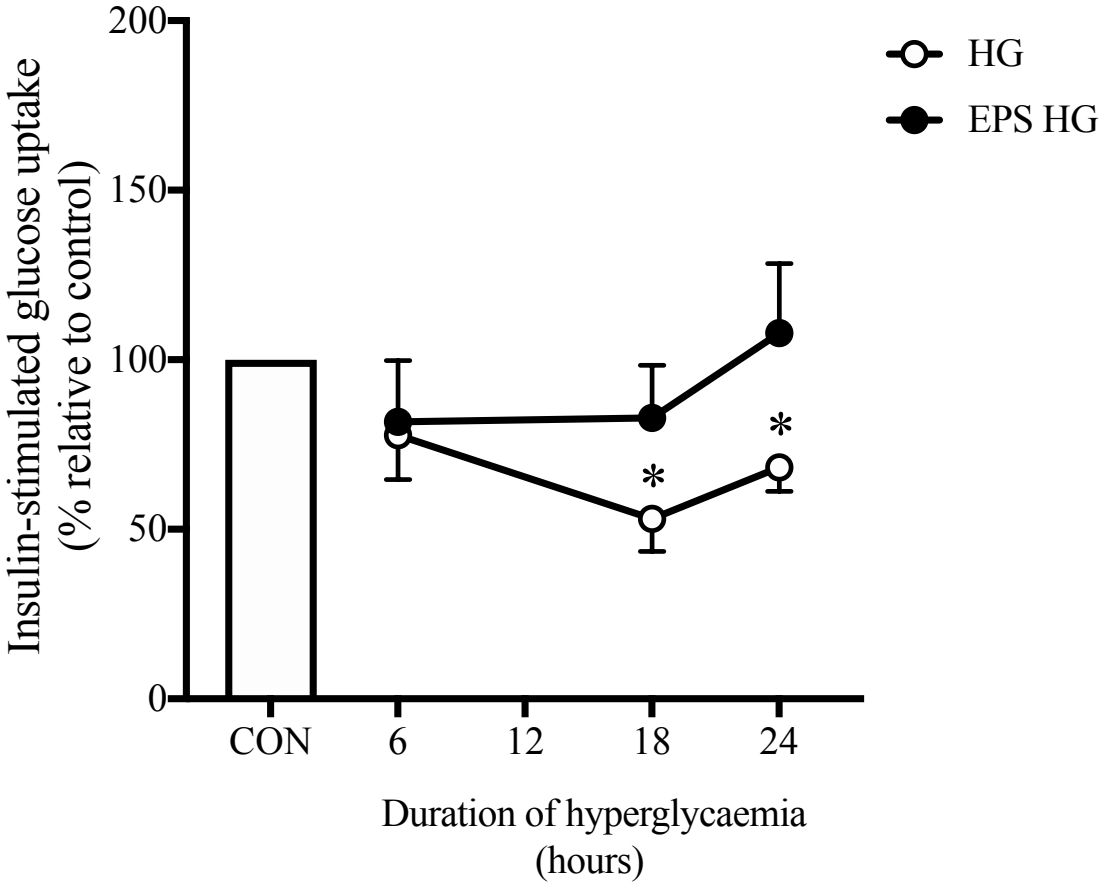



**Fig. 4.4 The effects of contraction and/or hyperglycaemia upon skeletal muscle insulin sensitivity.** Hyperglycaemia-induced changes in insulin-stimulated glucose uptake in myotubes treated with prior 'rest' (HG, white symbols) and contraction (EPS HG, black symbols). All values are presented relative to relevant time-matched CON as described above and are mean ± SEM ($n$ = 5; *$P$<0.05 *vs*. time-matched CON).

## 4.5 Discussion

Exercise confers vast health-promoting benefits and therefore forms the cornerstone in the prevention and management of a number of chronic diseases. Conversely, hyperglycaemia elicits deleterious effects upon metabolic health. Several of the benefits or impairments induced by exercise or hyperglycaemia, respectively, manifest as changes within skeletal muscle, such as responsiveness to insulin. In this study, separate models of *in vitro* contraction and hyperglycaemia-induced insulin resistance were established. These models

were then combined to assess whether skeletal muscle contraction *per se* protects against hyperglycaemia-induced insulin resistance.

Exercise *in vivo* increases skeletal muscle glucose uptake (27–29) and lactate production (30). Additionally, circulating IL-6 increases during exercise *in vivo*, attributable largely to IL-6 that is produced and released from contracting skeletal muscle *per se* (31–34). Accordingly, along with visual inspection of myotube contractility, IL-6 and lactate accumulation in conditioned medium and cellular glucose uptake were assessed as positive markers of exercise-like effects. Whilst media lactate accumulation did not differ between ± EPS conditions (our preliminary data suggest lactate concentration increases then plateaus in a time-dependent manner regardless of ± EPS exposure), the model of *in vitro* contraction significantly elevated skeletal muscle cell glucose uptake and increased IL-6 accumulation in conditioned media, without inducing cell death as demonstrated by no differences in media LDH (Fig. 4.2). It should be noted that whilst IL-6 concentrations observed in this study are comparable to those reportedly previously in EPS-treated C2C12 myotubes (35,36), concentrations are considerably higher than in healthy humans (by up to 150x). Cell membrane damage could result in passive leakage of intracellular proteins, such as IL-6, out of cells into the conditioned media. However, since EPS did not result in cell damage (indicated by similar media LDH levels) this is unlikely to explain this considerable discrepancy. Furthermore, IL-6 accumulation from human primary myotubes *in vitro* under control and EPS conditions can also reach between ~600 – 900 pg/ml (37,38), again far exceeding circulating concentrations in healthy humans *in vivo* meaning species differences *per se* are unlikely to account for this discrepancy *vs*. *in vivo* human physiology. Instead, high concentrations observed here and elsewhere (35–38) is perhaps indicative of inherent limitations of studying muscle cells *in vitro*. For example, the lack of

perfusion results in static accumulation of secreted factors in conditioned media, particularly if the very high rates of IL-6 turnover observed *in vivo* (31) are not evident *in vitro*. Additionally, conducting EPS for 24 h at 1 Hz (39) under normoglycaemic conditions (40) are conditions most conducive for potentiated EPS-induced IL-6 accumulation, possibly explaining discrepancies compared to lower EPS-induced IL-6 release from C2C12 myotubes reported previously (41,42). Nevertheless, visible myotube contraction, significantly increased glucose uptake and IL-6 secretion validate that the model induces exercise-like effects within skeletal muscle myotubes.

Moreover, improvements in contraction-mediated and insulin stimulated glucose uptake following exercise follow distinct time-dependent profiles, with initial increases in basal (non-insulin stimulated) glucose uptake followed by enhancements in insulin-stimulated glucose uptake (17). Serial assessments of basal and insulin-stimulated glucose uptake at 0, 6, 18 and 24 h following EPS within this study revealed similar time-dependent responses induced by the *in vitro* contraction model (Fig. 4.3). Specifically, compared to time-matched CON (i.e. no contraction), contraction-mediated glucose uptake (i.e. without insulin) was increased at 0 h post-EPS only (162 ± 28 % relative to CON, $P<0.05$), whereas insulin sensitivity was increased at 24 h post-EPS only (163 ± 35 % relative to CON, $P<0.05$).

The key mechanisms underpinning enhanced skeletal muscle glucose uptake and insulin sensitivity following exercise *in vivo* involve coordinated upregulation of glucose delivery (via increased microvascular perfusion), uptake and utilisation of delivered glucose by the skeletal muscle (via intracellular signalling) (43,44). In the current model, by design, skeletal muscle contraction *per se* has been isolated from other elements of *in vivo* exercise

physiology, including aforementioned changes in perfusion, meaning the current findings must be attributable to EPS-induced changes in glucose uptake/disposal by the contracting skeletal muscle cells, rather than changes in delivery.

Several intracellular signalling pathways combine to regulate exercise/contraction mediated enhancements in skeletal muscle glucose metabolism, primarily by promoting GLUT4 translocation to the plasma membrane (29,45,46). Contraction-induced GLUT4 translocation and resultant glucose uptake is driven by contraction-mediated stimulation of metabolic (activating the AMPK pathway (28) and downstream targets TBC1D1, TBC1D4 and Rab (47,48)), mechanical (partially mediated by Rac1 (49–52)) and calcium (activating CaMKII (47)) sensitive intracellular signalling pathways (13,53). Interestingly, elevated insulin-stimulated glucose uptake following exercise/contraction are not reliant on changes in the proximal steps of the insulin signalling pathway including the insulin receptor, insulin receptor substrate 1 (IRS1) or phosphatidylinositol 3-kinase (PI3K) since these are reportedly unchanged with exercise (43,54). Instead, insulin also promotes GLUT4 translocation via activation of several of the signalling molecules discussed above in the context of contraction, including AMPK (55), TBC1D1(48,56) and TBC1D4 (48,55), therefore suggesting that these converging points are likely to, at least partially, explain improved insulin action following exercise (13,43). Collectively, these contraction-responsive mechanisms may contribute to the EPS-induced improvements in skeletal muscle glucose uptake and insulin sensitivity seen here, although assessment of the phosphorylation signatures on AMPK, TBC1D1 and TBC1D4 via Western blot analyses, for example, would be required to confirm this suggestion.

The apparent delayed peak in EPS-induced improvements in insulin-stimulated glucose uptake (i.e. 24 h post-EPS only) compared to time-matched CON in the current study may be explained by evidence that the magnitude of EPS-induced upregulation of insulin-stimulated GLUT4 translocation is greatest >18 h post-EPS cessation (36). Insulin and contraction reportedly recruit distinct GLUT4 pools (57), and it has recently been shown that prior exercise promotes redistribution of intramuscular GLUT4 towards insulin-responsive GLUT4 storage vesicles thus enabling augmented insulin-stimulated GLUT4 translocation post-exercise (58). Collectively, one could speculate that if the regulation and recruitment of the insulin-responsive GLUT4 pool follows a different temporal profile (i.e. occurs later) to that of contraction responsive GLUT4 pools, this may contribute to the delayed peak in EPS-induced improvements in insulin sensitivity seen in this study. That said, further work characterising the temporal profile of these processes is required. Dissecting the underlying mediators controlling the distinct temporal effect of contraction upon muscle glucose metabolism could be targeted to optimise the efficacy of exercise in preventing insulin resistance and associated metabolic abnormalities. Therefore, in chapter 5 findings presented herein are extended to identify potential contraction-responsive transcriptional changes associated with these time-dependent metabolic effects.

Hyperglycaemia *in vivo* impairs several markers of glucose metabolism, including insulin sensitivity (7,8). A series of dose-response (as shown in chapter 3) and time-course experiments were conducted to establish conditions with which to induce similar effects *in vitro.* Whilst insulin sensitivity was preserved in cells exposed to 6 h hyperglycaemia *vs*. CON, 18 h and 24 h hyperglycaemia significantly impaired insulin-stimulated glucose uptake *vs*. time-matched normoglycaemic conditions. This hyperglycaemia-induced impairment agrees with previous evidence from hyperglycaemia treated skeletal muscle

cells (9–12), and may attributable to hyperglycaemia-induced impairments in GLUT4 translocation (59), oxidative (10) and nonoxidative glucose disposal (e.g. glycogen synthesis) (8) as well as elevated intracellular lipid accumulation (12). To understand whether contraction *per se* protects skeletal muscle from the aforementioned deleterious effects of hyperglycaemia, the two models were combined. The main finding from the current study was that insulin sensitivity was preserved under otherwise insulin resistance-inducing conditions. Specifically, whilst exposure to hyperglycaemia for 18 and 24 h (HG) impaired skeletal muscle insulin sensitivity, skeletal muscle contraction prior to hyperglycaemia exposure (EPS HG) prevented the impairment in insulin sensitivity seen in time-matched hyperglycaemic cells.

The contraction-mediated protection against hyperglycaemia-induced insulin resistance is similar to previous reports that similar *in vitro* contraction models confer protection against other insulin resistance inducing stimuli (namely, incubation in lipid or inflammatory cocktails). For example, incubation of human skeletal muscle myotubes in adipocyte-conditioned media and inflammatory adipokines such as chemerin or monocyte chemotactic protein (MCP) – 1 significantly impaired insulin-stimulated Akt phosphorylation whilst upregulating pro-inflammatory signalling (60). Concomitant EPS (i.e. during exposure to CM, chemerin or MCP-1) prevented these impairments, with authors suggesting that contraction prevented insulin resistance by blocking pro-inflammatory signalling pathways that were otherwise activated in the absence of contraction (60). Additionally, whilst lipid-induced insulin resistance manifests in non-contracted C2C12 myotubes at the levels of glucose uptake and canonical insulin signalling, prior contractile activity directly prevented these impairments in insulin sensitivity (61). The authors attributed this protection to contraction-mediated regulation of

both non-canonical (e.g. PI3Kα) and canonical activating pathways that were otherwise dysregulated by lipid exposure (61). Other existing evidence demonstrates EPS-induced contraction to prevents intramuscular lipid accumulation via enhanced fatty acid oxidation (62). Finally, muscle cells derived from exercise-trained/physically active individuals appear to be protected from lipid-induced (63) and ageing-associated (64) insulin resistance compared to myocytes from sedentary individuals.

The insulin resistance-inducing stimuli and/or timings relative to contraction differ between this study (i.e. contraction prior to hyperglycaemia exposure) compared to studies conducted by Lambernd et al. (60) (i.e. contraction during inflammatory adipokine exposure) and Nieuwoudt et al. (61) (i.e. contraction prior to lipid exposure). This precludes direct comparisons between studies, including directly transferring mechanistic findings of Lambernd et al. (60) and Nieuwoudt et al. (61) to explain the current data. Therefore, whilst contraction-mediated preservation of insulin sensitivity in otherwise insulin resistance inducing conditions (i.e. hyperglycaemia) may result from contraction-mediated improvements in glucose metabolism and/or attenuation of hyperglycaemia-induced dysregulation of glucose metabolism (both discussed above), the exact mechanisms underlying this protective effect specifically in the context of hyperglycaemia remain to be determined experimentally. Follow-up experiments, which aimed to advance knowledge in this area, are discussed in chapter 5.

Nevertheless, this data in combination with that of the studies discussed above (60,61) collectively emphasise the potency of skeletal muscle contraction alone in protecting against the development of insulin resistance that otherwise arises from a variety of stimuli. Future work to evaluate whether these protective effects are similarly preserved

even when exposed to all stimuli simultaneously (i.e. lipid, hyperglycaemia and inflammation) is warranted given that individuals with or at risk of developing conditions such as T2DM are likely to experience exposure to more than one of these simultaneously *in vivo*. Such knowledge will further our understanding of the contexts within which exercise exerts remedial effects. Equally, any potential scenarios where contraction alone is not sufficient and/or adaptation is impaired can be addressed, which in turn could be used to optimise the preventative and therapeutic effects of exercise.

## 4.6 Conclusion

In conclusion, data from this study characterises a model with which the opposite regulatory effects of hyperglycaemia and skeletal muscle contraction can be investigated. Similar to the *in vivo* situation, *in vitro* hyperglycaemia impaired skeletal muscle insulin sensitivity, whereas *in vitro* skeletal muscle contraction induced distinct temporal profiles for improvements in contraction-mediated glucose uptake and insulin sensitivity. Notably, the data demonstrate that skeletal muscle contraction *per se* protected skeletal muscle cells against hyperglycaemia-induced insulin resistance. This model will be used in chapter 5 to dissect potential transcriptional mediators of this contraction-mediated protective effect.

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# CHAPTER 5

## 5 CONTRACTION-MEDIATED PROTECTION AGAINST HYPERGLYCAEMIA-INDUCED INSULIN RESISTANCE COINCIDES WITH ATTENUATION OF HYPERGLYCAEMIA-RESPONSIVE TRANSCRIPTOMIC (DYS) REGULATION

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## 5.1 Abstract

**Background and aims:** Skeletal muscle contraction *per se* protects skeletal muscle cells from hyperglycaemia-induced insulin resistance (chapter 4). Molecular mediators controlling this protective effect could be targeted to prevent insulin resistance and associated metabolic abnormalities. Therefore, the aim of this study was to characterise the underlying transcriptional changes associated with these contraction-mediated protective effects.

**Methods:** C2C12 myotubes were exposed to 24 h of contractile activity (electrical pulse stimulation; EPS) or rest, followed by 0, 6, 18 or 24 h of normo- (5 mmol/L) or hyperglycaemic (25 mmol/L) conditions. An unbiased approach using RNA sequencing was utilised to characterise the transcriptional responses to these experimental treatments. Gene expression was assessed via qPCR to validate RNA sequencing-derived shortlisted transcripts.

**Results:** The enhancement of contraction-mediated (0 h post-EPS) and insulin-stimulated (24 h post-EPS) glucose uptake induced by EPS *vs*. CON coincided with differential expression of 143 and 145 transcripts, respectively, with notably minimal transcriptional overlap between these conditions. The impairment in insulin-stimulated glucose uptake induced by exposure to hyperglycaemia for 18 and 24 h coincided with differential expression of 115 transcripts in each condition. Notably, contraction-mediated attenuation of hyperglycaemia-induced insulin resistance in skeletal muscle cells coincided with prevented differential expression of 47 and 44 of the transcripts regulated by 18 and 24 h of hyperglycaemia, respectively. qPCR data support RNA sequencing expression profiles for miR-1a-1 and miR-194-1. Specifically, miR-1a-1 expression was significantly

upregulated in EPS HG *vs*. HG ($P<0.05$), and hyperglycaemia-induced downregulation of miR-194-1 expression in HG ($P<0.05$ *vs*. CON) was prevented by prior skeletal muscle contraction through significant opposite regulation ($P<0.05$ *vs*. CON and HG).

**Conclusion:** The present study characterised the time-course of hyperglycaemia- and contraction-responsive transcriptional changes in skeletal muscle cells. Data demonstrated that protection against hyperglycaemia-induced insulin resistance conferred by skeletal muscle contraction coincides with contraction-mediated normalisation of several hyperglycaemia-responsive transcriptional changes. Herein, miR-194-1 and miR-1a-1 are identified as possible novel contraction-responsive regulators of this contraction-mediated protective effect.

## 5.2 Introduction

Skeletal muscles represent a major site of glucose uptake and insulin serves as a potent stimulus to increase glucose uptake to maintain normoglycaemia (1,2). Maintaining skeletal muscle insulin sensitivity is important in the preservation of human metabolic health (3), and understanding how various stimuli differentially influence this key metabolic parameter remains vital in optimising interventions to improve glycaemic control. In chapter 4 it was shown that skeletal muscle contraction and hyperglycaemia elicit opposite regulatory effects on skeletal insulin sensitivity. These findings were extended to provide novel evidence that highlights a potent skeletal muscle contraction-mediated protective effect against hyperglycaemia-induced insulin resistance (chapter 4).

Exercise enhances skeletal muscle glucose uptake and insulin sensitivity via coordinated increased glucose delivery (via increased perfusion), uptake and utilisation (via intracellular signalling) (4–6). Effects demonstrated in chapter 4 are independent of changes in glucose delivery as, by design, skeletal muscle contraction *per se* was isolated from other elements of *in vivo* physiology, such as changes in perfusion. Accordingly, the contraction-mediated changes in glucose metabolism reported previously (chapter 4) must be attributable to changes within the contracting muscle, such as activation of various contraction-mediated signalling pathways, such as AMPK (7,8), TBC1D1 (8,9) and GLUT4 (10). Additionally, transcriptional changes within contracting muscle coincide with exercise-induced amelioration of glucose metabolism in muscle from individuals with (11) and without (12–14) metabolic conditions such as type 2 diabetes mellitus (T2DM). However, mechanisms underlying the protection that contraction *per se* directly confers against insulin-resistance inducing stimuli have only been characterised in a small number

of studies. For example, contraction directly prevented lipid-induced insulin resistance through attenuation of lipid-induced dysregulation of canonical and non-canonical signalling pathways (15), and inflammation-induced insulin resistance by attenuating inflammatory signalling (16). This suggests that the mechanism through which contraction prevents insulin resistance is context-dependent and/or stimuli-specific. Therefore, given the novelty of findings detailed in chapter 4, the precise mechanisms responsible for the contraction-mediated protection specifically against hyperglycaemia reported in chapter 4 remain to be determined.

Targeting the skeletal muscle transcriptome represents an emerging therapeutic option in the battle against metabolic diseases such as T2DM. As such, dissecting the key transcriptional regulators through which skeletal muscle contraction *per se* ameliorates hyperglycaemia-induced insulin resistance could facilitate the identification of novel exercise-responsive molecular targets that could be manipulated via alternative interventions, such as nutrition or pharmaceuticals, to improve skeletal muscle glucose metabolism. Such interventions could supplement exercise to improve exercise efficacy, which is particularly pertinent given the heterogeneity in the glucoregulatory effects of exercise (17–19). Furthermore, such interventions may provide 'exercise-like' benefits for individuals who are physically unable to exercise (long-term or temporarily as a result of illness or injury). Accordingly, the aim of this chapter was to characterise the transcriptional changes associated with the contraction-induced protection against hyperglycaemia-induced insulin resistance reported in chapter 4. It was hypothesised that hyperglycaemia and skeletal muscle contraction induce distinct transcriptional profiles in skeletal muscle cells. In particular, it was hypothesised that hyperglycaemia without prior

skeletal muscle contraction would induce a distinct transcriptional profile compared to hyperglycaemia with prior skeletal muscle contraction.

## 5.3 Research design and methods

### *5.3.1 Cell culture*

Mouse skeletal muscle C2C12 myoblasts (CRL-1772) were maintained in growth medium consisting of: Dulbeccos's Modified Eagles Medium (DMEM: GIBCO #11966025) containing 5 mmol/L glucose, 10% (v/v) fetal bovine serum (FBS), 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L GlutaMAX. For experiments, cells were seeded into 6-well plates at a density of 2 x $10^5$ cells/well in above growth medium. At ~90% confluence, medium was changed to differentiation medium consisting of: DMEM containing 5 mmol/L glucose, 2% (v/v) horse serum, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L GlutaMAX. Differentiation medium was replaced daily for 5 days until myotubes had formed, as confirmed via visual inspection using an inverted light microscope. Cell passages between 11 and 15 were used for experimentation.

