LOUGHBOROUGH UNIVERSITY

Metabolic responses to short-term high-fat overfeeding

A Doctoral Thesis

Siôn Adam Parry

March 2017

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University.

Abstract

The main aim of this thesis was to increase our understanding of the metabolic responses associated with short-term high-fat overfeeding. To this end, four separate studies are described in this thesis; each of which involved the provision of a high-fat, high-energy diet to young, healthy, lean individuals. The first of these experimental chapters (**Chapter 2**) determined the effects of a 7-day, high-fat (65%), high-energy (+50%) diet on postprandial metabolic and endocrine responses to a mixed meal challenge. This chapter demonstrates that 7-days of overfeeding impaired glycaemic control in our subject cohort but did not influence the response of selected gut hormones (acylated ghrelin, GLP-1 and GIP). In a mechanistic follow up study utilising stable isotope tracer methodology we then demonstrate that overfeeding-induced impairments in glycaemic control are attributable to subtle alterations in plasma glucose flux, rather than the overt tissue-specific adaptations (e.g. increased EGP, or reduced glucose disposal) that have previously been reported (**Chapter 3**). In an attempt to delineate the time-course of diet-induced impairments in glycaemic control, we then investigated the effects of 1-day of overfeeding (+80% energy with 73% of total energy coming as fat) (**Chapter 4**). Results demonstrate that a single day of overfeeding elicits responses which are comparable to 7-days of high-fat overfeeding; highlighting the rapidity with which excessive high-fat food intake can negatively influence glucose metabolism. In **chapter 5** we utilised stable isotope tracer and muscle biopsy techniques to demonstrate that 7-days of high-fat overfeeding impairs glycaemic control but does not influence the fed-state mixed muscle protein fractional synthesis rate (FSR). In conclusion, the findings of this thesis demonstrate that while short-term high-fat overfeeding negatively influences whole-body glucose metabolism, skeletal muscle protein metabolism appears to be relatively unaffected in young, lean, healthy humans.

Keywords: Overfeeding, high-fat diet, insulin sensitivity, glycaemic control, muscle protein synthesis.

Acknowledgements.

Firstly, I would like to thank my supervisor Dr. Carl Hulston without whom this PhD thesis would not have been possible. I am grateful for allowing me the opportunity to study for a PhD, and for the guidance, insight, and technical assistance you have provided throughout my studentship. I would also like to thank my internal moderator Dr. Richard Ferguson, for providing further advice and guidance during my PhD and your critical help in setting up the muscle biopsy technique in our laboratory. My gratitude is extended to Professor Mark Lewis, Dr. Lewis James, Dr. Stephen Mears, Dr Emma Watson and the many other academics I have had the pleasure of working alongside and learning from during my time in Loughborough. The technical skills of Professor Gerrit van Hall and his team at The Clinical Metabolomics Core Facility, Righospitalet have also been invaluable throughout the completion of this work. Additionally, I have to thank Professor Leanne Hodson for her guidance and support throughout my writing up period.

Particular thanks must go to current and past PhD students and LDC members for their advice, support, and friendship throughout; Joseph Harb, Danielle Evans, Ben Kelly, Conor Taylor, Mark Turner, Adam Fry, Andrew Shaw and the boys of Adam Dale, Neil Martin, Darren Player, David Clayton, Nanpantan and many, many more. I honestly doubt I could have done it without you all.

I cannot summarise my gratitude to a number of people in my life. Rob and Luke, you're both like family to me and your lifelong friendship and support is something that has greatly contributed not just to this thesis but my life in general. Kate, your love and constant support these last two years means more to me than I can ever express. I hope I will one day be able to contribute to your life as fully as you have mine. I love you all.

Finally, and most importantly I would like to thank my family; Dad, Mum, Stevie, my grandparents, aunties and uncles. You have all made me the man I am today and my goal is and has always been to make you proud. I am eternally grateful for all you have ever done for me. I dedicate this work in its entirety to the loving memory of my grandmother and best friend, Patricia Parry.