Experimental treatment consisted of exposing C2C12 myotubes to conditions of contraction (electrical pulse stimulation; EPS) *vs*. rest (no EPS), followed by normo- (5 mmol/L glucose) *vs*. hyperglycaemia (HG; 25 mmol/L glucose). The four experimental conditions are identified as follows: No EPS followed by normoglycaemia (CON); EPS followed by normoglycaemia (EPS); No EPS followed by hyperglycaemia (HG); EPS followed by hyperglycaemia (EPS HG). The experimental design is demonstrated in Fig. 5.1, with full description of experimental treatment and analysis provided in respective sections below.

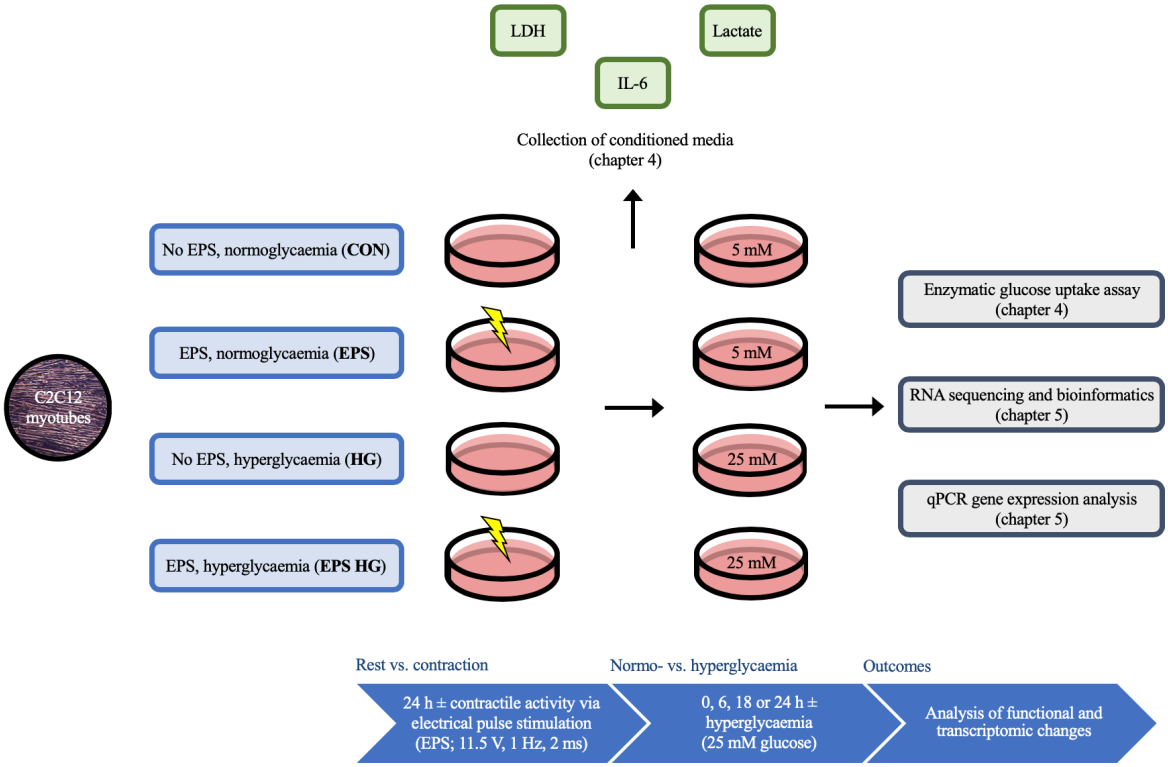



**Fig. 5.1 Schematic of experimental design.** C2C12 myotubes were exposed to 24 h of EPS *vs*. no EPS followed by 0, 6, 18 or 24 h normo- *vs*. hyperglycaemic conditions, after which functional (glucose uptake; chapter 4) or transcriptional (RNA sequencing and qPCR; this chapter) changes were assessed.

### *5.3.2 Electrical pulse stimulation*

On day 6 of differentiation, myotubes were washed into fresh differentiation medium, and exposed to 24 h rest (no EPS) or contraction (EPS; 11.5 V, 1 Hz, 2 ms pulses) using a C-Dish combined with a commercially available pulse generator (C-Pace EP, IonOptix, Westwood, CA, USA). In EPS treatment, C-Dish carbon electrodes were submerged into medium and power was applied. Muscle contraction was confirmed and documented via visual inspection and video recordings using an inverted light microscope. In rest (no EPS) treatment, C-Dish carbon electrodes were also placed into medium but without power as a sham control as recommended previously (20).

### *5.3.3 Normo- vs. hyperglycaemia exposure*

Immediately following ± EPS treatment, myotubes were washed into differentiation media containing either 5 mmol/L glucose (NG) or 25 mmol/L glucose (HG) and cultured for an additional 0, 6, 18 or 24 h. Transcriptome profiling (RNA sequencing and bioinformatics) in response to these conditions of rest *vs*. contraction and/or normo- *vs*. hyperglycaemia was completed. Finally, validation of RNA sequencing-derived expression profiles of shortlisted genes was completed using qPCR.

### *5.3.4 RNA sequencing and bioinformatics*

Transcriptome profiling of C2C12 myotubes exposed to 24 h of EPS *vs*. no EPS followed by 0, 6, 18 or 24 h normo- *vs*. hyperglycaemic conditions was conducted to characterise the time-course for transcriptional responses to different duration of a) rest following contraction cessation (0, 6, 18 and 24 h) and b) hyperglycaemia (6, 18 and 24 h). The overarching aim was to identify differentially expressed transcripts associated with the functional (glucose uptake) changes reported in chapter 4.

For RNA sequencing, total RNA from C2C12 myotubes was isolated with Trizol (Life Technologies) according to the manufacturer's instructions ($n$ = 3 per condition). Subsequent RNA sequencing and bioinformatics were conducted by BGI Genomics (Hong Kong) according to the analysis pipeline outlined in Fig. 5.2. Prior to sample processing RNA integrity number (RIN) for all samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) which assigns a RIN value from 1 (low integrity) to 10 (highest integrity), with a RIN of ≥ 7 required to proceed with subsequent analyses. RIN values confirmed that all RNA samples used for RNAseq had

high integrity (mean ± SEM: 9.76 ± 0.01; range: 9.60 to 9.90). Prior to quantification of gene expression sequencing data underwent rigorous quality control procedures following 1) data filtering (which removes 'dirty' reads that contain adaptors, high content of unknown bases or are of low quality to generate 'clean' reads) and 2) alignment to the reference genome (*mus musculus*). All samples passed these quality control measures allowing downstream analyses to proceed.

Transcript quantification was performed using the RNAseq by Expectation Maximization (RSEM) quantification tool (21) with normalised gene expression subsequently calculated using the fragments per kilobase per million reads (FPKM) method (22). This method eliminates the bias that sequencing depth and/or transcript length could otherwise introduce onto the calculation of gene expression if such correction was not used (23). Next, screening for differentially expressed genes (DEGs) within pairwise comparisons used the NOISeq method (23,24). This is a non-parametric approach that compares the signal (i.e. treatment *vs.* CON) to background noise to identify DEGs according to the following default criteria: Fold change ≥ 2 and diverge probability ≥ 0.8. In practise, a probability of 0.8 indicates a gene is four times more likely to truly be a DEG than not. Fold changes values are detailed as log2Ratio. Subsequently, clustering analysis of DEGs was used to arrange genes and conditions based on genes with similar fold change values (25).

Follow-up analysis was conducted to identify key biological processes and functions regulated by conditions of rest *vs.* contraction and/or normo- *vs.* hyperglycaemia. Specifically, gene ontology (GO; an international classification system used to comprehensively describe properties and functions of genes) annotation and Kyoto

Encyclopaedia of Genes and Genomes (KEGG; a reference database used to map genes to their biological functions) pathway enrichment analysis for DEGs was conducted using WEGO software (26,27), and the KEGG database (28), respectively. All pairwise comparisons were made relative to time-matched CON (no EPS, normoglycaemia) conditions.

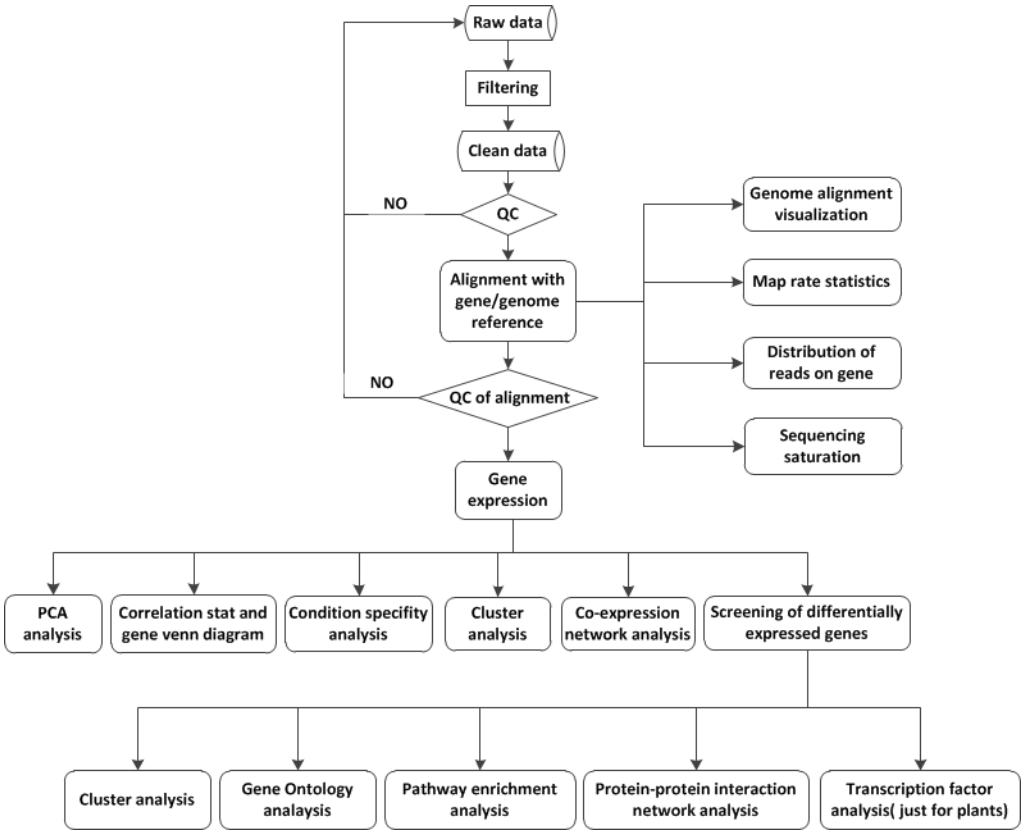



**Fig. 5.2 Bioinformatics pipeline completed by BGI Genomics.** Primary sequencing data (raw reads) underwent quality control (QC) followed by filtering to be aligned to the reference sequences (*mus musculus*). Resequencing and/or realignment was completed if necessary. Downstream analysis consisted of gene expression, clustering, GO annotation and KEGG pathway analysis. Schematic provided by BGI Genomics.

### *5.3.5 Validation of RNA sequencing results using qPCR*

Gene expression in C2C12 myotubes exposed to 24 h EPS or rest, followed by 24 h of normo- (5 mmol/L) or hyperglycaemic (25 mmol/L) conditions was assessed via qPCR to

validate shortlisted genes identified through RNA sequencing. For this, total RNA (including miRNAs) was extracted from C2C12 samples using mirVana miRNA isolation kit (Life Technologies) according to the manufacturer's instructions ($n$ = 5 per condition). Total RNA concentration and purity (260/280 ratio) were quantified using the LVis function of the FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany), with a 260/280 ratio of ≥ 1.7 indicating high integrity RNA. The 260/280 ratios confirmed that all RNA samples used for qPCR analyses had high integrity (mean ± SEM: 1.95 ± 0.01; range: 1.86 to 2.04).

RNA was reverse transcribed to cDNA using the Taqman Advanced miRNA cDNA synthesis kit (A28007) following the manufacturer's instructions. miRNA expression was determined using Taqman Advanced miRNA assays for target genes. Four endogenous reference genes (mmu-miR-186, mmu-miR-22hg, mmu-miR-17hg and mmu-miR-155hg) were identified as the miRNA most stably expressed across all experimental samples (i.e. lowest standard deviation) from aforementioned RNA sequencing analysis, and the mean Ct of these was used as the internal control (CV% of mean Ct across all samples = 5.36%). As exogenous control, a synthetic RNA oligonucleotide with a miRNA target sequence not expressed in skeletal muscle, namely ath-miR-159a, was added to all samples during the isolation procedure and expression analysis was completed to determine efficiency of extraction and sample handling procedures. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (29). Statistical analysis was conducted on ΔΔCt data. Identification of predicted targets (TargetScan and miRDB) and proposed functional classifications (miRbase, MGI and KEGG) of significantly regulated miRNAs was completed using freely available databases.

### *5.3.6 Statistical analysis*

For RNA sequencing experiments, DEGs were defined according to the following default criteria: Fold change ≥ 2 and diverge probability ≥ 0.8. GO terms were identified following Bonferroni correction for multiple comparisons with an adjusted $P<0.05$ considered to be statistically significant. Significantly enriched KEGG pathways were identified by a value of $q<0.05$.

For qPCR validation experiments, differences in gene expression (ΔΔCt data) across experimental conditions were determined using one-way repeated measures ANOVA followed by Bonferroni correction for multiple comparisons. All analyses were performed in GraphPad Prism 7.0 (La Jolla, CA) with a value of $P<0.05$ considered to be statistically significant. Data are presented as mean ± SEM, with replicate numbers indicated in figure legends.

## 5.4 Results

The *in vitro* models of skeletal muscle contraction and hyperglycaemia-induced insulin resistance used within this chapter are characterised fully in chapter 4.

### *5.4.1 EPS-induced enhancements in muscle glucose metabolism coincide with distinct temporal transcriptional profiles (EPS vs. CON)*

As shown in chapter 4, contraction of skeletal muscle cells induced distinct time-course profiles for changes in contraction-mediated (only significantly elevated immediately post-EPS) and insulin-stimulated (only significantly elevated 24 h post-EPS) glucose uptake in C2C12 myotubes. EPS-induced transcriptional changes were assessed at 0, 6, 18 and 24 h

post-EPS cessation. Collectively, RNA sequencing demonstrated that contraction induced a total of 567 DEGs across the four post-EPS conditions (Table 5.1). This included 374 individual genes, of which 144 were differentially expressed within 2 or more conditions, representing an overlap of 38.5% (Fig. 5.3A). Hierarchical clustering of the fold changes of genes identified as a DEG in at least one of the four post-EPS *vs*. time-matched CON comparisons demonstrates strikingly distinct temporal transcriptional profiles at these four time points post-EPS (Fig. 5.3B).

**Table 5.1 The time-course of changes in glucose uptake and the associated number of DEGs post-EPS relative to time-matched CON**

| | Contraction-mediated glucose uptake | Insulin-stimulated glucose uptake | DEGS | Up | Down |
|---|---|---|---|---|---|
| 0 h | ↑ | ↔ | 143 | 76 | 67 |
| 6 h | ↔ | ↔ | 141 | 74 | 67 |
| 18 h | ↔ | ↔ | 138 | 71 | 67 |
| 24 h | ↔ | ↑ | 145 | 71 | 74 |

↑; significantly increased *vs*. time-matched CON. ↓; significantly decreased *vs*. time-matched CON. ↔; no significant effect *vs*. time-matched CON. Glucose uptake data detailed fully in chapter 4.

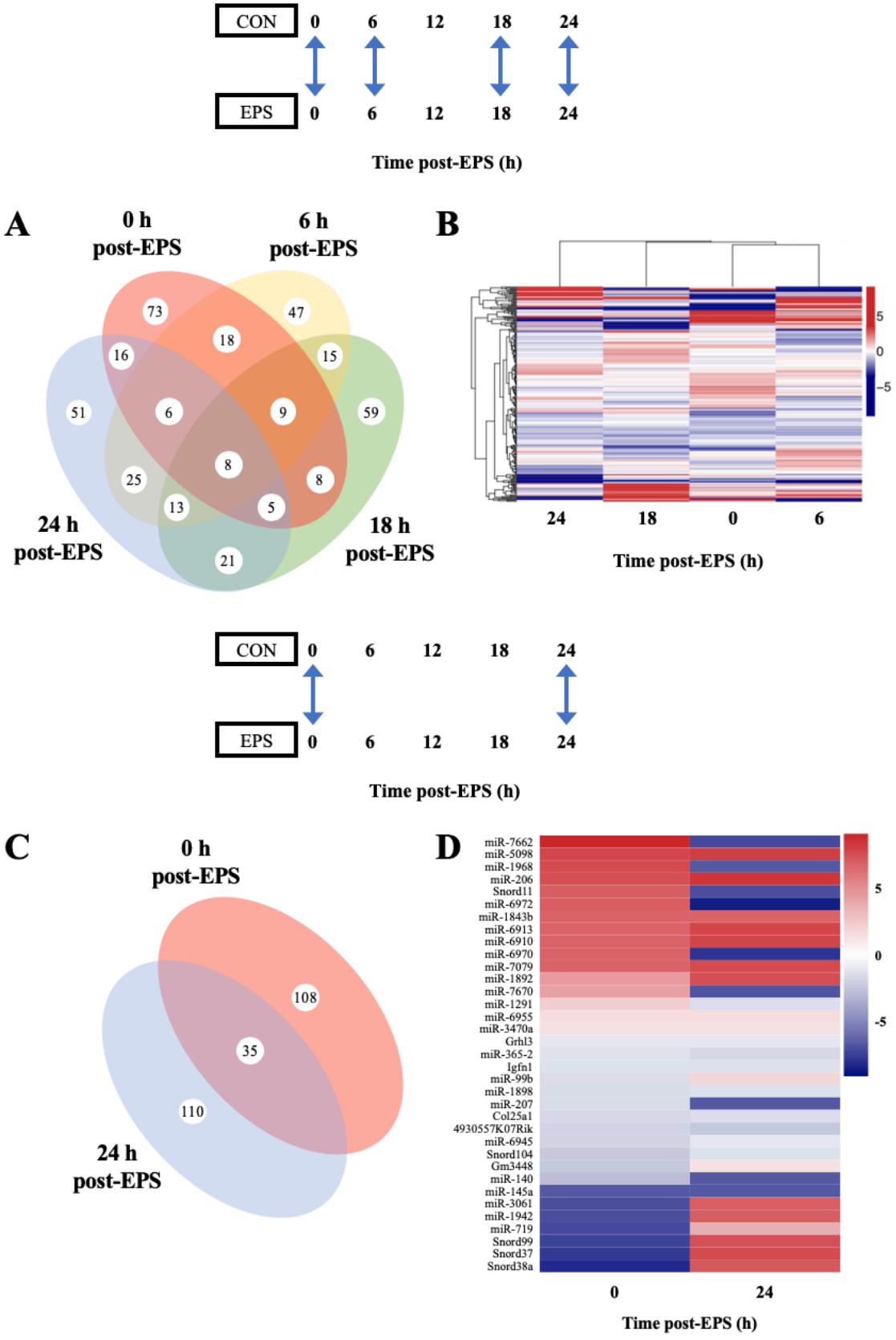



**Fig. 5.3 Transcriptional changes assessed at 0, 6, 18 and 24 h post-EPS relative to time-matched CON.** Immediately following ± EPS, cells were incubated in fresh

normoglycaemic differentiation media for indicated durations prior to subsequent RNA isolation. A) Venn diagram demonstrating overlap of DEGs between conditions of increasing duration post-EPS. B) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated within at least one of 0, 6, 18 or 24 h of post-EPS conditions *vs*. time-matched CON. C) Venn diagram demonstrating overlap of DEGs between 0 and 24 h post-EPS conditions. D) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated within at least one of 0 and 24 h post-EPS conditions *vs*. time-matched CON.

Notably, the distinct time-dependent changes in glucose metabolism following skeletal muscle contraction is associated with an equally distinct contraction-specific transcriptional profile. Focusing on the two time points with the most distinct differences in glucose uptake profile, namely 0 h (increased contraction-mediated) compared to 24 h (increased insulin sensitivity) post-EPS conditions, 143 DEGs coincided with improved contraction-mediated glucose uptake at 0 h post-EPS, whereas 145 DEGs coincided with improved EPS-induced improvements in insulin-stimulated glucose uptake at 24 h post-EPS (Table 5.1). Of the total 288 DEGs across these two time-points, 253 individual contraction-responsive genes were identified (Fig. 5.3C). Notably, only 35 genes were differentially expressed in both 0 and 24 h post-EPS conditions, of which only 20 genes were differentially expressed in the same direction within both time-points, and 15 were oppositely regulated (Fig. 5.3D). Similar to distinct DEG profiles, GO analysis highlights contraction-mediated activation of different GO pathways also occurs in a time-dependent manner (Fig. 5.4).

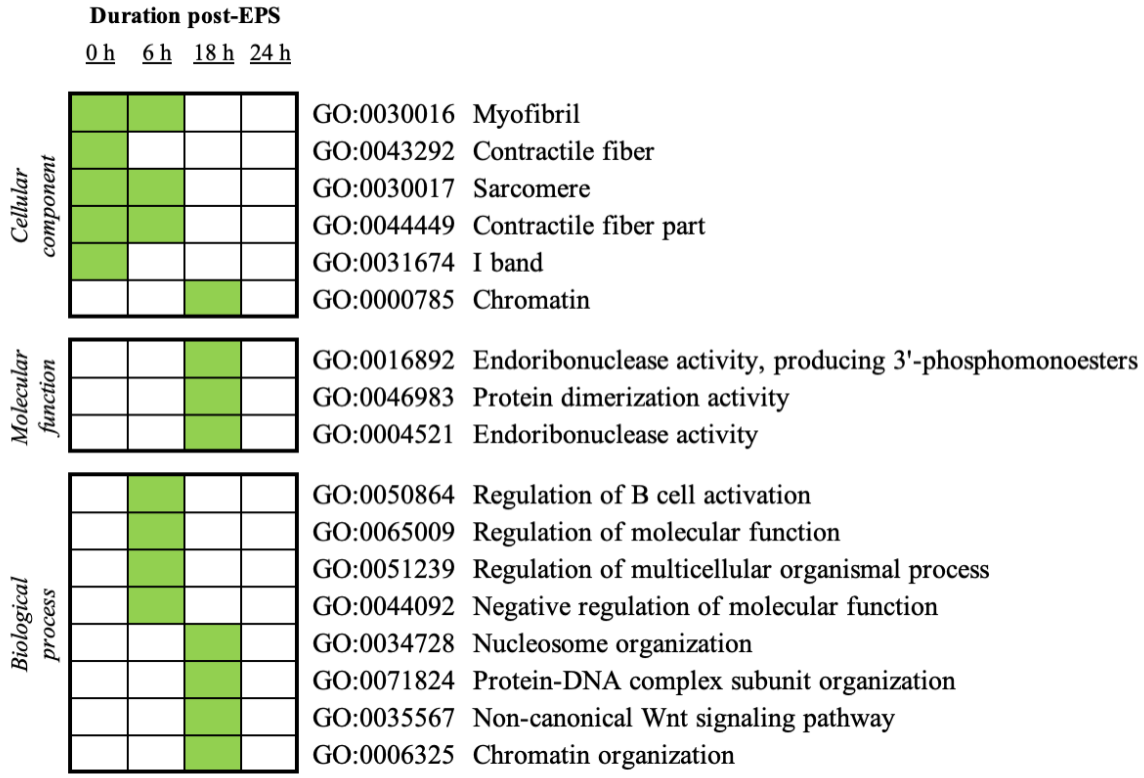



**Fig. 5.4 Functional characterization of the transcriptional signatures assessed at 0, 6, 18 and 24 h post-EPS relative to time-matched CON.** Significantly enriched GO term gene sets regulated within at least one of 0, 6, 18 or 24 h post-EPS conditions *vs.* time-matched CON. Green indicates significantly enriched GO terms (adjusted $P < 0.05$).

### *5.4.2 Hyperglycaemia-induced insulin resistance coincides with (dys) regulation of numerous transcripts (HG vs. CON)*

Insulin-stimulated glucose uptake was significantly impaired in skeletal muscle myotubes exposed to 18 and 24 h of hyperglycaemia *vs*. time-matched normoglycaemic conditions (chapter 4). Hyperglycaemia-induced transcriptional changes were assessed following 6, 18 and 24 h exposure to hyper- *vs*. normoglycaemia. A total of 375 DEGs were identified across the three hyperglycaemia durations (Table 5.2). This included 289 individual genes, of which 72 genes were differentially expressed within 2 or more conditions, representing an overlap of 24.9% (Fig. 5.5A). Hierarchical clustering of the fold changes of genes

identified as a DEG in at least one of the three hyperglycaemia *vs*. time-matched CON comparisons demonstrates the strikingly low degree of similarity between transcriptional profiles across these conditions (Fig. 5.5B).

**Table 5.2 The time-course of changes in insulin-stimulated glucose uptake and the associated number of DEGs following increasing durations of hyperglycaemia with (EPS HG) and without (HG) prior contractile activity relative to CON**

| | HG *vs*. CON | | | | EPS HG *vs*. CON | | | |
|---|---|---|---|---|---|---|---|---|
| | Insulin-stimulated glucose uptake | DEGs | Up | Down | Insulin-stimulated glucose uptake | DEGs | Up | Down |
| 6 h | ↔ | 145 | 83 | 62 | ↔ | 151 | 81 | 70 |
| 18 h | ↓ | 115 | 60 | 55 | ↔ | 142 | 77 | 65 |
| 24 h | ↓ | 115 | 58 | 57 | ↔ | 143 | 82 | 61 |

↑; significantly increased *vs*. time-matched CON. ↓; significantly decreased *vs*. time-matched CON. ↔; no significant effect *vs*. time-matched CON. Glucose uptake data detailed fully in chapter 4.

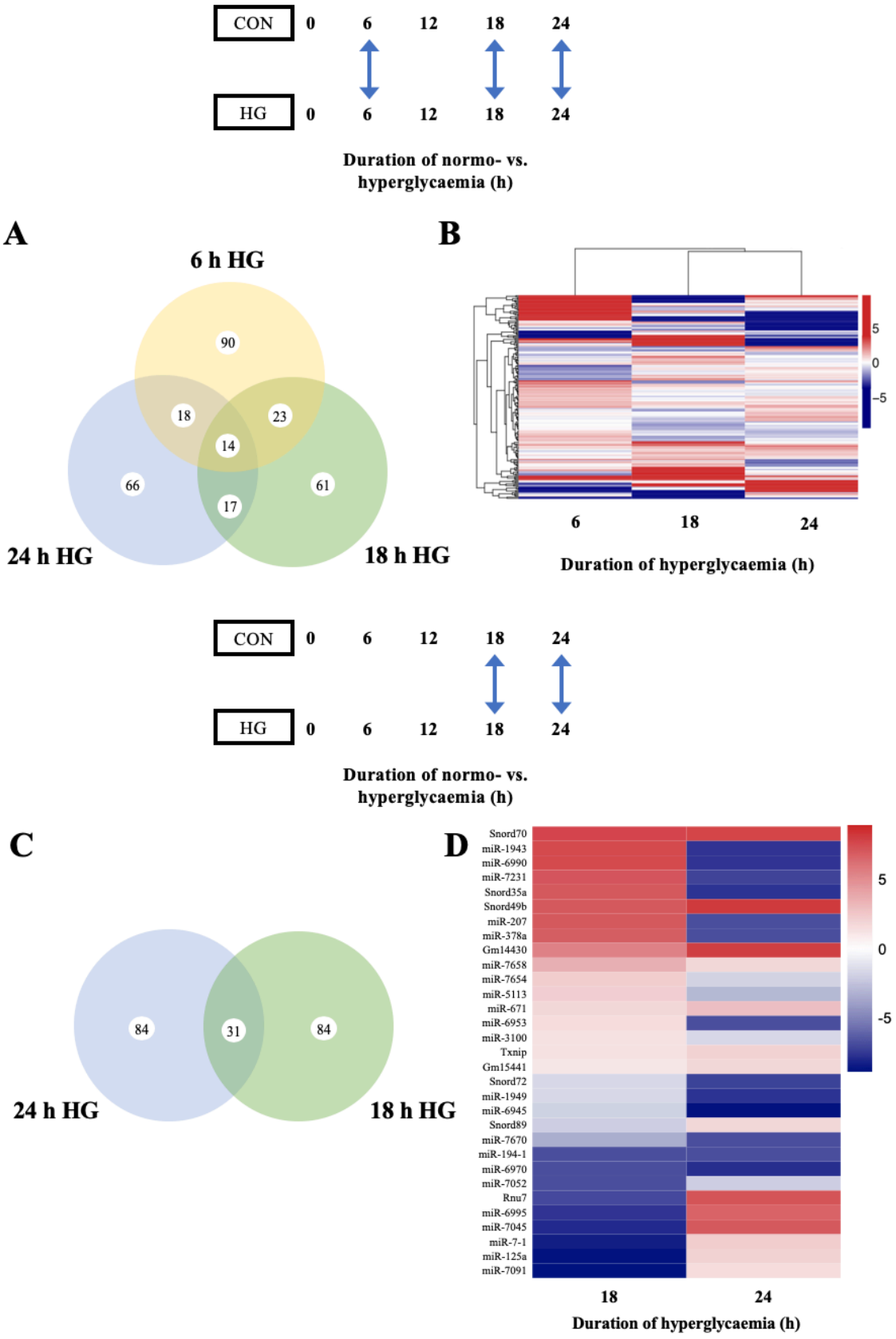



**Fig. 5.5 Transcriptional changes following increasing duration of hyperglycaemia relative to time-matched CON.** A) Venn diagram demonstrating overlap of DEGs

between conditions of increasing hyperglycaemia. B) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated by at least one of 6, 18 or 24 h of hyperglycaemia *vs*. time-matched CON. C) Venn diagram demonstrating overlap of DEGs between 18 and 24 h hyperglycaemia conditions. D) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated within at least one of 18 and 24 h hyperglycaemia conditions *vs*. time-matched CON.

RNA sequencing analysis demonstrates that hyperglycaemia-induced insulin resistance coincides with differential expression of 115 genes within each of 18 and 24 h hyperglycaemic *vs*. time-matched normoglycaemic conditions (Table 5.2). Of the total 230 DEGs across these two insulin resistance-inducing conditions, 158 individual hyperglycaemia-responsive genes were identified (Fig. 5.5C). Only 31 genes were differentially expressed within both conditions; 14 of which were the DEG in the same direction and 17 were oppositely regulated (Fig. 5.5D). Similarly, GO analysis of DEGs highlights a time-dependent effect of exposure to HG upon activation of different GO pathways (Fig. 5.6).

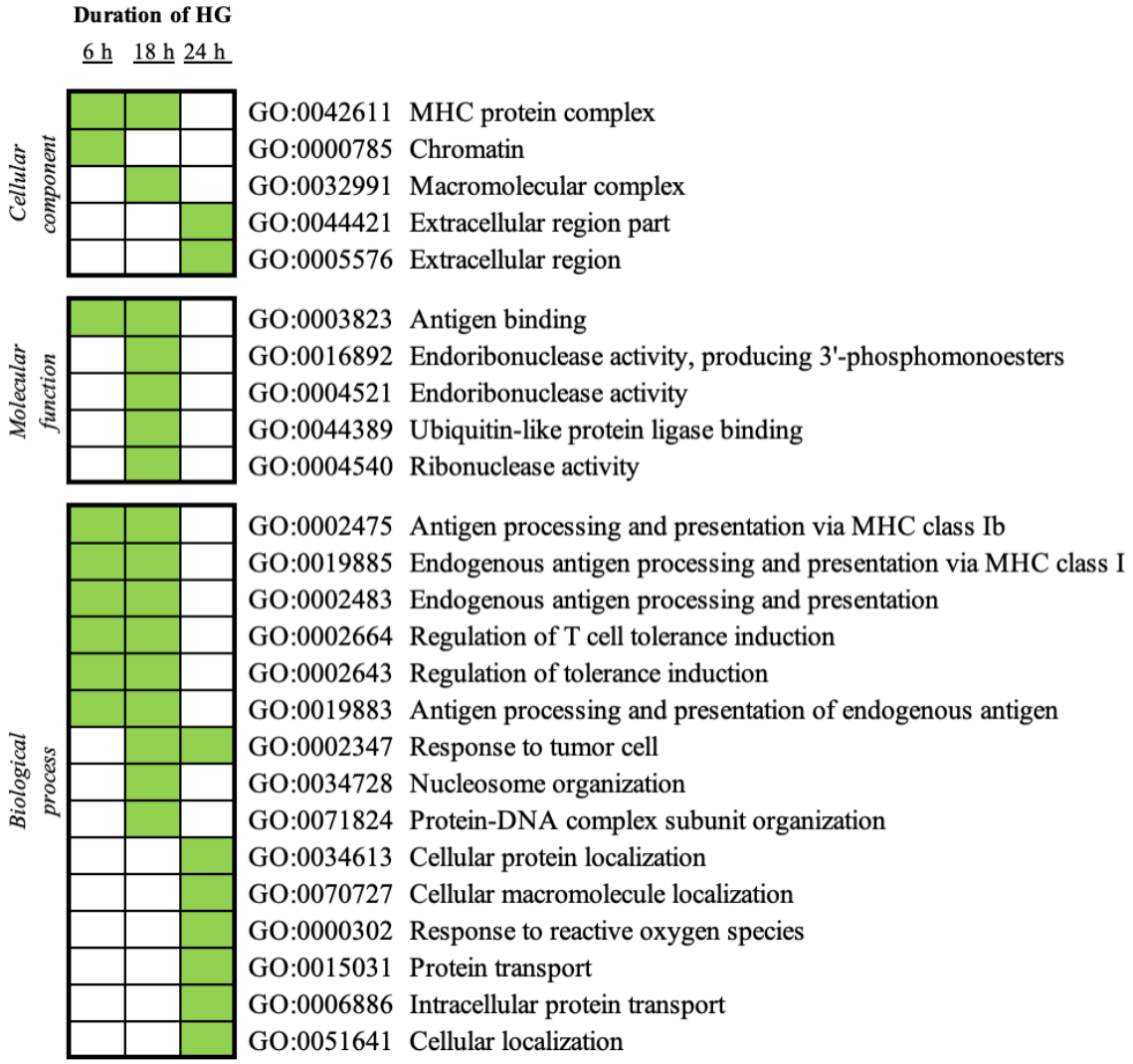


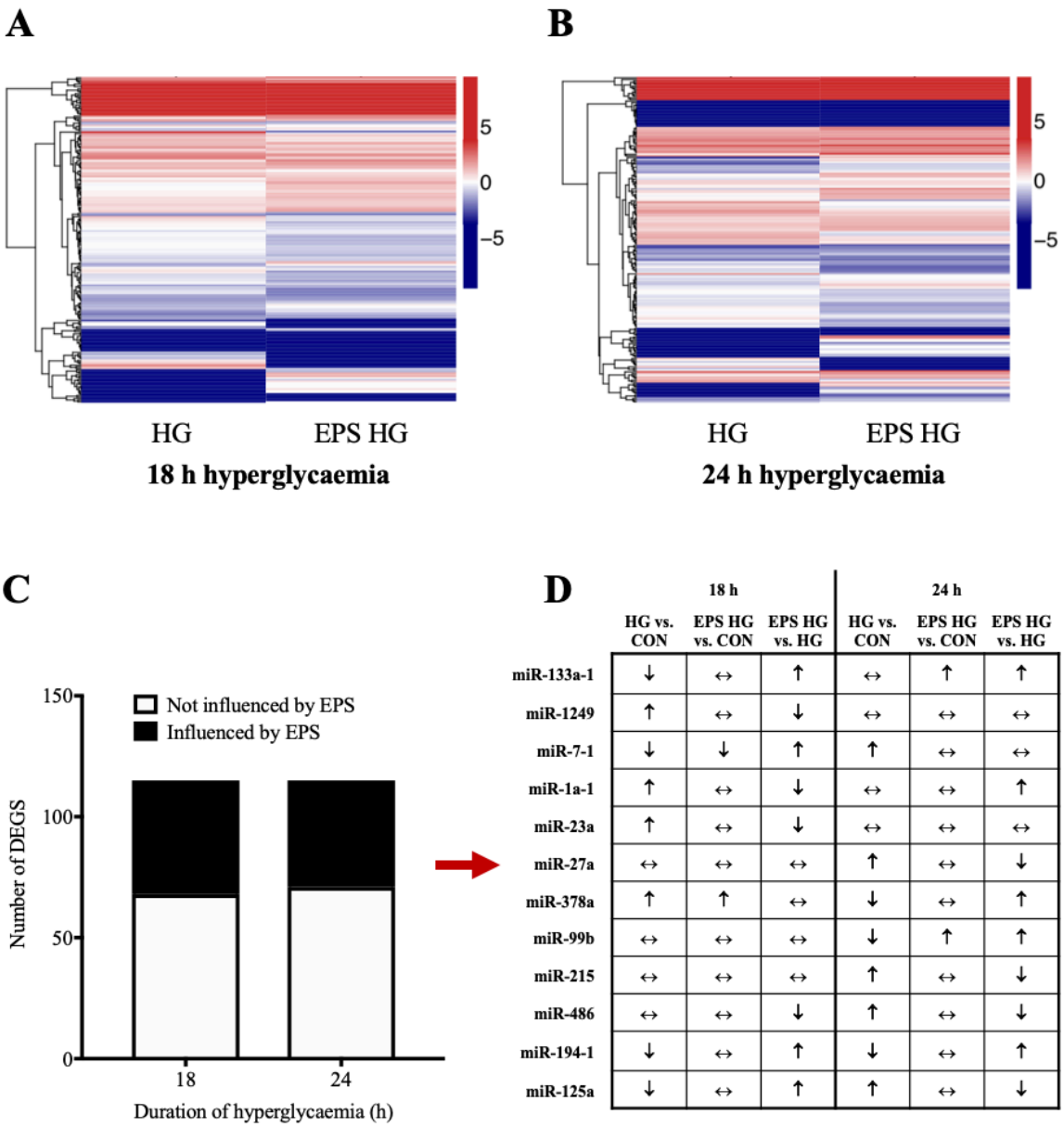

**Fig. 5.6 Functional characterization of the transcriptional signatures following increasing duration of hyperglycaemia relative to time-matched CON.** Significantly enriched GO term gene sets regulated within at least one of 6, 18 or 24 h hyperglycaemia *vs.* time-matched CON. Green indicates significantly enriched GO term (adjusted $P<0.05$).

### *5.4.3 Skeletal muscle contraction attenuates hyperglycaemia-induced insulin resistance and associated transcriptional changes (EPS HG vs. HG vs. CON)*

EPS-induced contraction attenuated hyperglycaemia-induced insulin resistance in C2C12 myotubes (chapter 4). RNA sequencing was used to identify potential transcriptional regulators of this contraction-mediated protection, with results demonstrating that EPS

prevented the (dys) regulation of multiple hyperglycaemia-responsive genes. Specifically, of the 115 hyperglycaemia-responsive genes within each of the 18 and 24 h conditions, prior contractile activity prevented the (dys) regulation of 47 and 44 genes, respectively (Fig. 5.7C). Hierarchical clustering of DEGs demonstrates the degree of opposite regulation induced by HG *vs*. EPS HG within 18 h (Fig. 5.7A) and 24 h (Fig. 5.7B) conditions. Of the 91 DEGs across both conditions, 81 individual genes were identified, consisting primarily of non-coding RNAs (54 x miRNAs; 18 x snoRNAs). Based on this, and the intention to maximise applicability to human muscle in the future, this list was condensed to include only miRNAs known to be expressed in human skeletal muscle for further validation within this experimental design. RNA sequencing derived expression of the final 12 miRNAs on this shortlist are presented in Fig. 5.7D.

Classification of enriched GO and KEGG terms for genes identified as DEGs was conducted to explore functional processes regulated by 24 h hyperglycaemia with (EPS HG) and without (HG) prior contractile activity (Fig. 5.8). These classification categories provide insight into global trends of functions and processes that are differentially regulated between hyperglycaemic cells that are insulin resistant (HG) or retain insulin sensitivity (EPS HG).




| | 18 h | | | 24 h | | |
|---|---|---|---|---|---|---|
| | HG vs. CON | EPS HG vs. CON | EPS HG vs. HG | HG vs. CON | EPS HG vs. CON | EPS HG vs. HG |
| miR-133a-1 | ↓ | ↔ | ↑ | ↔ | ↑ | ↑ |
| miR-1249 | ↑ | ↔ | ↓ | ↔ | ↔ | ↔ |
| miR-7-1 | ↓ | ↓ | ↑ | ↑ | ↔ | ↔ |
| miR-1a-1 | ↑ | ↔ | ↓ | ↔ | ↔ | ↑ |
| miR-23a | ↑ | ↔ | ↓ | ↔ | ↔ | ↔ |
| miR-27a | ↔ | ↔ | ↔ | ↑ | ↔ | ↓ |
| miR-378a | ↑ | ↑ | ↔ | ↓ | ↔ | ↑ |
| miR-99b | ↔ | ↔ | ↔ | ↓ | ↑ | ↑ |
| miR-215 | ↔ | ↔ | ↔ | ↑ | ↔ | ↓ |
| miR-486 | ↔ | ↔ | ↓ | ↑ | ↔ | ↓ |
| miR-194-1 | ↓ | ↔ | ↑ | ↓ | ↔ | ↑ |
| miR-125a | ↓ | ↔ | ↑ | ↑ | ↔ | ↓ |

**Fig. 5.7 Transcriptional changes coinciding with contraction-mediated protection against hyperglycaemia-induced insulin resistance.** A) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated by at least one of 18 h hyperglycaemia with (EPS HG) or without (HG) prior contraction *vs*. time-matched CON. B) Heat map of the fold increases (red) or decreases (blue) for DEGs regulated by at least one of 24 h hyperglycaemia with (EPS HG) or without (HG) prior EPS *vs*. time-matched CON. C) The proportion of hyperglycaemia responsive DEGs influenced by prior skeletal muscle contraction. D) Shortlisted miRNAs detailed were differentially expressed within both of the following pairwise comparisons at 18 and 24 h time points: i) HG *vs*. CON and ii) HG *vs*. EPS HG. ↑; significantly increased *vs*. time-matched CON. ↓; significantly decreased *vs*. time-matched CON. ↔; no significant effect *vs*. time-matched CON.