List of publications:

***Journal Articles*:**

Parry, S. A., Smith, J. R., Corbett, T. R., Woods, R. M. & Hulston, C. J. (2017). Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal-weight individuals. Br J Nutr, 117, 48-55

***Scientific meeting proceedings*:**

Parry, S.A., Smith, J.R., & Hulston, C.J. (2015) A seven-day high-fat, high-energy diet impairs glycaemic control but does not disrupt the postprandial acylated ghrelin response to a mixed meal. Published abstract; 20th annual congress of the European College of Sport Science, Malmö – Sweden

***In preparation*:**

Parry, S. A. Woods, R. M., Hodson, L. & Hulston, C. J. (2017). A single day of excessive dietary fat intake impairs whole-body glycaemic control in healthy, non-obese, young men. *In preparation*.

Parry, S. A., James, L.J., Ferguson, R.A., van Hall, G., & Hulston, C. J. (2017). Diet-induced impairments in insulin sensitivity do not influence the muscle protein synthetic response to carbohydrate and protein ingestion in young, healthy, non-obese individuals. *In preparation*

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CHAPTER 1

Introduction.

1.1 Background

Globally, the prevalence of overweight and obesity is increasing at an alarming rate (Joshi *et al.*, 2007). Dubbed "prosperity's plague" (Taubes, 2009), reports estimate that the proportion of individuals classed as overweight and obese (i.e. body mass index (BMI) > 25 kg/m^2) increased by 27.5% worldwide between 1980 and 2013 (Ng *et al.*, 2014); an increase which is projected to continue over the next two decades (Finkelstein *et al.*, 2012). In the United Kingdom it has been estimated that approximately two-thirds of the male, and over half of the female population are overweight or obese (Ng *et al.*, 2014). This is pertinent as obesity is strongly associated with a myriad of clinical problems, including non-alcoholic fatty liver disease (NAFLD) (Fabbrini *et al.*, 2010), type 2 diabetes mellitus (T2DM) (Steppan *et al.*, 2001), cardiovascular disease (CVD) (Poirier, & Eckel, 2002) and certain cancers (Bianchini *et al.*, 2002), amongst others. Thus, overweight and obesity are an important contributor to morbidity and mortality, and a reduced quality of life (Abdelaal *et al.*, 2017) and therefore represent one of the major healthcare challenges of the 21st Century.

The recent rise in overweight and obesity is attributable to both genetic and lifestyle factors; with genes predisposing individuals and lifestyle factors such as diet and physical inactivity providing the catalyst (Swinburn *et al.*, 2011). Based on the speed at which prevalence is increasing it would suggest that changes in lifestyle are the driving force, as genetic influences would not be expected to change over such a short period. This change in lifestyle is driven by the development of machines and technologies which have reduced daily physical activity levels (Booth *et al.*, 2008), and the broad availability of relatively inexpensive, highly palatable, energy-dense foods (Schrauwen, 2007). Thus, it is extremely likely that most individuals experience at least brief periods of positive energy balance (Hall *et al.*, 2012). This seems to be especially true during holiday periods or times of celebration (Cooper, & Tokar, 2016; Yanovski *et al.*, 2000). If these periods of positive energy balance are not counterbalanced by subsequent periods of negative energy balance (where energy expenditure exceeds intake), then individuals will begin to gain weight, of which approximately 60-80% will be attributable to increases in body fat (Hill, & Commerford, 1996). If this practice continues long-term then obesity will ensue.

A common feature underpinning many of the diseases associated with obesity is insulin resistance; a state in which normal or elevated concentrations of insulin elicit a subnormal biological response (DeFronzo, & Tripathy, 2009). Whilst it is clear that insulin resistance is