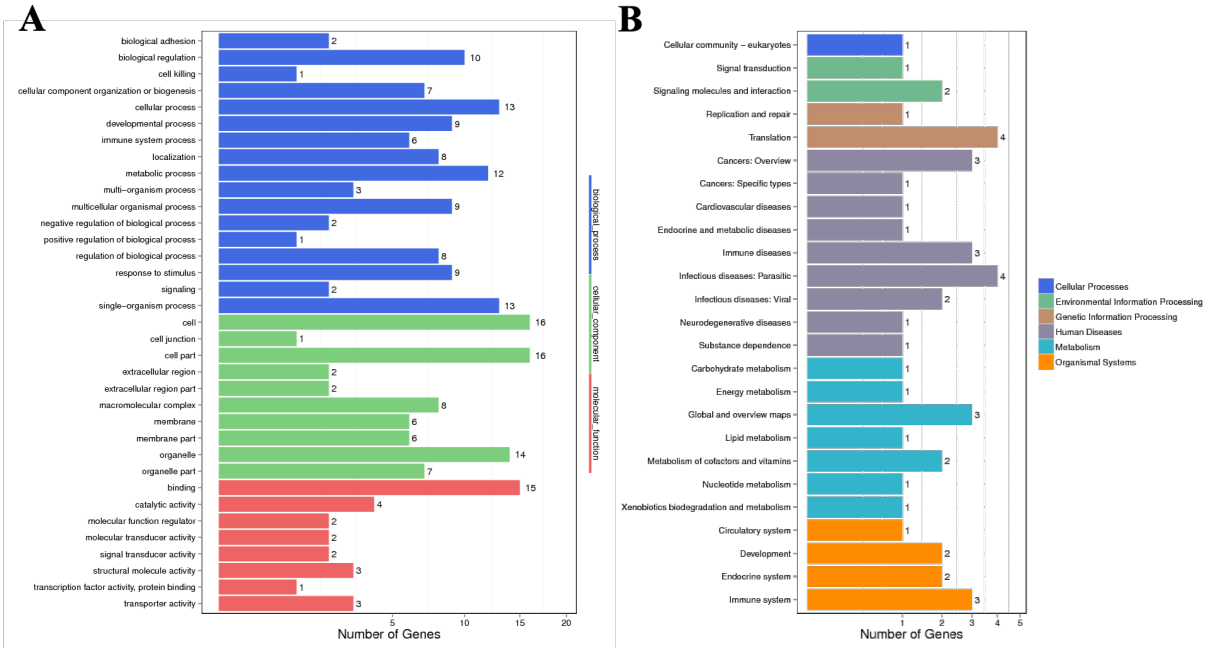



**Fig. 5.8 Functional characterization of the transcriptional signatures coinciding with contraction-mediated protection against hyperglycaemia-induced insulin resistance.** A) GO functional classification for DEGs regulated by 24 h hyperglycaemia with (EPS HG) or without (HG) prior EPS *vs.* time-matched CON. B) KEGG pathway classification for DEGs regulated by 24 h hyperglycaemia with (EPS HG) or without (HG) prior EPS *vs.* time-matched CON.

### *5.4.4 qPCR validation of RNAseq findings.*

The expression of the final 12 miRNAs was assessed by qPCR in independent validation experiments. Analysis revealed no significant effects of experimental treatments on 9 out of 12 targets – namely, miR-133a (P = 0.34, Fig. 5.9A), miR-7-1 (P = 0.30, Fig. 5.9C), miR-23a (P = 0.22, Fig. 5.9E), miR-27a (P = 0.50, Fig. 5.9F), miR-378a (P = 0.70, Fig. 5.9G), miR-99b (P = 0.08, Fig. 5.9H), miR-215 (P = 0.41, Fig. 5.9I), miR-486 (P = 0.71, Fig. 5.9J), miR-125a (P = 0.80, Fig. 5.9L). Significant effects of experimental treatments were seen for remaining 3 targets – namely, miR-1249 (Fig. 5.9B), miR-1a-1 (Fig. 5.9D) and miR-194-1 (Fig. 5.9K) (all $P<0.05$). Post-hoc analysis revealed no significant effect of EPS or HG in isolation upon miR-1249 or miR-1a-1 expression compared to CON ($P>0.05$); however, both were significantly upregulated in EPS HG compared to time-

matched HG ($P$<0.05). The expression of miR-194-1 following EPS alone was significantly higher than in HG alone ($P$<0.05), but not different *vs*. CON ($P$>0.05). Most notably, HG alone significantly downregulated miR-194-1 expression *vs*. CON ($P$<0.05), whereas this was prevented by prior contraction in EPS HG, which was significantly higher than both CON and HG ($P$<0.05). Expression profiles for miR-1a-1 and miR-194-1 following these conditions of contraction and/or hyperglycaemia are consistent between RNA sequencing and qPCR-derived expression analysis (Fig. 5.7D, Fig. 5.9D and Fig. 5.9K). Follow-up characterisation of proposed functional properties (GO and KEGG pathways) of these miRNAs is provided below (Table 5.3).

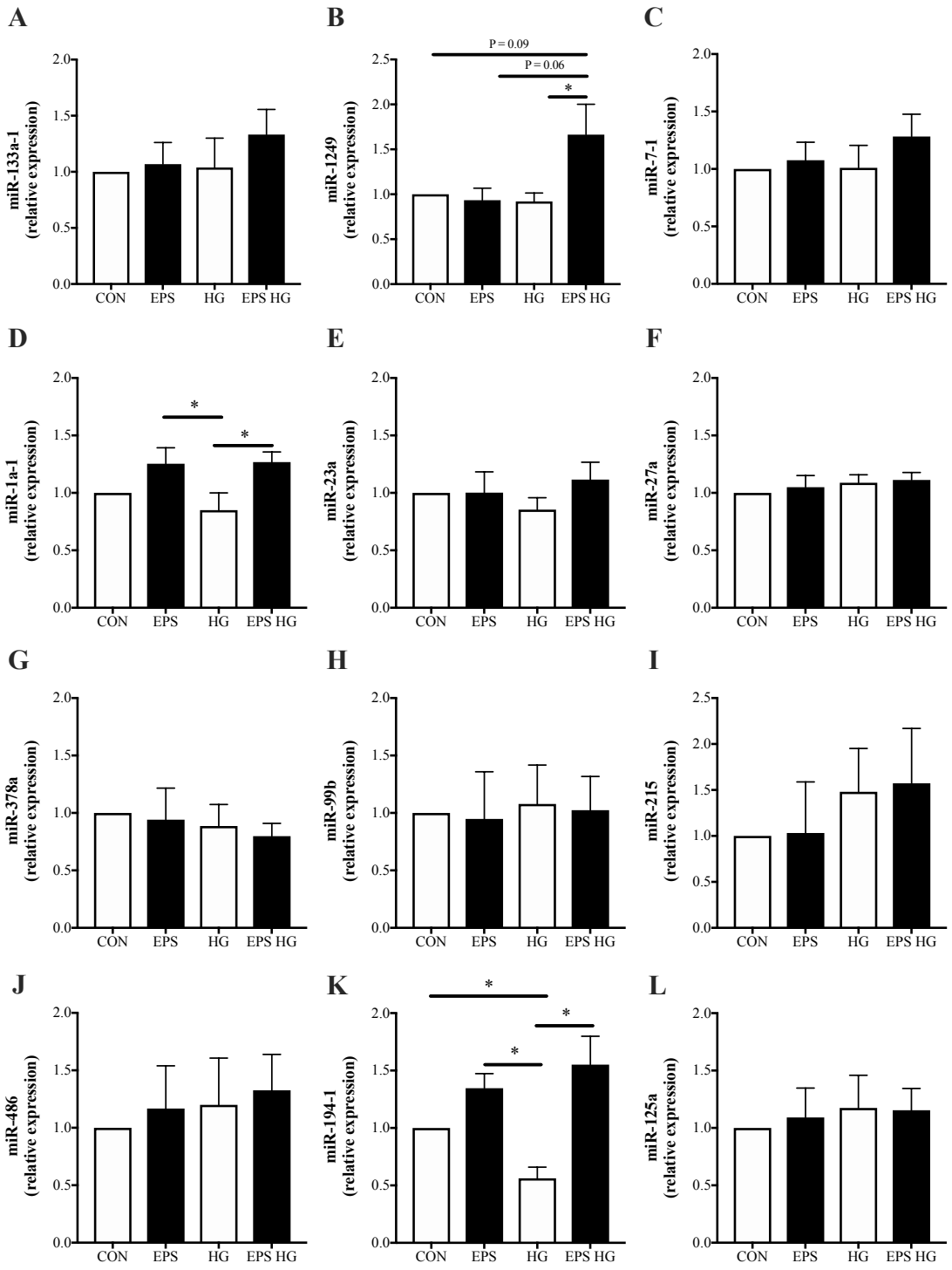



**Fig. 5.9 qPCR validation of differentially regulated miRNAs identified through RNA sequencing as potential regulators of contraction-mediated protection against hyperglycaemia-induced insulin resistance.** A – L) qPCR measurement of miRNA expression was assessed in independent validation experiments where C2C12 myotubes were exposed to 24 h EPS or rest, followed by 24 h of normo- (5 mmol/L) or hyperglycaemic (25 mmol/L) conditions. Data are mean ± SEM and expressed relative to time-matched CON ($n$ = 5; *$P$<0.05).

**Table 5.3 GO annotations and KEGG pathways assigned to miR-194-1 and miR-1a-1**

| | Pathway identity | Pathway term |
|---|---|---|
| miR-194-1 | GO:0016442 | RNA-induced silencing complex (RISC complex) |
| | GO:0071230 | Cellular response to amino acid stimulus |
| | GO:0071222 | Cellular response to lipopolysaccharide |
| | GO:0009617 | Response to bacterium |
| | K05206 | MicroRNAs in cancer |
| | K03100 | Non-coding RNAs |
| miR-1a-1 | GO:0016442 | RNA-induced silencing complex (RISC complex) |
| | GO:1990830 | Cellular response to leukaemia inhibitory factor |
| | GO:0035195 | Gene silencing by miRNA |
| | GO:0010629 | Negative regulation of gene expression |
| | GO:0017148 | Negative regulation of translation |
| | GO:0010608 | Posttranscriptional regulation of gene expression |
| | GO:0070884 | Regulation of calcineurin-NFAT signalling cascade |
| | GO:0010468 | Regulation of gene expression |
| | K05206 | MicroRNAs in cancer |
| | K03100 | Non-coding RNAs |
| | K04147 | Exosome - Exosomal RNA of muscle cells |

GO: prefix indicates gene ontology (GO) pathway and K prefix indicates Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway. Information retrieved from miRbase, MGI and KEGG.

## 5.5 Discussion

Hyperglycaemia and skeletal muscle contraction exert opposite regulatory effects upon skeletal muscle glucose metabolism, and contraction *per se* directly protects against hyperglycaemia-induced insulin resistance (Chapter 4). In the present study, RNA sequencing analysis demonstrated that this protective effect coincides with contraction-mediated normalisation of several hyperglycaemia-responsive transcriptional changes of several non-coding RNAs, particularly miRNAs. Follow-up validation experiments

highlighted miR-1249, miR-1a-1 and miR-194-1 as possible novel targets in the modulation/prevention of skeletal muscle insulin resistance.

Here, in an unbiased approach, RNA sequencing was used to characterise transcriptional regulators of the hyperglycaemia- and contraction-responsive changes in skeletal muscle cell glucose metabolism described in chapter 4. Firstly, the distinct time-dependent improvements in contraction-mediated glucose uptake (immediately post-EPS only) and insulin sensitivity (24 h post-EPS only) that occurs following skeletal muscle contraction (chapter 4) also coincides with temporal changes in skeletal muscle transcriptome (Table 5.1, Fig. 5.3). Notably, of the 253 individual contraction-responsive genes identified across 0 and 24 h post-EPS conditions, only 35 were differentially regulated at both time points (Fig. 5.3C). Of these, only 20 were regulated in the same direction, with the remaining 15 oppositely regulated (Fig. 5.3D). Interestingly, 25 of the 35 overlapping contraction-responsive transcripts were miRNAs. Given the minimal overlap of DEGs between conditions it is perhaps unsurprising that there is also minimal overlap of significantly enriched GO pathways between the four conditions (only 3 out of 17 terms; Fig. 5.4). Overall, these data demonstrate strikingly dissimilar transcriptional profiles across these different post-contraction time points, particularly 0 and 24 h post-EPS (Fig. 5.3D, Fig. 5.4). Therefore, the distinct temporal profiles for contraction-mediated glucose uptake and insulin sensitivity seen following contraction in chapter 4 are associated with an equally distinct contraction-specific transcriptional profile. From a methodological standpoint, these findings emphasise the importance of including time-course experiments incorporating serial sample harvests following treatment cessation to ensure changes with different kinetics (e.g. rapid, transient or delayed) are not missed.

Next, the insulin resistance that manifested in skeletal muscle cells following 18 and 24 h of hyperglycaemia (chapter 4) coincides with differential expression of 115 and 115 genes, respectively (Table 5.2). Interestingly, the majority of DEGs identified across the increasing durations of hyperglycaemia were only significantly regulated at one time point (Fig. 5.5A), demonstrating a dynamic, time-specific transcriptional response to hyperglycaemia. Notably, despite both conditions inducing insulin resistance in skeletal muscle cells, 18 and 24 h hyperglycaemia induced distinctly different DEG (Fig. 5.5C-D) and GO pathway enrichment (Fig. 5.6) profiles with minimal overlap. The small number of overlapping genes is particularly striking considering the wide coverage of transcripts (i.e. > 20,000 genes) assessed within the RNA sequencing analysis within this study. Collectively, this data suggests progression towards insulin resistance in skeletal muscle is associated with a myriad of transcriptional pathways that are regulated in a dynamic, temporal manner.

The main finding of these experiments is that the skeletal muscle contraction-mediated protection against hyperglycaemia-induced insulin resistance reported in chapter 4 coincides with contraction-mediated normalisation of several hyperglycaemia-responsive transcriptional changes. Specifically, analysis of RNA sequencing data highlighted a small subset of genes that were differentially expressed within both of the following pairwise comparisons at 18 and/or 24 h time points: i) HG *vs*. CON and ii) HG *vs*. EPS HG. This identified a subset of 81 genes, within which there were notably few DEGs from the muscle coding RNA, including mRNA. Instead, the majority of highlighted candidates were non-coding RNAs, including miRNAs. For example, prior contractile activity prevented the hyperglycaemia-induced regulation of several non-coding RNAs that were otherwise upregulated (such as miR-125a) or downregulated (such as miR-194-1) in the

model of insulin resistance. Interestingly, Gallagher et al. (30) found that there were few distinguishable differences in the skeletal muscle coding transcriptome (e.g. mRNA) between insulin resistant *vs*. healthy control subjects, whereas non-coding transcriptome profiling revealed that ~1/3 of the muscle expressed miRNAs assessed were altered with changing insulin sensitivity. Data from the current study supports findings of Gallagher et al. (30), and corroborates recent accumulating evidence supporting the key role of miRNAs in regulating skeletal muscle glucose metabolism (31–34).

The primary purpose of this investigation was to characterise transcriptional regulators of contraction-mediated protection against hyperglycaemia, as opposed to either contraction or hyperglycaemia in isolation. Addressing this aim, RNA sequencing revealed 81 unique transcripts, expression of which were oppositely regulated in rested *vs*. contracted hyperglycaemia cells. Given the aforementioned emerging support for an integral regulatory role of miRNAs in skeletal muscle, along with the desire to maximise applicability to human muscle metabolism in the future, the subset of 81 was condensed to include only miRNAs known to be expressed in human skeletal muscle, leaving a final shortlist of 12 miRNAs.

Prior skeletal muscle contraction prevented hyperglycaemia-induced insulin resistance (chapter 4). Within independent qPCR validation experiments, treatments had significant effects upon relative expression of three of the shortlisted miRNAs, namely miR-1249, miR-1a-1 and miR-194-1 (Fig. 5.9 B, D and K, respectively). Specifically, contraction-mediated protection against hyperglycaemia appears to coincide with significantly elevated expression of miR-1249, miR-1a-1 and miR-194-1 in contracted (EPS HG) *vs*. rested (HG) skeletal muscle cells exposed to 24 h hyperglycaemia. Notably, the expression profiles of

miR-1a-1 and miR-194-1 from qPCR are strikingly consistent with RNA sequencing findings. In particular, whilst miR-194-1 was significantly downregulated by HG compared to CON, prior contraction prevented this downregulation to an extent that final miR-194-1 expression levels were significantly higher than both CON and HG.

The potency of miRNAs to influence metabolic regulation result largely from their multivalent influence over posttranscriptional control of a large number of mRNA. Specifically, miRNAs interact with ~30% of all genes, and by interacting with mRNA a single miRNA is capable of altering the expression of hundreds of proteins (35,36). For example, miR-194-1 has been predicted to interact with 428 (TargetScan) or 592 (miRDB) transcripts, such as IGF1r and members of Rab and TBC1D families. Additionally, miR-1a has been predicted to interact with 729 (TargetScan) or 766 (miRDB) transcripts, including members of Rab and TBC1D families. For example, miR-1a-1 reportedly interacts with TBC1 domain family member 15 (Tbc1d15), which has recently been suggested as a regulator of GLUT4 translocation and thus GLUT4-mediated glucose uptake in L6 muscle cells (37). Similarly, miR-1a-1 reportedly interacts with Rab8a, which in turn contributes to GLUT4 translocation in C2C12 myotubes following contraction (38). Further characterisation of proposed functional effects of miR-194-1 and miR-1a-1 are detailed in Table 5.3.

Unfortunately, the functional impact of alterations in miR-194-1 and miR-1a-1 on the contraction-mediated protection against hyperglycaemia-induced insulin resistance was not causally determined in the current study and requires further experimentation, including gain and loss of function experiments (as discussed further in chapter 7). Nevertheless, it is possible that contraction-mediated regulation of miR-194-1 and miR-1a-1 may regulate

vital parts of the insulin signalling pathway in a way that attenuates the (dys) regulation induced by hyperglycaemia. Indeed, miRNA expression has been demonstrated to interact with key proteins in the insulin signalling cascade, particularly in the early steps (39), and consequently determine degree of insulin sensitivity/resistance in skeletal muscle (34). Likewise, expression profiles of multiple miRNAs have been associated with insulin sensitivity *in vivo* (30,40). Interestingly, similar to hyperglycaemia-induced downregulation of miR-194 expression in C2C12 myotubes reported here, skeletal muscle miR-194 expression has also been shown to be significantly downregulated in muscle from insulin resistant (T2DM) individuals *vs*. healthy controls (41). Currently, the effects of exercise on expression of miR-194-1 *in vivo* has only been addressed in a very small number of studies with inconsistent findings but inactivity, which is associated with insulin resistance, may downregulate miR-194-1 expression (14). Furthermore, lower levels of miR-1a-1 in insulin resistant cells (HG) *vs*. cells with improved insulin sensitivity (EPS HG) is consistent with lower miR-1a-1 expression in skeletal muscle from insulin resistant (T2DM) individuals *vs*. healthy controls (33).

It should be noted that ~26.5% of analysed genes were not annotated to defined GO pathways, and ~22% of genes were not assigned to a KEGG pathway, perhaps highlighting a limitation of the bioinformatics process used here. In particular, only ~ 10% of the miRNAs analysed were annotated to defined GO and/or KEGG pathways at the time of analysis. Therefore, since miRNAs feature heavily in DEG lists, functional characterisation of the DEGs in the current study may be incomplete. Species differences in miRNA expression profiles between murine and human myotubes, such as during myocyte differentiation (42), should also be noted and may preclude direct application of findings to human skeletal muscle metabolism. Additionally, for reasons discussed above, identified

genes returned 81 possible targets, 49 of which were deemed to not be expressed in human muscle and therefore not pursued further. The possibility that any of these excluded genes may elicit greater regulatory control over the functional responses seen in this experimental model than any of those genes selected cannot be ruled out. Similarly, whilst the current study demonstrates that distinct metabolic changes induced by conditions of rest *vs.* contraction and/or normo- *vs.* hyperglycaemia coincided with equally distinct transcriptional changes in skeletal muscle cells, the role of other biological processes at the protein or metabolite level, for example, in regulating the metabolic responses to these experimental treatments should not be overlooked. Additionally, there can be discord between transcriptional changes and protein expression (43,44) meaning definitive conclusions regarding changes in molecular functionality of cells, which are heavily reliant on protein abundance and/or activity (43), cannot be made from transcriptomics alone. A comprehensive multi-omics approach incorporating proteomics and metabolomics alongside the transcriptomics methodology used here would have provided a more integrative assessment of responses incorporating these important exercise-responsive processes, but such approach was not possible within this thesis. Nevertheless, there are methodological strengths of the current study that have proven particularly important given the dynamic, temporal profile of changes in glucose metabolism induced by the experimental treatments (chapter 4). Most notably, the multiple time point analysis permitted evaluation of transcriptional responses to conditions of contraction *vs*. rest and/or normoglycaemia *vs*. hyperglycaemia with greater temporal resolution than is otherwise possible in single-/endpoint studies.

Recent studies have demonstrated that experimentally manipulating miRNA expression in skeletal muscle *per se* directly influences skeletal muscle glucose metabolism (31,41,45–

50). For example, gain and loss of function experiments revealed that miR-29 regulated insulin-stimulated glucose metabolism and lipid oxidation of skeletal muscle myotubes (31). Additionally, inhibition of miR-106b attenuated insulin resistance that was otherwise induced by exposing C2C12 myotubes to TNF-α (46). As a corollary, our results suggest that manipulating skeletal muscle expression of miR-194-1 and/or miR-1a-1 could be a novel avenue to improve muscle glucose metabolism, and protect against hyperglycaemia-induced insulin resistance (Fig. 5.10). Such interventions could be used alone or in combination with exercise to improve glycaemic outcomes. For example, hyperglycaemia and insulin resistance have been linked to smaller exercise-induced improvements in peripheral insulin sensitivity and postprandial glucose control (51). Manipulating miRNAs to ameliorate insulin sensitivity/protect against hyperglycaemia-induced insulin resistance prior to/during/after exercise training may contribute to overcoming the exercise response heterogeneity seen in these population (17,18) and potentiate exercise-induced benefits. Equally, protecting skeletal muscle from hyperglycaemia-induced insulin resistance may be particularly important in the absence of muscle contraction, such as experienced during periods of inactivity enforced by serious illness or musculoskeletal injury. Whilst targeting skeletal muscle miRNA to ameliorate glucose metabolism *in vivo* remains in a nascent stage, the rapidly accumulating evidence of their regulatory control over skeletal muscle metabolism certainly highlights this as an appealing future therapeutic option in the battle against metabolic diseases such as T2DM (39,52).