strongly associated with obesity, it would appear that visceral adiposity (i.e. an accumulation of adipose tissue underneath the abdominal muscle wall) is particularly deleterious to insulin and glucose metabolism (Amati *et al.*, 2012; Hayashi *et al.*, 2008; Indulekha *et al.*, 2011; McLaughlin *et al.*, 2011; Preis *et al.*, 2010; Wagenknecht *et al.*, 2003). A prime example of this can be seen in individuals with genetic defects who are characterised by partial whole-body, or depot-specific lipodystrophy. These individuals exhibit an increased visceral fat depot with little or no change in subcutaneous adiposity alongside severe insulin resistance (Arioglu *et al.*, 2000; Huang-Doran *et al.*, 2010; Reitman *et al.*, 2000). However, the molecular mechanisms through which increases in fat, visceral or other, cause insulin resistance are yet to be clarified. This is due to ethical considerations which make it difficult, nigh on impossible, to study the progression of obesity and/or metabolic disease. Thus, much of the knowledge regarding the development of insulin resistance in humans is inferred from animal studies (Han *et al.*, 2013; McManaman *et al.*, 2013; Tsai *et al.*, 2016) which cannot be directly transferred into our understanding of human metabolic dysfunction. With regards to human research, there is a vast amount of cross-sectional research in the literature (i.e. obese and/or insulin resistant *vs*. healthy controls) (McLaughlin *et al.*, 2016; Mitrakou *et al.*, 1992; Prager *et al.*, 1986), and epidemiological reports (Marshall *et al.*, 1991; Martin *et al.*, 1992; Pereira *et al.*, 2005). While these observational studies are invaluable with regards characterising obesity and insulin resistance, they are unable to illuminate causality or offer any insight into the developmental time-course. In order to overcome this limitation, researchers have employed intervention strategies such as intravenous (iv) lipid infusions, bed rest/relative physical inactivity models, and high-fat and/or high-energy diets. These interventions are designed to impair glycaemic control/reduce insulin sensitivity, and thus provide information on the metabolic responses which underpin the development of insulin resistance in humans. However, these early-phase responses are yet to be fully elucidated.

The primary aim of this thesis is to provide a greater insight into the early metabolic responses associated with short-term high-fat overfeeding. To this end, the following sub-chapters will provide a brief overview of insulin and its role in metabolism. Subsequently, this chapter will attempt to outline current knowledge regarding the pathophysiology and pathogenesis of whole-body insulin resistance in humans. A critical review of current literature examining metabolic responses to short-term high-fat overfeeding interventions will also be presented. This review will serve to highlight gaps in the literature which this thesis will hopefully address.

1.2 Physiological role of insulin

Despite intermittent ingestion of dietary carbohydrates, in healthy individuals circulating glucose concentrations are maintained within narrow limits (~4-6.0 mmol/L) (Saltiel, & Kahn, 2001). This requires the concerted actions of several different tissues which govern the interplay between the rate of glucose entering the circulation (rate of appearance [R_a]), and that being removed from the circulation (rate of disappearance [R_d]) (Thorens, 2015; Utzschneider *et al.*, 2006; Woerle *et al.*, 2003). Insulin is a critical regulator in this metabolic transition from the fasted to the fed state (Saltiel, & Kahn, 2001). Insulin secretion occurs in response to numerous stimuli such as amino acids (especially, leucine and lysine), the incretin hormones (glucagon-like peptide-1 [GLP-1] and gastric inhibitory polypeptide [GIP]), and sulphonylureas (Joshi *et al.*, 2007). However, the most potent stimulus of insulin secretion is glucose. In order to sense changes in plasma glucose or other secretory stimuli, islets continually sample blood from the branches of the splenic and pancreaticoduodenal arteries (Newsholme *et al.*, 2014). When elevations in secretory stimuli are detected, insulin is released. In the instance of glucose, this occurs when circulating blood glucose concentrations are ≥ 3.3 mmol/L, above which the secretion of insulin is increased in proportion to the degree of glucose perturbation (i.e. the greater the glucose concentration, the greater the insulin response) (Gerich, 1993). Insulin secretion occurs from the islet cells into the portal vein in a pulsatile manner, requiring coordinated secretory bursts from millions of cells. The secretion of insulin is characteristically biphasic in nature. The initial rapid (5-10 min) release of pre-synthesised insulin is mediated by an increase in the β-cell adenosine triphosphate (ATP):adenosine diphosphate (ADP) ratio and the subsequent intracellular flux of calcium ions. Whereas, the second more prolonged secretion of insulin (30-60 min) requires increased synthesis of insulin and is mediated by mitochondrial metabolism and the increase in tricarboxylic acid (TCA) cycle intermediates, protein kinase C (PKC) signalling, and increased calcium flux (Keane, & Newsholme, 2014; Newsholme *et al.*, 2014; Newsholme, & Krause, 2012).