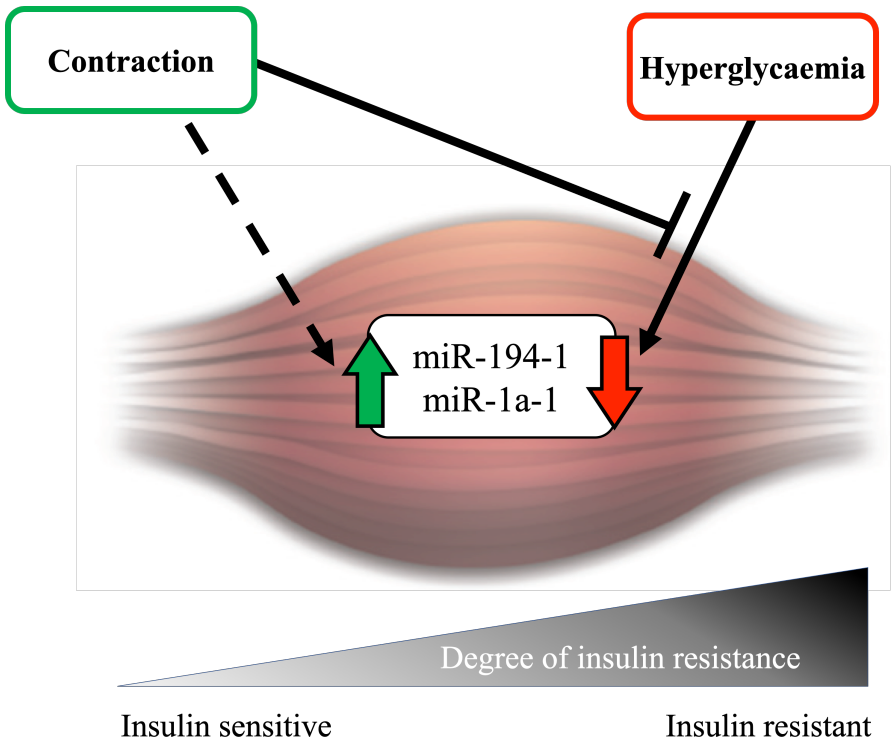



**Fig. 5.10 A model to depict how miR-194-1 and miR-1a-1 are contraction-responsive transcripts associated with contraction-mediated protection against hyperglycaemia-induced insulin resistance in skeletal muscle cells.**

## 5.6 Conclusion

Herein, findings characterise the hyperglycaemia- and contraction-responsive transcriptome to identify underlying transcriptional regulators of the hyperglycaemia- and contraction- induced changes in skeletal muscle glucose metabolism. The findings demonstrate that the underlying transcriptional changes contributing to improvements in glucose uptake in the immediate post-exercise period are likely to be very different to the transcriptional changes contributing to improved insulin sensitivity that manifests hours – days after exercise. Additionally, prior contractile activity prevented the hyperglycaemia-induced (dys) regulation of several non-coding RNAs that were otherwise up-regulated or down-regulated by insulin resistance-inducing conditions. Increased miR-194-1 and miR-

1a-1 expression in contracted, insulin sensitive (EPS HG) *vs*. rested, insulin resistant (HG) cells exposed to hyperglycaemia suggests that miR-194-1 and miR-1a-1 are contraction-responsive miRNAs that may confer protection against hyperglycaemia induced-insulin resistance. Future investigation involving gain/loss of function experiments to define possible causal roles of these miRNAs would establish whether they can be targeted to modulate skeletal muscle insulin sensitivity in human skeletal muscle.

## 5.7 References


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# CHAPTER 6

# 6 EXERCISE-INDUCED IMPROVEMENTS IN POSTPRANDIAL GLUCOSE RESPONSE ARE BLUNTED BY PRE-EXERCISE HYPERGLYCAEMIA

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## 6.1 Abstract

**Background and aims:** Exercise potently improves glycaemic control in individuals with hyperglycaemia-related conditions, such as type 2 diabetes. However, the magnitude, and in some cases, the direction of this effect is variable. Isolating factors contributing to this exercise response heterogeneity is vital to optimising the glucoregulatory effects of exercise, and recent evidence implicates the degree of hyperglycaemia as a possible contributor. Accordingly, the current study investigated whether pre-exercise hyperglycaemia *per se* impacts the beneficial effect of a single exercise bout upon glycaemic control.

**Methods:** Twelve healthy males (age: 23.6 ± 1.5 years, BMI: 22.7 ± 0.7 kg/m$^2$, HbA1c: 5.4 ± 0.1%, $VO_2$max: 41.5 ± 3.3 mL/kg/min) underwent four experimental trials in a randomised order. Each trial consisted of a 24 h pre-trial monitoring period, a 7 h experimental trial and a 24 h post-trial monitoring period. Glycaemic control was assessed under strict dietary control but otherwise free-living conditions by continuous glucose monitoring for 24 h following no exercise (CON), or following 45 mins of cycling exercise (70% HRmax) that was preceded by 3.5 h of either normoglycaemia (NG-Ex), steady-state hyperglycaemia (HG-Ex; constant rate glucose infusion) or fluctuating glycaemia (FG-Ex; repeated glucose bolus infusions, with total glucose load matched to HG-Ex). Data are presented as mean ± SEM.

**Results:** Pre- and post-trial physical activity levels and dietary intake were similar between trials, and exercise intensity, work done and energy expenditure were matched between exercise trials (all $P$>0.05). During the 3.5 h ± infusion period, average blood glucose levels were higher in HG-Ex (7.2 ± 0.4 mmol/L) and FG-Ex (7.3 ± 0.3 mmol/L) compared

to CON (4.8 ± 0.2 mmol/L) and NG-Ex (5.0 ± 0.2 mmol/L) trials ($P$<0.01), and glycaemic variability was greatest in FG-Ex ($P$<0.01 *vs.* all other trials). Postprandial glucose response, measured as the sum of post-meal glucose iAUC, was significantly reduced by exercise in NG-Ex compared to CON (321.1 ± 38.6 mmol/L/8h *vs*. 445.5 ± 49.7 mmol/L/8h, $P$<0.05). However, such benefits were blunted when exercise was preceded by steady hyperglycaemia (425.3 ± 45.7 mmol/L/8h) and fluctuating glycaemia (465.5 ± 39.3 mmol/L/8h) in HG-Ex and FG-Ex, respectively (both $P$>0.05 *vs*. CON).

**Conclusion:** Pre-exercise hyperglycaemia blunted the glucoregulatory benefits of acute exercise upon postprandial glucose response, suggesting that exposure to hyperglycaemia directly contributes to exercise response heterogeneity. Clinically, coinciding exercise sessions with periods of improved glucose control (i.e. lower, more stable levels) may be a necessary strategy to optimise the remedial effects of exercise in individuals with and at risk of hyperglycaemia-related conditions, such as type 2 diabetes.

## 6.2 Introduction

The worldwide prevalence of type 2 diabetes mellitus (T2DM) continues to rise, meaning increasing proportions of the global population are at risk of or living with a range of serious microvascular (e.g. retinopathy, neuropathy and nephropathy) and macrovascular (e.g. myocardial infarction and stroke) complications (1–3). Moreover, risk of Alzheimer's disease (4,5) and some cancers may also be increased. Consequently, the economic cost associated with managing this condition continues to rise, and thus place an increasing burden on healthcare systems worldwide. Clearly, optimising interventions to improve glycaemic control remains a clinical necessity.

The level of glycaemia, and particularly postprandial glucose (PPG) exposure (6–8), has been implicated in the manifestation of the aforementioned diabetic complications in patients with T2DM, as well as being predictive of a worsening glycated haemoglobin (HbA1c) level in non-diabetic individuals (9). Therefore, reducing PPG exposure is particularly important for the prevention and long-term management of worsening glycaemia in individuals with or at risk of T2DM.

Exercise training exerts potent glucoregulatory effects in those with and at risk of developing T2DM. For example, exercise training reduces HbA1c and fasting blood glucose (10,11) as well as increases peripheral insulin sensitivity (12–14) and β-cell insulin secretory function (15,16). A single exercise bout also potently improves glucose uptake (immediately post-exercise), insulin sensitivity (for up to ~48 h post-exercise) (17) and β-cell insulin secretory function (18) in the hours – days following each individual exercise bout. Similarly, and of particular relevance to the current study, reduced (i.e. improved) PPG response is also among the glucoregulatory effects of a single exercise bout (19–23).

Many of these benefits are gained by individuals with and without T2DM, but the transient nature of benefits means exercise must be repeated on a regular basis to preserve metabolic health. Accordingly, regular exercise and physical activity forms the cornerstone in the prevention and management of hyperglycaemia-related conditions, including T2DM (24).

While the potent glucoregulatory effects of exercise are unequivocal, the magnitude and direction of change following both acute exercise (18,25,26) and exercise training (27,28) in hyperglycaemic individuals vary considerably. Isolating factors contributing to this exercise response heterogeneity is vital to optimise the glucoregulatory effects of exercise in this population and have been discussed in several recent reviews (29–32). In free-living environments, factors such as diet and exercise characteristics/adherence and exercise-medication interactions (33,34) likely contribute to variability in this setting (32). Interestingly, evidence from recent well-controlled studies, where free-living sources of heterogeneity (e.g. exercise – drug interaction, exercise adherence, diet) are controlled, implicates the degree of hyperglycaemia as one possible contributor to heterogeneity following single exercise bouts as well as exercise training (29,30,32). That said, equivocal conclusions have been made in studies to date, with both blunted (acute (18) and chronic (35–37) exercise) and potentiated (acute exercise (25,26)) exercise-induced glucoregulatory benefits associated with higher baseline fasting plasma glucose and/or HbA1c. Furthermore, the evidence to date is also largely correlational in nature, meaning whether hyperglycaemia *per se* directly impacts the exercise-mediated improvements in glycaemic control following acute exercise remains to be tested experimentally. Accordingly, the current study investigated the impact of pre-exercise hyperglycaemia on the response to a single exercise bout in healthy participants. It was hypothesised that hyperglycaemia *per se* blunts the glucoregulatory effects of acute exercise.

## 6.3 Methods

### *6.3.1 Participants*

Healthy, recreationally active males ($n$ = 12; Table 6.1) were recruited from the local community to participate in the current study. Potential participants underwent an initial screening visit to determine their eligibility for the study. Individuals were excluded from participation if they 1) smoked, 2) were obese (BMI >30 kg/m$^2$) and 3) demonstrated any history of cancer, haematological, pulmonary, cardiac, hepatic, renal, metabolic or gastrointestinal diseases. All participants were informed about the nature of the current study and any risks associated with the experimental procedures prior to gaining their written informed consent. Formal sample size calculations were made using G*Power Version 3.1.7 (38) to maintain statistical power for CGM-derived assessment of PPG control. Ethical approval was obtained through the West Midlands - South Birmingham Research Ethics Committee (16/WM/0242) and sponsored by the University of Birmingham Research Governance. The study is a registered trial (ClinicalTrials.gov; NCT03284216).

### *6.3.2 Screening and habitual monitoring*

All participants completed a general health questionnaire, as well as had their body composition (height, weight, BMI, waist circumference) and HbA1c analysed to confirm eligibility for the current study. Subsequently, participants performed a maximal incremental exercise test on a cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine their maximum workload capacity ($W$max), maximal oxygen consumption ($\dot{V}O_2$max) and maximal heart rate (HRmax). After 5 mins warm-up at 50 W, workload was increased by 25 W/min until exhaustion. Oxygen consumption was assessed

continuously during exercise via indirect calorimetry (Vyntus CPX, Jaeger, CareFusion, Germany), with heart rate (Polar Wearlink, Polar Electro Oy, Kempele, Finland, and ratings of perceived exertion (RPE; 6 – 20 Borg scale) monitored throughout. Following screening, participants completed a 7-day habitual monitoring, during an accelerometer (Actigraph wGT3X-BT, Pensacola, FL) and diet logs were used to assess habitual physical activity and diet, respectively.

### *6.3.3 Study Design*

Participants underwent four experimental trials in this randomised crossover study, with trials separated by ~1 week, during which time participants were instructed to maintain habitual physical activity and diet. Each experimental trial consisted of a 24 h pre-trial monitoring period, a 7 h experimental intervention period and a 24 h post-trial monitoring period (Fig. 6.1). The four 7 h interventions were completed in a randomised order (determined using http://www.randomization.com/) and consisted of 1) normoglycaemia, no exercise (CON), 2) normoglycaemia, exercise (NG-Ex), 3) steady hyperglycaemia, exercise (HG-Ex) and 4) fluctuating glycaemia (FG-Ex).

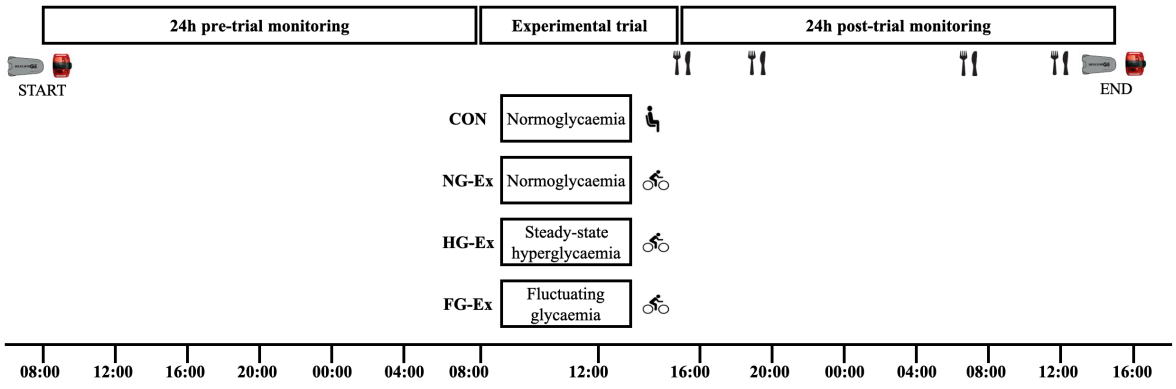



**Fig. 6.1 Schematic overview of the study design.** Pre-trial monitoring involving physical activity ( ) and CGM-derived glycaemic control ( ) began 24 h prior to each experimental trial. During experimental trials, participants were exposed to differing

glycaemic profiles followed by 45 mins of rest ( 🪑) or exercise (🚴). Post-trial glycaemic control was assessed under strict dietary control (🍴) but otherwise free-living conditions. A full description is provided in the text.

### *6.3.4 Study protocol*

**Pre-trial standardisation and monitoring period.** In the afternoon of day 0, participants reported to the laboratory for insertion of the continuous glucose monitoring (CGM) glucose sensor (Dexcom G5 mobile, Camberley, UK) into the abdominal subcutaneous adipose tissue, and to receive an accelerometer and diet records. Data was not collected on this day; instead, the purpose of this visit was to allow time for CGM calibration (which required ~2 – 3 h) in preparation for data collection to commence on day 1. The CGM was calibrated twice daily using a glucose meter (Contour Next One, Ascensia Diabetes Care UK, Berkshire, UK) on fingertip capillary blood samples. Participants were provided with detailed written instructions, including refraining from strenuous physical activity and the consumption of alcohol and caffeine from day 0 to day 3, inclusive.

On day 1 (24 h pre-trial), diet record (food type, amount and time of ingestion), accelerometry-derived physical activity and CGM data collection commenced and continued until 24 h post-exercise (i.e. days 1 – 3, inclusive). Participants consumed a self-selected diet prior to trial 1, and then replicated this chosen diet on day 1 of subsequent trials. Similarly, participants were instructed to replicate as closely as possible activity patterns (e.g. mode of transportation) during the 24 h prior to each trial. This approach to pre-trial standardisation is similar to other studies assessing the acute effects of exercise on glucose metabolism (39–41). Repeated verbal and written reminders of the importance of adhering to these standardisations were provided throughout.

**Experimental trial.** On day 2, participants reported to the laboratory at 08:00, after an overnight fast (≥ 10 h, except water). Body weight, height and waist circumference were measured by standard procedures, and bilateral antecubital venous lines were placed for glucose infusion and blood sampling. The four trials were identical except for a) the pre-exercise glycaemic intervention and b) rest *vs.* exercise conditions. Specifically, after baseline measurements, one of four 3.5 h pre-exercise glycaemic interventions commenced: CON) no infusion; NG-Ex) no infusion; HG-Ex) 1.2 g/kg glucose infusion distributed via constant infusion rate across 3.5 h (5.71 mg/kg/min) and FG-Ex) 1.2 g/kg glucose infusion distributed via 8 equal boluses every 30 mins across 3.5 h (equivalent of 0.15 g/kg per bolus infused over 3.5 mins at rate of 42.86 mg/kg/min) interspersed with periods of no infusion. Glucose (20% w/v glucose monohydrate; Baxter FKB0213B) infusion was administered using a volumetric infusion system (Infusomat Space pump, B. Braun Medical Ltd, Sheffield, UK).

Sixty minutes after cessation of the respective glycaemic intervention (to allow for normalisation of glucose levels), participants completed either a 45 mins rest period (CON; remain seated) or a 45 mins exercise bout (NG-Ex, HG-Ex and FG-Ex; stationary cycling at 70% HRmax). Indirect calorimetry, heart rate and power output were measured continuously throughout the exercise/rest period. The end of the 45 mins ± exercise period marked the start of the 24 h post-trial monitoring period.

**Post-trial standardisation and monitoring period.** For the remainder of day 2 until ~15:00 on day 3 (i.e. the 24 h post-exercise period), CGM, accelerometry and diet record monitoring continued in free-living conditions. During this time, participants were

instructed to minimise any unnecessary physical activity (e.g. walking around at home was permitted, but exercise was not) and to eat only the standardised meals provided.

The standardised 24 h diet consisted of four meals ingested at predetermined time points (lunch at 15:00, dinner at 19:00, breakfast at 07:00, lunch at 12:00). The first meal (lunch) was consumed after 30 mins seated rest following the cessation of the ± exercise period, after which participants were free to leave the laboratory. The standardised 24 h diet provided 2467.6 ± 4.6 kcals and consisted of a mixed macronutrient composition (54.9 ± 0.1% of energy from carbohydrates, 31.6 ± 0.1% from fat and 13.5 ± 0.1% from protein). Participants were instructed to consume meals within a 20 mins timeframe and recorded the exact meal ingestion start and end times to allow specific data to be extracted from CGM data files. This approach ensured that the dietary intake (food type, amount and timing) was standardised across all four trials within subjects; an important approach frequently adopted when using CGM to evaluate exercise-induced changes in measures of glycaemic control in free-living settings (22,23,40,42–45). In the afternoon of day 3 (≥ 24 h following exercise cessation), participants reported to the laboratory for removal of CGM, accelerometer and collection of diet records and activity logs, marking the end of the trial.

It should be noted that no additional calories were provided to replace those expended during exercise in exercise trials (NG-Ex, HG-Ex, FG-Ex) compared to CON (Table 6.3), or to account for extra calories gained from glucose infusions in HG-Ex and FG-Ex (83.5 ± 2.8 g of glucose infused, equating to 333.8 ± 11.2 kcals). Resultant energy balance induced by experimental interventions (i.e. crudely calculated as: kcal gained during infusion – kcal expended during exercise) is as follows: CON = 61.7 ± 3.6 kcals deficit; NG-Ex = 326.1 ±

18.8 kcals deficit; HG-Ex = 10.9 ± 20.5 kcals surplus; FG-Ex = 9.8 ± 17.7 kcals surplus). Although caloric imbalances between trials may be a confounder (46), accounting for imbalances between trials with provision of food, for example, would result in differences in macronutrient provision between trials, which in itself would also be a confounding variable. Therefore, the meals were kept constant across all conditions.

### *6.3.5 Blood sample analyses*

Whole blood HbA1c was determined in capillary blood samples during the screening visit using a fully automated point-of-care system (HemoCue HbA1c 501, Radiometer, Copenhagen, Denmark). Venous blood samples were collected in pre-chilled EDTA-coated tubes and centrifuged at 2000g for 15 mins at 4ºC, with resulting plasma samples stored at -80 ºC until analysis. Plasma insulin (DINS00), plasma IL-6 (HS600C) and plasma CRP (DCRP00) concentrations were determined using solid-phase sandwich ELISAs (Quantikine, Biotechne, R&D). All kits were analysed according to manufacturer's instructions, and the concentration of the analyte quantified relative a standard curve of known concentration of the respective analyte. Plasma glucose concentrations were determined using a point-of-care analyser (HemoCue Glucose 201+, Radiometer, Copenhagen, Denmark), low and high calibration of which was completed before each use with Glucotrol level 2 and 4 (Radiometer, Copenhagen, Denmark), respectively.

### *6.3.6 Calculations*

Diet records were analysed using the UK food database within MyFitnessPal (Under Armour, Baltimore, MD). The 24 h pre-trial glucose and physical activity data were

derived from CGM and Actigraph data, respectively, from 08:00 on day before trial to 08:00 on day of the trial. The trial glucose data were extracted from CGM data based on time stamps marking the start and completion of the 3.5 h ± infusion period and 45 mins ± exercise period. The 24 h post-trial glucose and physical activity data were derived from CGM and Actigraph data, respectively, from end of exercise until 24 h later. Glycaemic variability measures were calculated by entering raw CGM data into EasyGV© (47).

Postprandial glucose (PPG) control was defined as the incremental area under the curve (iAUC) above pre-meal glucose level (average of 30 mins pre-meal) over the 2 h postprandial period for four individual meals (lunch, 15:00; dinner, 19:00; breakfast, 07:00; snack, 12:00). Total PPG iAUC was also calculated as the summation of iAUCs from all four meal periods. Such approach has been used frequently when assessing the effects of exercise on PPG outside of the laboratory (20,22,23).

Post-absorptive insulin sensitivity was estimated using the homeostasis model assessment (HOMA-IR), calculated as: HOMA-IR = (FPI X FPG) / 22.5). In this equation, FPI is fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/L) measured in samples from each participants' CON trial. Values derived from this equation are strongly correlated with gold standard assessment of insulin sensitivity using hyperinsulinemic – euglycaemic clamp, particularly in normal glucose tolerant individuals (48).

Whole body substrate oxidation rates were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using the equation of Frayn (49) for rest, and the moderate – high intensity exercise equation of Jeukendrup and Wallis (50) for exercise, with the assumption that protein oxidation made negligible contribution in both cases.

### *6.3.7 Statistical analysis*

Differences between non time-dependent variables (e.g. 24 h glucose parameters, PPG iAUC, exercise response variables) were assessed by one-way repeated measures ANOVA, with the experimental condition as within-subject factor. In case of significant main effects, pairwise comparisons with Bonferroni correction were applied to locate differences between treatments. For time-dependent variables (e.g. plasma insulin, plasma IL-6 and plasma CRP), two-way repeated-measures ANOVAs were used, with time and condition set as the within-subject factors. In case of significant interaction effects, pairwise comparisons with Bonferroni correction were applied to locate differences between treatments. All analyses were performed in GraphPad Prism 7.0 (La Jolla, CA) with a value of $P<0.05$ considered to be statistically significant. Data are presented as mean ± SEM.

## 6.4 Results

### *6.4.1 Participant characteristics.*

Table 6.1 presents characteristics and habitual physical activity levels of participants recruited to complete the study.

**Table 6.1 Participant characteristics**

| | |
|---|---|
| *N* | 12 |
| Age, years | 23.6 ± 1.5 |
| Height, cm | 175.1 ± 1.7 |
| Weight, kg | 69.5 ± 2.3 |
| BMI, $kg/m^2$ | 22.7 ± 0.7 |
| Waist circumference, cm | 76.5 ± 1.5 |
| HbA1c, % | 5.4 ± 0.1 |
| HbA1c, mmol/mol | 35.1 ± 1.0 |
| Fasting plasma glucose, mmol/L | 4.9 ± 0.2 |
| HOMA-IR | 1.1 ± 0.1 |
| Systolic blood pressure, mmHg | 121 ± 3 |
| Diastolic blood pressure, mmHg | 76 ± 3 |
| $\dot{V}O_2max$, ml/kg/min | 41.5 ± 3.3 |
| $\dot{V}O_2max$, L/min | 2.8 ± 0.2 |
| $W_{max}$, W | 267.8 ± 13.9 |
| $W_{max}$, W/kg | 3.8 ± 0.4 |
| Habitual step count, steps/day | 11452 ± 1578 |
| Habitual energy expenditure, kcals/day | 635.1 ± 103.0 |
| Habitual sedentary time, mins/day | 726.9 ± 47.2 |
| Habitual MVPA time, mins/day | 72.1 ± 11.4 |

Data are means ± SEM.

### *6.4.2 Pre-trial standardisation*

Adherence to pre-trial diet and activity standardisations was confirmed by evaluation of diet record and accelerometry data, respectively. There were no between-trial differences in dietary intake, physical activity or CGM-derived glycaemic control variables collected during 24 h pre-trial (Table 6.2). There were also no significant changes in body composition (body mass, BMI and waist circumference) between trials ($P$>0.05, data not shown).

**Table 6.2 Physical activity, dietary intake and free-living CGM variables measured for 24 h prior to each experimental trial**

| | Pre-trial standardisations | | | |
|---|---|---|---|---|
| | **CON** | **NG-Ex** | **HG-Ex** | **FG-Ex** |
| **Dietary intake** | | | | |
| Energy intake, kcals | 2250.3 ± 164.1 | 2116.3 ± 141.1 | 2043.8 ± 155.4 | 2080.5 ± 169.0 |
| Carbohydrate intake (% of kcals) | 47.0 ± 4.0 | 49.4 ± 4.6 | 47.0 ± 3.7 | 48.2 ± 4.3 |
| Fat intake (% of kcals) | 35.9 ± 2.0 | 33.5 ± 2.4 | 35.7 ± 2.1 | 34.7 ± 2.2 |
| Protein intake (% of kcals) | 17.1 ± 2.5 | 17.1 ± 2.4 | 17.3 ± 2.1 | 17.0 ± 2.4 |
| **Physical activity** | | | | |
| Step count, steps | 10715 ± 1893 | 10722 ± 1725 | 10032 ± 1746 | 10229 ± 2048 |
| Energy expenditure, kcals | 587.9 ± 132.9 | 554.4 ± 115.1 | 529.1 ± 109.3 | 562.4 ± 122.3 |
| Sedentary time, mins | 726.0 ± 67.6 | 619.1 ± 86.8 | 685.8 ± 72.4 | 753.1 ± 71.7 |
| MVPA time, mins | 72.3 ±17.2 | 70.8 ± 14.7 | 66.3 ± 13.9 | 68.9 ± 18.0 |
| **CGM-derived variables** | | | | |
| Mean 24 h glucose, mmol/L | 5.2 ± 0.3 | 5.4 ± 0.1 | 5.5 ± 0.2 | 5.2 ± 0.1 |
| Glucose variability, SD | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.1 |
| Glucose variability, CV% | 17.0 ± 1.4 | 14.7 ± 0.8 | 16.4 ± 1.7 | 13.7 ± 0.9 |

Data are presented as mean ± SEM. No significant between-trial differences.

### *6.4.3 Experimental trial characteristics*

*Blood biochemistry during the trials.* The glucose infusion protocol successfully induced three distinct glycaemic profiles (Fig. 6.2A). The first aim of this infusion protocol was to establish two normoglycaemic (CON and NG-Ex) and two hyperglycaemic conditions (HG-Ex and FG-Ex). Average glucose concentration (Fig. 6.2B) and glucose iAUC (Fig. 6.2D) during the 3.5 h ± infusion period were significantly higher during HG-Ex and FG-Ex compared to CON and NG-Ex ($P$<0.05), with no differences between CON and NG-Ex ($P$>0.05), thus meeting this first aim. The second aim was for glycaemia during one of the hyperglycaemic trials to follow an unstable, fluctuating profile (FG-Ex). Measures of glycaemic variability, namely CV% (Fig. 6.2C), were significantly higher in FG-Ex than all other trials ($P$<0.05), thus meeting this second aim.

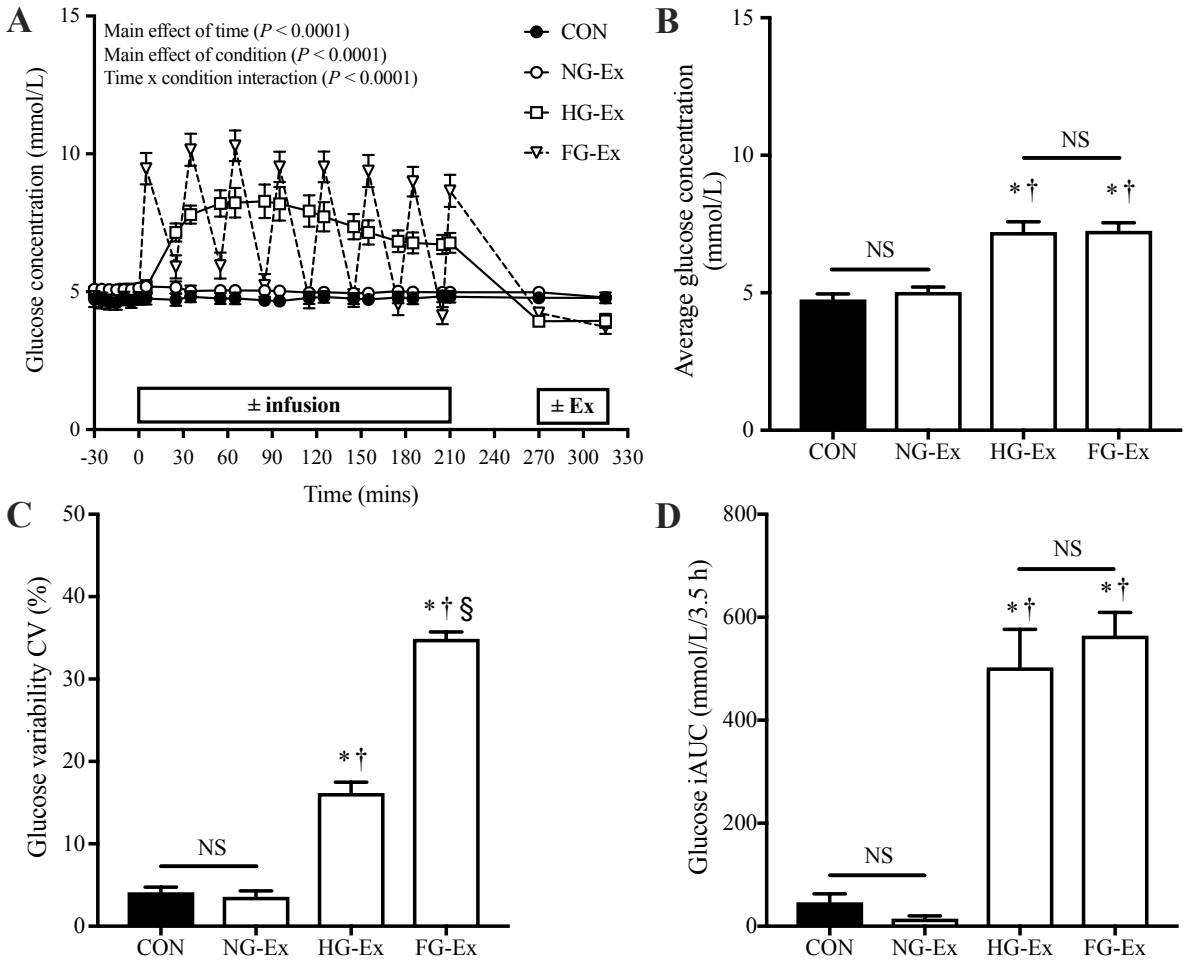



**Fig. 6.2 Glucose control during conditions of ± glucose infusion and ± exercise.** A) Time-course for glucose concentration (mmol/L) during each experimental trial. B) The mean glucose concentration (mmol/L) during each 3.5 h ± infusion period. *$P$<0.05 *vs.* CON. †$P$<0.05 *vs.* NG-Ex. C) Glycaemic variability (coefficient of variation; CV%) during each 3.5 h ± infusion period. *$P$<0.05 *vs.* CON. †$P$<0.05 *vs.* NG-Ex. §$P$<0.05 *vs.* HG-Ex. D) Glucose iAUC during each 3.5 h ± infusion period. *$P$<0.05 *vs.* CON. †$P$<0.05 *vs.* NG-Ex.

Two-way repeated-measures ANOVA revealed significant interaction effects of experimental treatment upon plasma insulin concentration (Fig. 6.3A). Post-hoc Bonferroni corrected pairwise comparisons identified that plasma insulin concentration was significantly increased post-infusion *vs*. pre-infusion in both HG-Ex and FG-Ex ($P$<0.0001 *vs*. respective baseline), but not NG-Ex and CON ($P$>0.05 *vs*. respective baseline). Post-

infusion values in HG-Ex and FG-Ex were significantly higher than time-matched values in NG-Ex and CON ($P$<0.0001 *vs*. time-matched values). Two-way repeated-measures ANOVA revealed no significant interaction effect of experimental treatments upon plasma IL-6 (Fig. 6.3B) or plasma CRP (Fig. 6.3C) concentration ($P$>0.05).

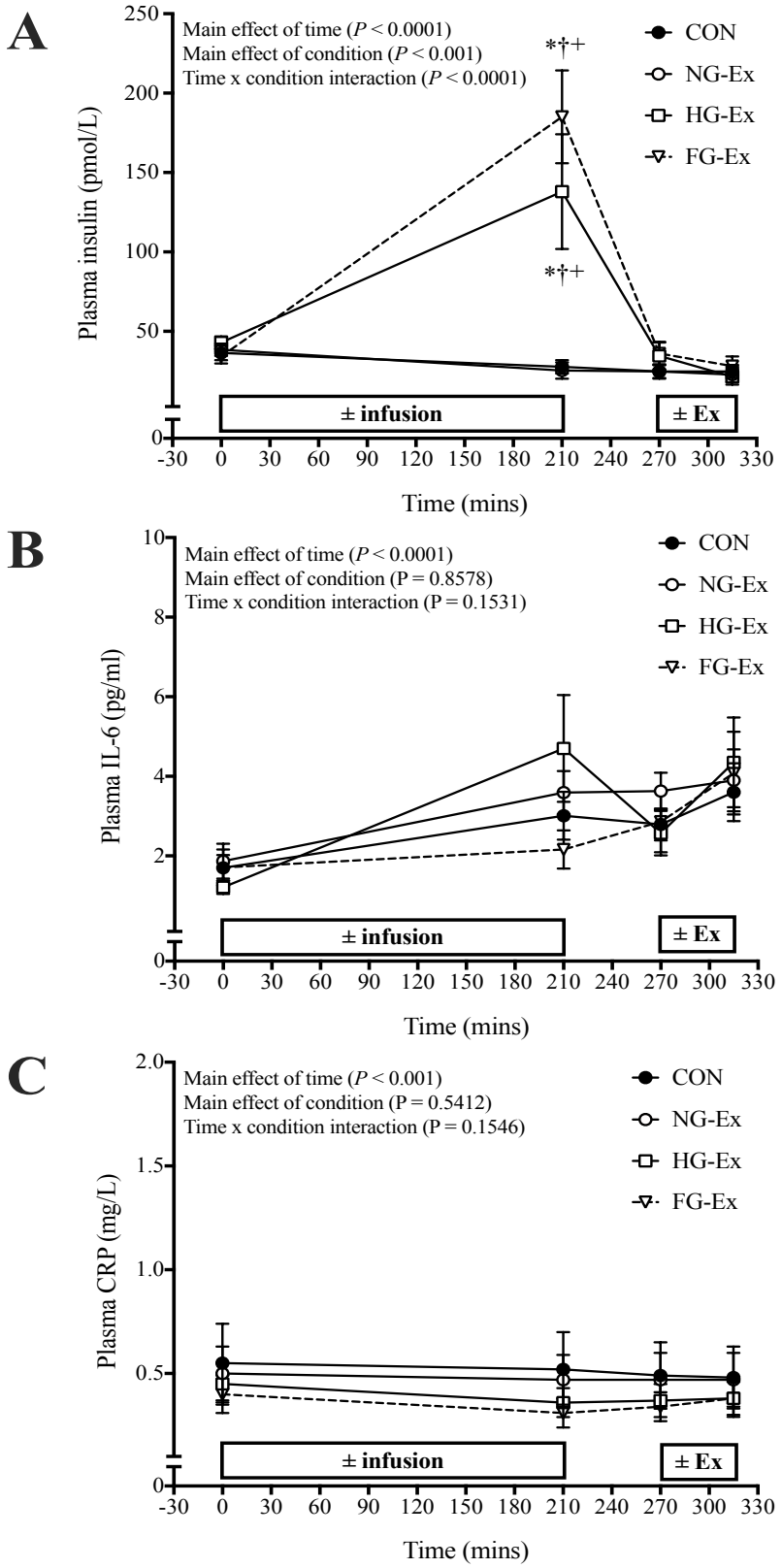



**Fig. 6.3 Time-course for changes in plasma insulin, IL-6 and CRP.** A) Time-course for plasma insulin during each experimental trial. *$P$<0.0001 *vs.* CON; †$P$<0.0001 *vs.* NG-Ex; +$P$<0.0001 *vs*. pre-infusion. B) Time-course for plasma IL-6 during each experimental trial. C) Time-course for plasma CRP during each experimental trial.

### *6.4.4 Physiological responses to exercise during the trials*

The exercise/rest period lasted 44.6 ± 0.4 mins and characteristics of these periods are shown in Table 6.3. Heart rate, power output, $\dot{V}O_2$, work done and energy expenditure were similar between the three exercise trials ($P$>0.05) and significantly higher than during the resting trial ($P$<0.05).

**Table 6.3 Physiological responses to acute exercise intervention**

| | CON | NG-Ex | HG-Ex | FG-Ex |
|---|---|---|---|---|
| Duration, mins | 44.6 ± 0.4 | 44.6 ± 0.4 | 44.6 ± 0.4 | 44.6 ± 0.4 |
| Heart rate, bpm | 61 ± 2 | 132 ± 2* | 131 ± 2* | 130 ± 2* |
| Heart rate, %HRmax | 32.2 ± 1.2 | 69.5 ± 0.4* | 68.7 ± 0.7* | 68.1 ± 0.7* |
| Power output, W | 0.0 ± 0.0 | 88.6 ± 4.8* | 88.0 ± 5.1* | 90.3 ± 4.7* |
| Power output, %Wmax | 0.0 ± 0.0 | 33.1 ± 0.7* | 32.9 ± 1.3* | 33.9 ± 1.2* |
| $\dot{V}O_2$, ml/min/kg | 4.0 ± 0.3 | 22.0 ± 1.6* | 21.5 ± 1.2* | 21.5 ± 1.1* |
| $\dot{V}O_2$, %$\dot{V}O_2$max | 10.0 ± 0.5 | 55.0 ± 3.5* | 53.3 ± 2.4* | 54.7 ± 4.0* |
| Work done, J | 0.0 ± 0.0 | 3884.7 ± 209.2* | 3875.3 ± 223.3* | 3950.7 ± 214.6* |
| Energy expenditure, kcals | 61.7 ± 3.6 | 326.1 ± 18.8* | 322.9 ± 15.0* | 324.0 ± 14.6* |
| RER, a.u. | 0.82 ± 0.01 | 0.90 ± 0.01* | 0.94 ± 0.01*† | 0.94 ± 0.01*† |

Data are mean ± SEM. *$P$<0.05 *vs*. CON. †$P$<0.05 *vs*. NG-Ex.

RER was significantly higher during the three exercise trials *vs.* CON ($P$<0.05; Table 6.3), and also significantly higher in HG-Ex and FG-Ex compared to NG-Ex ($P$<0.05; Table 6.3). Furthermore, carbohydrate oxidation rates during exercise were significantly higher in all three exercise trials (NG-Ex, HG-Ex and FG-Ex) compared to CON ($P$<0.05), with rates during HG-Ex and FG-Ex also significantly higher than NG-Ex ($P$<0.05; Fig. 6.4A). Fat oxidation rates during exercise in NG-Ex were significantly higher than all other trials ($P$<0.05), with no further between-condition differences ($P$>0.05; Fig. 6.4B).

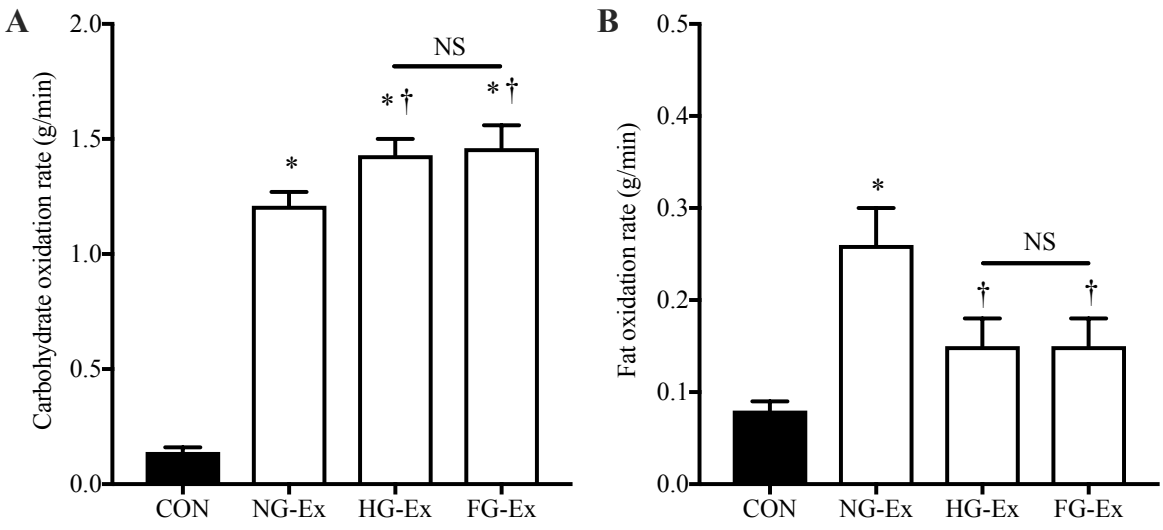



**Fig. 6.4 Rates of substrate oxidation during exercise.** A) Carbohydrate oxidation rates during the 45 mins ± exercise period within the four experimental trials. **P*<0.05 *vs.* CON. †*P*<0.05 *vs.* NG-Ex. B) Fat oxidation rates during the 45 mins ± exercise period within the four experimental trials. **P*<0.05 *vs.* CON. †*P*<0.05 *vs.* NG-Ex.

### *6.4.5 Post-trial standardisation*

Table 6.4 presents dietary intake and physical activity data collected during the 24 h post-trial period of each experimental trial. Post-trial physical activity was similar between trials (*P*>0.05), and by design, dietary intake was identical (*P*>0.999).

**Table 6.4 Physical activity, dietary intake and free-living CGM variables measured for 24 h following each experimental trial**

| | Post-trial standardisations | | | |
|---|---|---|---|---|
| | **CON** | **NG-Ex** | **HG-Ex** | **FG-Ex** |
| **Dietary intake** | | | | |
| Energy intake, kcals | 2467.6 ± 4.6 | 2467.6 ± 4.6 | 2467.6 ± 4.6 | 2467.6 ± 4.6 |
| Carbohydrate intake (% of kcals) | 54.9 ± 0.1 | 54.9 ± 0.1 | 54.9 ± 0.1 | 54.9 ± 0.1 |
| Fat intake (% of kcals) | 31.6 ± 0.1 | 31.6 ± 0.1 | 31.6 ± 0.1 | 31.6 ± 0.1 |
| Protein intake (% of kcals) | 13.5 ± 0.1 | 13.5 ± 0.1 | 13.5 ± 0.1 | 13.5 ± 0.1 |
| **Physical activity** | | | | |
| Step count, steps | 8729 ± 1333 | 8895 ± 853 | 7355 ± 998 | 8661 ± 1260 |
| Energy expenditure, kcals | 476.4 ± 63.8 | 474.7 ± 50.1 | 397.3 ± 60.0 | 473.6 ± 76.9 |
| Sedentary time, mins | 711.5 ± 75.3 | 733.8 ± 66.7 | 764.7 ± 60.7 | 709.5 ± 64.5 |
| MVPA time, mins | 56.8 ± 10.4 | 55.2 ± 6.5 | 42.5 ± 7.7 | 55.6 ± 10.9 |
| **CGM-derived variables** | | | | |
| Mean 24 h glucose, mmol/L | 5.3 ± 0.2 | 5.2 ± 0.1 | 5.3 ± 0.2 | 5.3 ± 0.2 |
| Glucose variability, SD | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| Glucose variability, CV% | 14.1 ± 0.8 | 14.1 ± 1.1 | 13.8 ± 0.9 | 14.3 ± 1.3 |

Data are presented as mean ± SEM. No significant differences.