1.2.1 Skeletal muscle

Skeletal muscle represent one of the primary target tissues for insulin. The principal role of insulin in skeletal muscle is to stimulate glucose uptake. This occurs by insulin binding to its receptor, which is part of a subfamily of receptor tyrosine kinases. The insulin receptor is a heterotetrameric glycoprotein with two extracellular α-subunits and two transmembrane β-

subunits. Insulin binds to the α-subunits which leads to auto-phosphorylation of tyrosine residues on the β-subunits. This process promotes the translocation of the insulin receptor substrates (IRS) to the plasma membrane where the interaction with the insulin receptor causes it to also undergo tyrosine phosphorylation (Defronzo, 2009). There exist 13 different IRS's, however, in skeletal muscle it is IRS-1 and IRS-2 which are most important. The activation of IRS-1, and to a lesser extent IRS-2, leads to the subsequent activation of phosphotidylinositol (PI)-3-kinase (PI3-K). PI3-K consists of a regulatory subunit, p85, and a catalytic subunit, p110, both of which exist in multiple isoforms (Shepherd *et al.*, 1998). The regulatory p85 subunit maintains the p110 catalytic subunit in a low-activity state until stimulation by insulin whereby p110 binds to IRS-1 activating PI3-K. PI3K catalyses the conversion of phosphatidylinositol (4,5)bis-phosphate (PI(4,5)P 2), to phosphatidylinositol (3,4,5)tris-phosphate (PI(3,4,5)P3) (Siddle, 2011). PI(3,4,5)P 3 serves to anchor PI3K and the 3-phosphoinositide dependent protein kinase-1 (PDK-1) to the plasma membrane (Shepherd *et al.*, 1998). PDK-1 acts to phosphorylate and activate protein kinase B (Akt) which acts downstream to phosphorylate a variety of substrates, including glycogen synthase kinase-3 (GSK-3), phosphofructokinase-2 (PFK-2), and Akt substrate of 160 kDa (AS160). Phosphorylation of these signalling intermediates, results in the translocation of glucose transporter 4 (GLUT-4) to the cell membrane permitting glucose entry into the cell (Siddle, 2011). It has been reported that skeletal muscle accounts for approximately 60-70% of insulin mediated glucose uptake (Smith, 2002). Once within the cell glucose is either stored as glycogen or metabolised; glucose is predominantly metabolised through glycolysis from which the pyruvate produced can be converted to lactate and released into the blood, or be decarboxylated and enter the TCA cycle for complete oxidation (Holloszy, & Coyle, 1984; Krebs, 1979).

Insulin has also been shown to be a key regulator in skeletal muscle protein turnover. However, the precise role of insulin in this process is complex and a topic of debate. Early reports from arterio-venous difference studies demonstrated that an increase in circulating insulin concentrations resulted in a reduction in muscle protein breakdown (MPB) (Fryburg *et al.*, 1990; Gelfand, & Barrett, 1987; Pozefsky *et al.*, 1969). While these early observations have remained true to this day, there exist mixed reports regarding the ability of insulin to stimulate muscle protein synthesis (MPS); some studies demonstrate increased MPS with increased plasma insulin concentrations (Biolo *et al.*, 1995; Biolo, & Wolfe, 1993), whereas others show no change (Chow *et al.*, 2006; Louard *et al.*, 1992). Much of this confusion is

likely related to alterations in amino acid concentrations; increased insulin levels reduce MPB and thus suppress amino acid availability reducing the available substrate for MPS. In a relatively recent study, Greenhaff *et al.* (2008) demonstrated that amino acids *per se* are a potent anabolic stimulus, able to induce a substantial upregulation of MPS even under basal (5 μU/mL) insulin concentrations. Furthermore, in the presence of fixed amino acid availability, stepwise elevations in insulin concentration did not further increase MPS, but did reduce MPB, a finding which is supported by a number of other studies (Bell *et al.*, 2006; Fujita *et al.*, 2006a; Fujita *et al.*, 2006b; Rasmussen *et al.*, 2006; Wilkes *et al.*, 2009). Thus, it would appear that insulin itself is not anabolic, but it is definitely anti-proteolytic. However, the general consensus is that insulin has a permissive role in MPS, and a small amount of insulin is necessary to prime the system (Cuthbertson *et al.*, 2005). Thus, it would appear that while insulin itself does not stimulate MPS, it is pro-anabolic through its permissive role in MPS and potent ability to reduce MPB, which act in synergy to increase net MPS.