### *6.4.6 Post-trial glycaemic control*

*24 h glycaemic control.* There were no significant between-condition differences in mean 24 h glucose concentrations during the post-exercise period ($P$>0.05; Table 6.4). Since the regulatory mechanisms (e.g. changes in glucose uptake and insulin sensitivity) of exercise-induced glucoregulatory benefits follow time-dependent profiles (17), the 24 h post-exercise period was broken down to discreet periods, namely: 0 – 6, 0 – 12, 0 – 18, 6 – 12, 6 – 18, 6 – 24, 12 – 18, 12 – 24, 18 – 24. However, there were no significant between-condition differences in mean glucose during these periods ($P$>0.05).

Similarly, there were no significant between-condition differences in prevalence of hyperglycaemia or hypoglycaemia ($P$>0.05; data not shown). Measures of 24 h glycaemic variability, namely SD and CV% (Table 6.4), as well as CONGA, MAGE, LGBI, HGBI, J Index, GRADE, LI, M Value and MAG, were also not significantly affected by

experimental conditions within this study (all $P>0.05$; data not shown). Heterogeneity in exercise response existed for these variables, with 9, 7 and 7 out of 12 individuals experiencing improved 24 h mean glucose, glucose SD and glucose CV%, respectively.

*Postprandial glycaemic control.* There were no significant between-condition differences in mean glucose or glucose iAUC in the 2 h postprandial period following the four meals provided when analysed per meal ($P>0.05$). However, as presented in Fig. 6.5, total postprandial glucose response, measured as the sum of post-meal glucose iAUC, was significantly reduced by exercise in NG-Ex compared to CON (321.1 ± 38.6 mmol/L/8h *vs*. 445.5 ± 49.7 mmol/L/8h, $P<0.05$). However, such benefits were blunted when exercise was preceded by steady hyperglycaemia (425.3 ± 45.7 mmol/L/8h) and fluctuating hyperglycaemia (465.5 ± 39.3 mmol/L/8h) in HG-Ex and FG-Ex, respectively (both $P>0.05$ *vs*. CON).

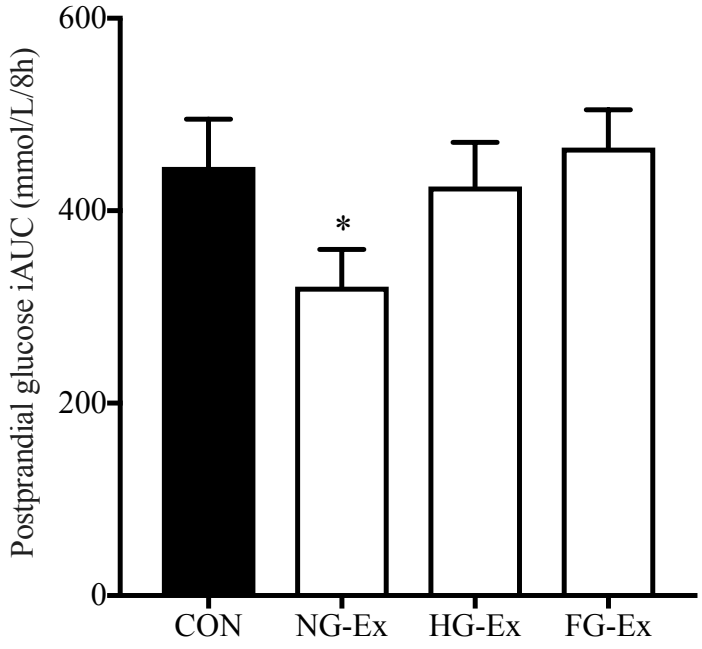



**Fig. 6.5 The effects of the four interventions on total postprandial iAUC.** Total postprandial glucose response (the sum of post-meal glucose iAUC across four standardised meals) was measured using CGM following four experimental trials. Data are mean ± SEM. *$P<0.05$ *vs.* CON.

## 6.5 Discussion

The current study is the first to experimentally investigate whether hyperglycaemia *per se* directly influences exercise-induced benefits in glucose control. The findings demonstrate that pre-exercise hyperglycaemia *per se* blunts the exercise-induced improvement in postprandial glucose response following a single exercise bout in healthy individuals.

Excessive PPG exposure contributes to the worsening of HbA1c in non-diabetic individuals (9), as well as exacerbating the risk of developing diabetic complications and premature mortality (6,8). Accordingly, reducing PPG is a prime therapeutic target in the prevention and management of hyperglycaemia-related conditions, such as T2DM. In this study, when exercise was preceded by normoglycaemia (i.e. healthy, stable blood glucose levels), total PPG iAUC was significantly reduced compared to the non-exercise CON condition (Fig. 6.5). Whilst clinical targets for some CGM-derived outcomes have been defined for patient populations (51,52), a standardised reference range for what constitutes a clinically important change specifically in CGM-derived PPG control does not currently exist. This precludes definitive conclusions regarding the degree to which observed reductions in PPG may reduce the risk of developing hyperglycaemia-related conditions and/or associated complications, as well as complicating direct efficacy comparisons to other established interventions that also benefit CGM-derived PPG control, such as medication (53–55). Nevertheless, the exercise-induced improvement in PPG response in the current study (Cohen's $d = 0.81$) is in agreement with previous research also demonstrating the potency of exercise in improving this specific CGM-derived measure of glycaemic control (20,22,23). However, this beneficial effect was blunted when the same individuals were exposed to steady hyperglycaemia (HG-Ex) and fluctuating glycaemia

(FG-Ex) prior to the exercise bout (Fig. 6.5). This is the first experimental evidence demonstrating a direct effect of pre-exercise hyperglycaemia upon the potency of the exercise to induce glucoregulatory benefits, and thus supports claims that hyperglycaemia *per se* contributes to the heterogeneity of metabolic response in individuals with hyperglycaemia, such as individuals with T2DM.

The exact mechanism by which pre-exercise hyperglycaemia blunted exercise-induced improvements in PPG cannot be fully determined from the current study. That said, it is known that, along with meal characteristics (e.g. caloric content, macronutrient and carbohydrate composition), the magnitude and profile of an individual's PPG response is determined by multiple factors including tissue glucose uptake, insulin sensitivity, pancreatic β-cell insulin secretory function, glucose effectiveness, as well as hepatic and renal glucose handling (56–58); all of which are regulated by exercise. Therefore, the exercise-induced enhancements in PPG response in NG-Ex are likely attributable to coordinated exercise-mediated enhancements in any of the above factors that are otherwise not gained in sedentary CON conditions. As a corollary, if pre-exercise hyperglycaemia impairs the adaptive process underlying the exercise-induced improvements in any of these factors, such as skeletal muscle insulin sensitivity and β-cell secretory capacity, this would feasibly blunt resultant improvements in PPG response. This is supported by previous evidence demonstrating that chronic hyperglycaemia is associated with smaller exercise-induced improvements in peripheral insulin sensitivity, which in turn was linked with blunted exercise-induced improvements in PPG response (59).

Hyperglycaemia *per se* impairs skeletal muscle cell insulin sensitivity (60–63), pancreatic β-cell insulin secretory capacity (64,65) and endothelial cell function (66,67) within *in*

*vitro* experimental models. Likewise, skeletal muscle (68,69) and hepatic (70) insulin sensitivity, insulin secretory capacity (68) and vascular function (71) are impaired by exposure to experimental hyperglycaemia *in vivo*, even in healthy individuals with normal glucose tolerance. Such hyperglycaemia-induced impairments alone could directly contribute to poorer PPG response following hyperglycaemic trials (HG-Ex and FG-Ex). Impaired function of these key glucoregulatory tissues may also contribute indirectly to poorer PPG response following HG-Ex and FG-Ex since poorer glucoregulatory function of key tissues (e.g. pancreas, muscle) at baseline is also associated poorer outcomes following exercise. For example, blunted improvements in insulin sensitivity in individuals with baseline insulin resistance following *in vivo* exercise (59), as well as blunted *in vitro* contraction-induced improvements in myocellular glucose metabolism (including insulin sensitivity) in human primary myotubes from insulin resistant (e.g. obese and/or T2DM) *vs*. healthy donors (72,73) support this suggestion. Similarly, poorer baseline pancreatic β-cell function is predictive of poorer exercise responsiveness (35). Collectively, applied to the current study it is possible that pre-exercise hyperglycaemia impaired glucoregulatory function of key tissues (e.g. pancreas, muscle) that a) was not reversed/overcome by exercise, b) subsequently inhibited the ability to respond to exercise or c) a combination of both. This would feasibly contribute to blunted exercise effects compared to exercise under normal conditions whereby function and/or adaptation is unhindered.

Markers of inflammation and oxidative stress such as IL-6 and CRP are elevated in T2DM (74–76) and are increased by experimental hyperglycaemia (71). Since excessive levels of inflammation and oxidative stress can impair exercise adaptations (77), if pre-exercise hyperglycaemia induced excessive inflammation and oxidative stress this may influence exercise-induced benefits. Therefore, similar to previous studies using glucose infusion to

induce experimental hyperglycaemia (78,79), plasma IL-6 and CRP were assessed to determine whether hyperglycaemia induced systemic inflammation. However, no significant interaction effect in two markers of systemic inflammation (IL-6 and CRP; Fig. 6.3) across trials suggest inflammation *per se* may be unlikely to explain findings within the current study. This contrasts previous evidence demonstrating elevated circulating IL-6 concentration during experimental hyperglycaemia in healthy individuals (78). That said, elevations peaked 1 h into glucose infusion, and returned to baseline at 3 h despite continued infusion (78). Therefore, given the poorer temporal resolution of IL-6 analysis in the current study (i.e. pre and post) compared to hourly during glucose infusion (78), the possibility that IL-6 (and CRP) had increased and returned to baseline within the 3.5 h infusion period cannot be excluded.

Furthermore, IL-6 can exert beneficial as well as deleterious effects (80,81), with the direction of effect likely influenced by the stimulus (e.g. exercise, immune response), source (i.e. the cell type that releases it) as well as context (e.g. underlying health) of release. In the context of exercise, increased IL-6, which is largely muscle-derived, can elicit beneficial metabolic effects such as mediating inter-organ cross-talk (82,83). Conversely, as detailed above, in the context of hyperglycaemia-related conditions such as T2DM (74–76) and experimental hyperglycaemia (78,79), IL-6 is released from multiple cell types and the resultant elevated circulating IL-6 is considered to represent an unfavourable, inflammatory response that contributes to the pathophysiology of hyperglycaemia-related conditions and associated complications (75,76,78). In this study, IL-6 release is examined under conditions of normo- *vs.* hyperglycaemia and/or rest *vs.* exercise, meaning the differences in stimuli, source and context must be acknowledged when interpreting whether changes in circulating IL-6 reflect a beneficial or deleterious

response. Furthermore, whether different responses would be seen in alternative systemic or tissue markers and/or in a patient population cannot be established from the current study.

Pre-exercise hyperglycaemia appeared to promote carbohydrate utilisation (Fig. 6.4A) and suppress fat utilisation (Fig. 6.4B) during exercise compared to exercise under normoglycaemic conditions despite matched exercise stimuli (Table 6.3). Such alterations in substrate use during exercise may contribute to altered glucoregulation during the post-exercise period. An interesting anecdotal observation is that pre-exercise hyperglycaemia in HG-Ex and FG-Ex reduced the participants' enjoyment during exercise *vs*. NG-Ex. While the matched exercise stimuli exclude this as a contributor to effects in the current study, reduced enjoyment during exercise could feasibly impact exercise adherence, and therefore variability in exercise response, in a free-living setting.

In contrast to improvements in PPG with exercise, there were no significant effects of exercise upon mean 24 h glucose concentration, glucose SD or glucose CV%. This contradicts the improvements in 24 h glycaemic control seen in hyperglycaemic participants (22,40,42,84). The smaller margin for improvement in the normal glucose tolerant participants in the current study (HbA1c 5.4 ± 0.1% and CGM-derived glycaemic variability within healthy range (47)) compared to the aforementioned studies in hyperglycaemic individuals may contribute to this discrepancy. Interestingly, there were also no significant effects of HG-Ex or FG-Ex on these markers of 24 h glycaemic control compared to either normoglycaemic condition, highlighting that the ability of healthy humans to regulate glucose homeostasis under a challenge from distinctly different stimuli (i.e. exercise and hyperglycaemia) is remarkably well-preserved.

Given the aim of the current study to investigate possible contributing factors to response heterogeneity in glycaemic improvements following exercise, it would be remiss to not discuss variability in outcomes. Inter-individual heterogeneity showed that 9, 7 and 7 out of 12 individuals experienced an improvement in 24 h mean glucose, glucose SD and glucose CV%, respectively, following exercise compared to sedentary CON. This variability in responses likely contributes to the lack of significance seen for these outcomes. That said, this study was perhaps underpowered to detect significant differences in 24 h mean glucose, glucose SD and glucose CV%. Indeed, based on the observed effect size calculated in the ANOVA for 24 h mean glucose (partial eta squared, $\eta^2 = 0.0128$) in the current study a total of 33 participants would be required to detect a statistically significant effect of condition in this outcome in a future study, for example. Therefore, the possibility that the lack of statistical significance is due to insufficient statistical power for this outcome cannot be excluded. Nevertheless, ethical obligations precluded recruitment of additional participants beyond that dictated by the *a priori* power calculations.

In the present study, healthy, recreationally active, non-diabetic individuals were exposed to blood glucose profiles similar to those seen in T2DM (i.e. elevated and unstable) for 3.5 h. It is important to note that the short-term, acute nature of this likely represents a different metabolic and physiological challenge compared to the chronic state of hyperglycaemia and/or glycaemic instability in T2DM. Nevertheless, the approach used enabled us to isolate the effect of acute hyperglycaemia *per se* from/avoid other symptoms or comorbidities of T2DM (e.g. chronic low-grade inflammation, dyslipidaemia, impaired insulin sensitivity and/or secretion, etc.), whilst also avoiding changes in circulating incretin hormones (e.g. GLP-1) otherwise induced by oral glucose ingestion (85), thus justifying the study design used. That said, since pancreatic clamp conditions were not

employed during glucose infusion in the current study, the possibility that increased plasma insulin in hyperglycaemic (HG-Ex and FG-Ex) *vs.* normoglycaemic (NG-Ex and CON) conditions (Fig. 6.3A) contributed to the responses seen cannot be excluded. Furthermore, since exercise in the current study is acute and of moderate-intensity, whether exercise within training regimes and/or of greater intensity elicits the same outcomes remains to be determined. Future studies should also aim to quantify the long-term impact of changes in CGM-derived outcomes on the risk of developing hyperglycaemia-related conditions/complications.

It should also be noted that experimental conditions of normo- *vs.* hyperglycaemia and/or rest *vs.* exercise introduced caloric and carbohydrate imbalances between trials. Specifically, there was an energy deficit in CON (-61.7 ± 3.6 kcals) and NG-Ex (-326.1 ± 18.8 kcals) and a slight energy surplus in HG-Ex (+10.9 ± 20.5 kcals) and FG-Ex (+9.8 ± 17.7 kcals). Similarly, whilst more carbohydrate was oxidised during exercise within the infusion trials compared to NG-Ex (Fig. 6.4A), this difference does not account for the amount of glucose infused during HG-Ex and FG-Ex. Whilst the thermic effect of glucose infusion itself, which increases resting energy expenditure (by ~5 – 11%) (86–88) and carbohydrate oxidation (89), may attenuate these between-trial imbalances to some extent, the possibility that they contributed to findings cannot be excluded. Excess glucose may have increased liver (90) and skeletal muscle (69) glycogen storage which may impact the capacity for postprandial glucose disposal during the post-exercise period in the infusion trials only. That said, whilst glucose infusion to maintain glucose concentrations at +2.5 mmol/L above fasting for 72 h doubled muscle glycogen (69), the short-term, acute nature of infusion in the current study (3.5 h) is unlikely to induce such a stark increase. Additionally, moderate-intensity exercise still reduces muscle glycogen under conditions

of experimental hyperglycaemia (via glucose infusion) (91) and when pre-exercise muscle glycogen is elevated (92). Furthermore, post-exercise skeletal muscle glycogen storage is not solely reliant on changes at the muscle itself, but also splanchnic bed responses that increase the rate of oral glucose appearance in the circulation (93).

Compensating for the aforementioned imbalances with altered food provision without several additional control trials would introduce other confounding variables (e.g. differences in macronutrient provision), meaning imbalance of some form is somewhat inevitable. Therefore, in line with studies evaluating the effects of normo- *vs*. hyperglycaemia (68,69) or rest *vs*. exercise (22,23,40) upon glucose metabolism, compensation of these caloric and carbohydrate imbalances were not included, with the meals kept constant across all conditions. Moreover, this should not detract from the significance of the findings since the exercise-induced improvements in function of key glucoregulatory tissues (e.g. muscle, pancreas, liver, gastrointestinal tract, etc.) that contribute to improved PPG control result from more than simply an energy and glycogen deficit.

Along with several possible clinically important implications of findings (discussed below), a key methodological strength of the current study is the balance between having strict control over a number of vital variables (e.g. pre-trial and post-trial standardisations, glycaemic interventions, exercise interventions, dietary control), whilst monitoring clinically relevance outcomes (i.e. CGM-derived glycaemic control) in an otherwise free-living, ecologically valid setting. Additionally, unlike some previous exercise *vs*. control studies using CGM-derived outcomes (20,94,95), trial order was randomised to minimise the risk of bias. The consumption of the same, controlled diet and no significant

differences in physical activity across all 24 h post-trial periods increases the confidence that observed effects upon PPG are likely attributable to differences in the experimental treatments (i.e. ± glucose infusion, ± exercise) during the respective trials. Not all studies report physical activity data (23) or control over dietary intake (41,96) during the post-exercise period.

Evidence from this study implicates a direct inhibitory effect of pre-exercise hyperglycaemia upon exercise-induced improvements in postprandial glucose control. Clinically, resolving hyperglycaemia prior to exercise and/or coinciding exercise sessions with periods of improved glucose control (i.e. lower, more stable levels) may be a necessary strategy to optimise the therapeutic effects of exercise in individuals with hyperglycaemia-related conditions, such as T2DM. It is unlikely that results from this study represent a baseline glycaemia cut-off point beyond which improvements in glycaemia would be blunted, since individuals with glucose levels similar to the levels induced in this study (i.e. ~7.5 mmol/L average) frequently benefit greatly from exercise in most instances. Instead, an absolute (mmol/L) or relative (% of fasting) increase above basal values may be a key determinant. For example, average glucose across the infusion period was ~ 2.5 mmol/L above basal, representing ~50% increase. Therefore, in a hyperglycaemic individual with a basal glucose concentration of ~7.5 mmol/L but also periods ~10 mmol/L (or ~11 mmol/L based on % increase), it could be advisable to schedule exercise to coincide with glycaemia closer to basal and therefore avoid periods where glycaemia peaks. Anti-hyperglycaemic medication and/or dietary interventions may provide viable options in this regard – that said, both diet and medication have themselves been implicated in variable exercise responsiveness (29,32). Therefore, whilst an individualised approach using CGM would likely be a helpful starting point to identify

candidate exercise periods, the best approach to resolve hyperglycaemia before/during exercise and still gain the optimal exercise-induced benefits, as well as how these findings translate to a period of exercise training, remains to be determined. Of course, further work quantifying whether normalising hyperglycaemia potentiates subsequent glucose-lowering effects of exercise is also required before results of this study can be extrapolated to individuals with T2DM in the context of disease management. Nevertheless, large portions of the worldwide population are non-diabetic meaning these results hold clinical significance and can readily be applied in the context of disease prevention.

## 6.6 Conclusion

Our study adds to evidence demonstrating the potency of exercise in reducing PPG in the post-exercise period compared to sedentary conditions. Furthermore, this study provides the first experimental evidence that pre-exercise hyperglycaemia *per se* can blunt the glucoregulatory benefits of acute exercise, suggesting that hyperglycaemia *per se* contributes to exercise response heterogeneity. Accordingly, resolving hyperglycaemia prior to exercise and/or coinciding exercise sessions with periods of improved glucose control may be vital to overcome exercise non-response/maximise the remedial effects of exercise in individuals with and at risk of hyperglycaemia-related conditions, such as T2DM.

## 6.7 References


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# CHAPTER 7

## 7 GENERAL DISCUSSION

Hyperglycaemia *per se* impairs insulin sensitivity in humans *in vivo* (1,2) and in skeletal muscle cells *in vitro* (3–6). In contrast, exercise potently improves skeletal muscle insulin sensitivity in healthy as well as insulin resistant individuals (7,8). Repeated exposure to either of these stimuli makes significant contributions to determining an individual's long-term metabolic health. Understanding how these two opposite regulatory stimuli (i.e. hyperglycaemia and exercise) interact to impact glucose metabolism could facilitate optimisation of interventions to ensure preservation of healthy glycaemic control. However, whether one stimulus directly influences the impact of the other upon resultant glucose metabolism remains to be determined experimentally. Therefore, the overarching aim of this thesis was to investigate how hyperglycaemia and exercise interact to regulate important glucose metabolism-related outcomes.

To address this, an integrative, translational approach was used combining *in vivo* human and *in vitro* skeletal muscle cell experiments. Specifically, *in vitro* approaches were applied to investigate whether muscle contraction *per se* protects against the hyperglycaemia-induced insulin resistance (chapters 4 and 5). An *in vivo* human study was also conducted to determine whether hyperglycaemia *per se* influences the glucoregulatory effects of a single exercise bout (chapter 6). This discussion will summarise and contextualise the data presented within this thesis.

## 7.1 Skeletal muscle contraction prevents hyperglycaemia-induced insulin resistance (chapter 4)

Hyperglycaemia and exercise oppositely regulate skeletal muscle insulin sensitivity. However, whether exercise *per se* protects against hyperglycaemia-induced impairments remained unknown.

Many of the detrimental effects of hyperglycaemia and beneficial effects of exercise originate/manifest within skeletal muscle. Therefore, in chapter 4, *in vitro* experimental models of hyperglycaemia-induced insulin resistance and 'exercise' were developed and characterised. Specifically, skeletal muscle myotubes were exposed to increasing durations of hyperglycaemia to induce graded skeletal muscle insulin resistance, and electrical pulse stimulation (EPS) as a means to induce skeletal muscle contraction. Applying these models together enabled us to examine the hyperglycaemia – contraction interaction with a level of abstraction not possible *in vivo* in humans. Results from this chapter provide the first experimental evidence that when skeletal muscle contraction precedes hyperglycaemia, insulin resistance induced by hyperglycaemia in non-contracted cells is attenuated (Fig. 4.4). From this, it was concluded that skeletal muscle contraction *per se* confers protection against insulin resistance otherwise induced by hyperglycaemia.

## 7.2 Contraction responsive regulation of miRNAs may contribute to protection against hyperglycaemia (chapter 5)

The mechanisms underlying contraction-mediated protection against insulin resistance-inducing stimuli appear context/stimuli specific, with different mechanisms contributing to inflammation-induced (9) and lipid-induced (10) insulin resistance. Therefore, given the

novelty of the findings in chapter 4, the precise mechanisms responsible for the contraction-mediated protection specifically against hyperglycaemia reported in chapter 4 remained to be determined.

Targeting the skeletal muscle transcriptome offers an appealing therapeutic option in the battle against hyperglycaemia-related conditions. Typically, research has addressed the possibility of targeting coding RNA (particularly mRNA) in this pursuit, but recent evidence highlights the key regulatory role that non-coding RNA (particularly miRNAs) also play in the control of skeletal muscle glucose metabolism (11–14). Therefore, to encompass both coding and non-coding RNA, unbiased RNA sequencing with follow-up qPCR validation was applied to characterise transcriptional changes associated with contraction-mediated protection specifically against hyperglycaemia. The qPCR validation experiments revealed increased miR-194-1 and miR-1a-1 expression in contracted, insulin sensitive (EPS HG) *vs*. rested, insulin resistant (HG) cells exposed to hyperglycaemia (Fig. 5.9). From this, it was concluded that miR-194-1 and miR-1a-1 are contraction-responsive miRNAs that may contribute to contraction-mediated protection against hyperglycaemia induced-insulin resistance.

## 7.3 Transcriptional regulators of temporal improvements in glucose metabolism following muscle contraction (chapter 4 and 5)

Exercise-induced improvements in skeletal muscle glucose metabolism follow a distinct time-course profile, with immediate increases in glucose uptake followed by improved insulin sensitivity (7). Data presented in chapter 4 demonstrates that the model of *in vitro* skeletal muscle contraction used resembles this important aspect of *in vivo* exercise by evoking a similarly distinct temporal response in changes in skeletal muscle glucose

metabolism (chapter 4). Specifically, under normal conditions (i.e. without subsequent hyperglycaemia exposure), contraction-mediated glucose uptake was significantly elevated immediately following contraction cessation only, whereas insulin sensitivity was significantly elevated 24 h post-contraction only (Fig. 4.3). Interestingly, RNA sequencing identified an equally distinct contraction-specific transcriptional profile (chapter 5). When comparing the two time points with the most distinct profile of changes in glucose metabolism, namely 0 h post-EPS and 24 h post-EPS, there was minimal transcriptional overlap (Fig. 5.3). Notably, of 35 transcripts differentially regulated within both timepoints, only 20 were regulated in the same direction (e.g. miR-206, miR-207), with the remaining 15 oppositely regulated (e.g. miR-99b, miR-6790) (Fig. 5.3). From this, it was concluded that the underlying transcriptional changes contributing to improvements in glucose uptake in the immediate post-exercise period are likely to be very different to the transcriptional changes contributing to improved insulin sensitivity that manifests hours – days after exercise.

### 7.4 Pre-exercise hyperglycaemia blunts exercise-induced glucoregulatory benefits (chapter 6)

Exercise is amongst the most potent interventions to improve glycaemic control in healthy individuals, as well as individuals with or at risk of hyperglycaemia-related conditions, such as type 2 diabetes mellitus (T2DM). Accordingly, regular exercise forms the cornerstone in the prevention and management of T2DM (15). That said, in the context of T2DM the full remedial potential of exercise is not always gained, with the magnitude and direction of this effect demonstrating distinct variability (16–20). Isolating factors contributing to this exercise response heterogeneity is vital to optimise the glucoregulatory

effects of exercise, and recent evidence implicates the degree of hyperglycaemia as one possible contributor (21–26). However, previous evidence was equivocal and largely correlational in nature. Therefore, whether hyperglycaemia *per se* directly influences exercise-induced glucoregulatory effects remained unknown.

In chapter 6, healthy, non-diabetic individuals received intravenous glucose infusion to induce blood glucose profiles similar to those seen in T2DM (i.e. elevated and unstable/fluctuating) followed by a moderate-intensity exercise bout. This approach isolated hyperglycaemia from other aspects of the diabetic phenotype/condition (e.g. chronic low-grade inflammation, dyslipidaemia, duration since diagnosis, medication, etc.), thus enabling us to examine the effects of hyperglycaemia *per se* upon subsequent exercise response. The data shows that exercise under normal conditions (i.e. without prior glucose infusion) significantly improved postprandial glucose (PPG) response (Fig. 6.5). Since excessive PPG is associated with hyperglycaemia-related complications as well as worsening of HbA1c (27), these exercise-induced reductions in PPG emphasise the importance of regular exercise in preventing the progression towards, and complications of, hyperglycaemia-related conditions such as T2DM. However, when hyperglycaemia (induced via intravenous glucose infusion) precedes exercise, the improvements in PPG control conferred by exercise were blunted (Fig. 6.5). The profile of hyperglycaemia (i.e. steady-state *vs*. fluctuating) did not influence this response. This is the first experimental evidence addressing this topic, and it was concluded that these data confirm hyperglycaemia *per se* as a source of variability in the glucoregulatory benefits of exercise.

## 7.5 Recommendations

Overall, findings from the aforementioned chapters promote the importance of regular exercise/muscle contraction in improving glucose metabolism by preventing metabolic disturbances induced by hyperglycaemia (chapter 4) and improving postprandial glucose control (chapter 6). This agrees with a vast amount of previous literature (far too many to cite).

The evidence presented within this thesis also provides novel information highlighting how the timing of exercise in relation to exposure to hyperglycaemia alters the resultant changes in glucose control and metabolism. Overall, the stimuli (exercise/contraction or hyperglycaemia) that is experienced first blunted the changes in glucose metabolism that are induced by the second stimuli. Specifically, when skeletal muscle contraction precedes exposure to hyperglycaemia, the deleterious effects of hyperglycaemia are blunted (a favourable response since insulin sensitivity is preserved). When the order of exposure is switched, namely exposure to hyperglycaemia precedes exercise, the beneficial effects of exercise are blunted (an unfavourable response since glycaemic control is not improved). Collectively, the most obvious recommendation (aside from avoiding hyperglycaemia completely) would be to tailor interventions to ensure exercise precedes exposure to acute hyperglycaemia, since doing so would not only avoid the detrimental effect of hyperglycaemia on the glucoregulatory effects of acute exercise, but also confer protection against hyperglycaemia-induced adverse effects.

Furthermore, the evidence presented in this thesis can be harnessed to optimise future glycaemic control interventions in a number of ways. Firstly, chapter 5 identified miR-194-1 and miR-1a-1 as contraction-responsive transcriptional targets that could be manipulated

via alternative means (e.g. nutritional or pharmaceutical interventions) to potentially confer contraction-like improvements in skeletal muscle glucose metabolism even under conditions when contraction is not possible. This may be particularly useful to preserve skeletal muscle glucose metabolism in circumstances where long-term injury or illness preclude regular exercise. Whilst targeting skeletal muscle miRNA to ameliorate glucose metabolism *in vivo* remains in a relatively nascent stage, the rapidly accumulating evidence supports this as an appealing future therapeutic option (28,29), and data in chapter 6 suggest that miR-194-1 and miR-1a-1 should be investigated in this regard.

Secondly, chapter 6 promotes coinciding exercising with periods of improved glycaemic control (i.e. lower, more stable levels) to maximise exercise-induced benefits. This information may be particularly pertinent in circumstances where exercise does not confer the desired glucoregulatory benefits. An important clarification is that this finding does not preclude individuals with hyperglycaemia benefitting from exercise, nor do the findings identify specific glycaemic cut-off points beyond which individuals would not benefit. Instead, rather than the absolute level of glycaemia, the relative increase above basal may be a more influential determinant of the blunted benefits. Therefore, findings suggest that coinciding exercise with periods of improved glycaemic control (i.e. avoiding peaks and/or unstable glycaemic profiles) would be conducive to maximise the glucoregulatory effects of exercise.

Finally, the above findings could be combined to speculatively devise an approach to optimise the remedial effects of exercise in managing hyperglycaemia. Skeletal muscle insulin sensitivity is one key determinant of PPG glucose response (30,31). Exercise potently improves skeletal muscle insulin sensitivity, and such improvements likely

contribute to exercise-induced improvements in PPG response (32). Conversely, hyperglycaemia *per se* impairs skeletal muscle insulin sensitivity (1,2), and hyperglycaemia (and associated insulin resistance) is linked with lower exercise-induced improvements in insulin sensitivity, which turn in coincides with smaller exercise-induced improvements in PPG response (33). Therefore, the impaired exercise-induced improvements in PPG response seen following acute hyperglycaemia in chapter 6 may be explained, at least in part, by hyperglycaemia-induced insulin resistance and/or blunted exercise-induced improvements in insulin sensitivity (Fig. 7.1 pathway A). By extension, ameliorating muscle insulin sensitivity and/or protecting skeletal muscle from hyperglycaemia prior to exercise may maximise exercise-induced improvements in PPG response. Speculative combination of findings from the three experimental chapters within this thesis suggest miRNAs identified in chapters 4 and 5 (namely miR-194-1 and miR-1a-1) could be targeted to ameliorate skeletal muscle insulin sensitivity/protect against hyperglycaemia-induced insulin resistance. This in turn could overcome the hyperglycaemia-induced blunting of exercise-induced improvements in PPG response shown in chapter 6 (Fig. 7.1 pathway B), perhaps towards levels achieved under normoglycaemic conditions (Fig. 7.1 pathway C). As stated, this proposal is rather speculative and based on a number of assumptions (including that the 3.5 h glucose infusion models induced insulin resistance). Nevertheless, this suggestion does demonstrate how data from *in vitro* and *in vivo* models such as within this thesis can be integrated to stimulate new hypotheses to progress towards novel optimised interventions to improve glycaemic control.

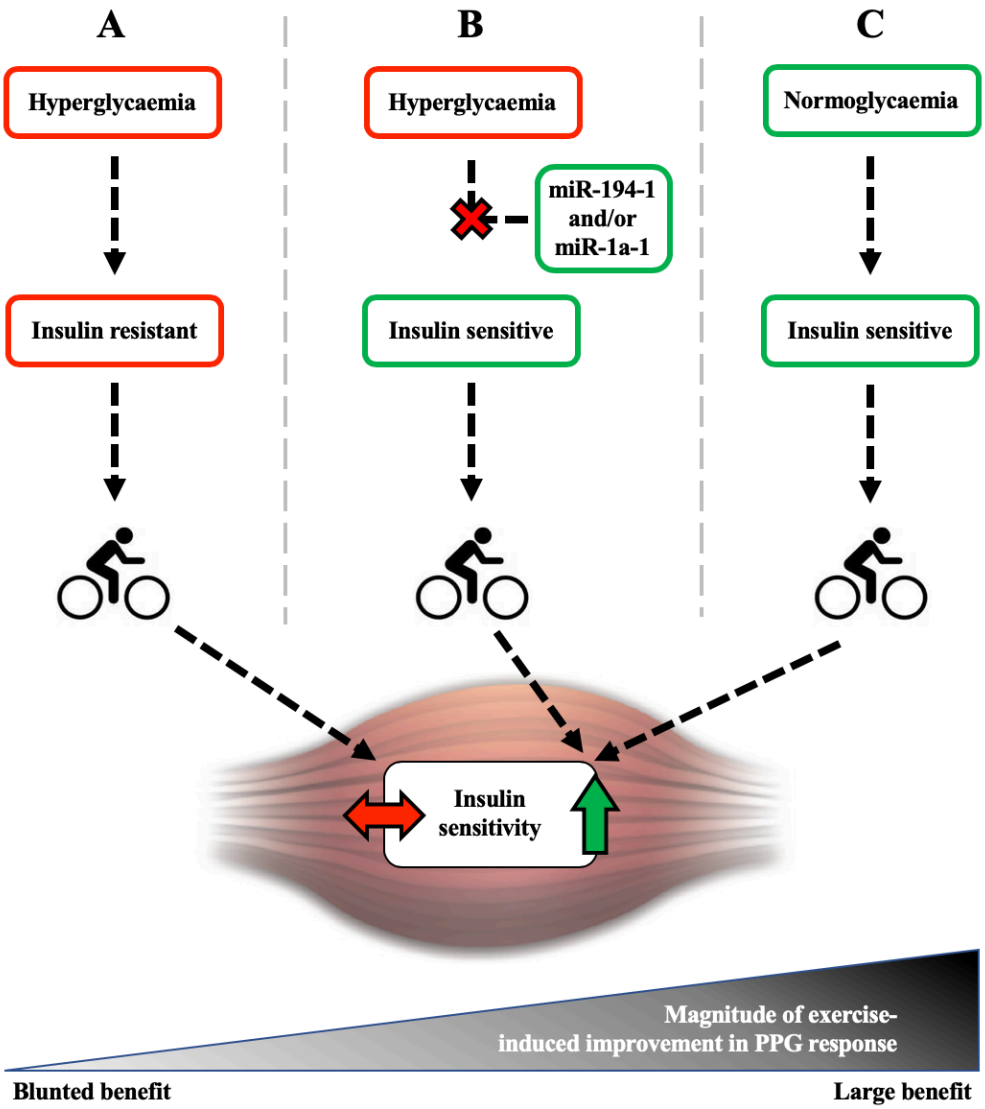



**Fig. 7.1 A model to depict how data from *in vitro* and *in vivo* models within experimental chapters 4 – 6 could be integrated to design optimised interventions to improve glycaemic outcomes following exercise.** Hyperglycaemia impairs skeletal muscle insulin sensitivity. Since insulin sensitivity is an important determinant of PPG control, ameliorating insulin sensitivity/preventing hyperglycaemia-induced insulin resistance by targeting miRNA expression profiles may overcome hyperglycaemia-induced blunting of exercise-induced improvements in PPG. Full details of this proposed model are provided within the text.

## 7.6 Limitations

In chapters 4 and 5, C2C12 cells were used as the model of skeletal muscle *in vitro*. C2C12 cells are a robust cell line with predictable growth/differentiation patterns, and the

myogenesis and differentiation processes recapitulate processes seen in human muscle cells. Therefore, C2C12 cells provide an appropriate and convenient model that is widely used to investigate the effects of EPS-induced contraction (34). However, excessive culture durations and handling can make cell lines, like C2C12, prone to deviate from normal function. Additionally, C2C12 myotubes are notably more glycolytic than healthy human muscle. This is demonstrated by lactate concentrations of less than 7 mmol/L accumulating over 96 h in conditioned media from human primary cells (35) compared to ~8 mmol/L accumulating within 24 h in C2C12 cells in chapter 4 (Fig. 4.2D). Therefore, these disparities urge caution before direct application of findings to human skeletal muscle glucose metabolism. Accordingly, whilst use of human primary cells introduces its own limitations when using EPS to induce contraction – namely, distinct donor-specific variability in contractility and resultant metabolic responses (36–40) – translation of findings to human muscle is ultimately required to maximise impact of findings. Nevertheless, as discussed in chapter 2, extensive efforts were made to make the C2C12 model used throughout this thesis as physiologically relevant as possible. In particular, C2C12 cells are typically cultured in insulin resistance-inducing conditions (namely 25 mmol/L glucose), which is inappropriate particularly when trying to mimic healthy (including being insulin sensitive) skeletal muscle. Therefore, despite the limitations of the C2C12 cell line, routine culture in 5 mmol/L at least ensured the model used was more representative of a normoglycaemic phenotype. That said, despite being planned, follow-up gain and loss of function experiments were unfortunately not possible within this thesis due to financial/administrative restraints towards the end of the project. Therefore, the direct role of miR-194-1 and miR-1a-1 expression in the regulation of contraction-

mediated protection against hyperglycaemia was not causally determined. Outlines of proposed follow-up experiments to address this are detailed below.

In chapter 6, the human exercise metabolism study is acute in nature, meaning results should not be directly extrapolated to assume similar responses would be seen during exercise training. Therefore, long-term training studies would ultimately be required to understand whether the hyperglycaemia-induced blunting of benefits following a single exercise bout translate to blunted benefits within a chronic exercise training programme. Similarly, moderate-intensity cycling was used throughout, meaning the possibility that different intensities and/or modalities of exercise would induce different responses cannot be excluded. Additionally, young males only were recruited, therefore the possibility that age and/or gender differences exist cannot be excluded. Finally, the direct translation of findings may only be appropriately applied to non-diabetic individuals at this stage, with caution required for extrapolation to individuals with T2DM. Nevertheless, since non-diabetic individuals represent ~90% of global population, findings hold potential significance for a large proportion of individuals.

## 7.7 Future research

As detailed above, novel evidence generated during this thesis addresses multiple scientific gaps in previous research to extend prior knowledge regarding the hyperglycaemia – exercise interaction. That said, the findings also stimulate additional questions that warrant further investigation. Specifically, it could be hypothesised that a) miR-194-1 and miR-1a-1 could be targeted to ameliorate skeletal muscle glucose metabolism (based on chapters 4 and 5) and b) coinciding exercise with periods of improved glycaemic control may potentiate exercise-induced glucoregulatory benefits (based on chapter 6). To address these

new questions and provide natural progression to data presented herein, the following experiments would be warranted.

Firstly, gain and loss of function experiments to investigate whether overexpression or inhibition of miR-194-1 and miR-1a-1 causally regulates skeletal muscle glucose metabolism under conditions of normo- *vs*. hyperglycaemia and/or 'rest' *vs*. contraction are required. Assessment of protein expressions of purported protein targets of miR-194-1 and mir-1a-1 would further our understanding of the role of miRNA – protein interactions in this response. Applying this design to human primary myotubes with wide-ranging insulin sensitivity (e.g. exercise trained *vs*. healthy control *vs*. T2DM) would maximise impact of findings. Such experiments would progress towards defining possible causal roles for these novel contraction-responsive transcripts, and establish whether they can be targeted to modulate skeletal muscle insulin sensitivity in contexts of widely varying insulin sensitivity. This would address questions posed by data generated in chapter 4 and 5.

Secondly, the natural progression to data presented in chapter 6 would involve the following investigations: a) whether the effects of hyperglycaemia on responses to a single exercise bout translate to a period of exercise training and b) whether resolving hyperglycaemia in diabetes patients directly potentiate exercise-induced benefits. Importantly, such studies should include additional measures to quantify changes in the risk of developing hyperglycaemia-related conditions or complications to fully understand the clinical relevance of findings. If indeed coinciding exercise with improved glycaemic control (i.e. lower and/or more stable levels) translates to potentiated exercise training-induced glucoregulatory effects, further investigation to identify the best adjunct lifestyle or pharmaceutical approach to maximise exercise response would be the natural research

progression. For example, as speculated above (Fig. 7.1), it will be important to investigate whether improving skeletal muscle insulin sensitivity by targeting miRNA expression prior to exercise improves resultant exercise-induced benefits. Knowledge gained from these studies would inform and enhance current clinical guidelines regarding the best way to manipulate the hyperglycaemia – exercise interaction to ultimately optimise interventions to prevent and manage hyperglycaemia.

## 7.8 Conclusions

Herein, an integrative approach was applied to provide a novel insight into how hyperglycaemia and exercise interact to influence glucose metabolism. Firstly, prior skeletal muscle contraction blunts hyperglycaemia-induced impairments in insulin sensitivity, and this contraction-mediated protection coincides with increased miR-194-1 and miR-1a-1 expression. In contrast, prior hyperglycaemia blunts exercise-induced improvements in glycaemic control *in vivo*. This data clearly highlights that the order of exposure to these two opposite regulatory stimuli (exercise/contraction and hyperglycaemia) is important in determining the resultant impact upon glucose metabolism, with the first stimuli blunting the impact of the second stimuli in both cases. Therefore, the best approach (aside from avoiding hyperglycaemia completely) may be to schedule exercise to precede hyperglycaemia since doing so would prevent the adverse effects that hyperglycaemia exerts on a) basal (non-exercised) glucose metabolism and b) exercise-induced improvements in glycaemic control. Identification of miR-194-1 and miR-1a-1 as potential mediators of contraction-mediated protection against hyperglycaemia-induced insulin resistance suggest that targeting these transcripts may be a novel approach to modify the hyperglycaemia – exercise interaction. Collectively, this

information can be harnessed to optimise future interventions aiming to improve human glycaemic control.

## 7.9 References


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