1.2.2 Liver

A second major target tissue for insulin is the liver. In health, increased circulating insulin concentrations stimulate glucose uptake and glycogen synthesis. This effect is mediated via the same canonical signalling pathway as muscle (described above), although in this instance glucose entry is facilitated by GLUT-2 transporters (Thorens, 2015). The liver has been reported to be responsible for approximately 30% of glucose uptake (Smith, 2002). In addition, increased insulin concentrations also suppress glycogenolysis and gluconeogensis (hereafter combined and referred to as endogenous glucose production [EGP]) leading to a reduction in glucose output. (Cersosimo *et al.*, 1994; Saltiel, & Kahn, 2001; Samuel *et al.*, 2004). This is thought to occur via direct and indirect mechanisms. Directly, activation of Akt phosphorylates and inactivates forkhead box protein 01 (FOXO1), reducing the transcription of gluconeogenic enzymes (Samuel, & Shulman, 2016), indirectly insulin inhibits adipose tissue lipolysis (see below), limiting the availability of substrate for glucose conversion (Perry *et al.*, 2014; Previs *et al.*, 1999).

A further process within the liver in which insulin plays a role is the transcriptional regulation of *de novo lipogenesis* (DNL); the biochemical process of synthesising fatty acids from non-lipid precursors (Strable, & Ntambi, 2010). Increased insulin signalling upregulates the sterol regulatory element binding protein 1c (SREBP1c) pathway which increases the expression of lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC),

resulting in a sequence of events which converts acetyl-CoA to malonyl-CoA to the 16 carbon fatty acid palmitate (Hellerstein *et al.*, 1996; Leavens, & Birnbaum, 2011; Sanders, & Griffin, 2016). The primary route of disposal of *de novo* synthesised fatty acids is secretion in triglyceride rich very low density lipoprotein (VLDL) (Sanders, & Griffin, 2016).

1.2.3 Adipose tissue

In adipocytes insulin stimulates both fatty acid and glucose uptake. Glucose uptake is mediated via a Glut 4 dependent mechanism (Ducluzeau *et al.*, 2002), whereas fatty acid uptake involves the activation of lipoprotein lipase (LPL) (Sadur, & Eckel, 1982). Adipose tissue has been estimated to be responsible for only 2-6% of whole-body glucose uptake (Marin *et al.*, 1987; Virtanen *et al.*, 2002), but is the predominant site of dietary-derived fatty acid uptake (Nestel *et al.*, 1962). Glucose can be metabolised via glycolysis or stored as intracellular lipid via the action of lipogenic enzymes, including pyruvate dehydrogenase (PDH), FAS and ACC (Saltiel, & Kahn, 2001). Fatty acids are then re-esterified using glycerol 3-phosphate (G3P) (derived from glucose metabolism) as a backbone to form triglycerides within adipocytes (Clifton-Bligh, & Galton, 1976).

Insulin also profoundly inhibits adipose tissue lipolysis, suppressing fatty acid and glycerol turnover. This occurs by decreasing cyclic adenosine monophosphate (cAMP) levels, through the phosphorylation and subsequent activation of a cAMP-specific phosphodiesterase (Degerman *et al.*, 1990), which ultimately leads to the inhibition of hormone sensitive lipase (HSL) (Anthonsen *et al.*, 1998).

1.3 Heterogeneity of insulin resistance

Due to the widespread effects of insulin described above, insulin resistance may vary in both its cause and effect depending on the physiological function of the tissues in which it is manifest. However, defects in any of these tissues can have a profound impact on whole-body metabolic control, leading to impaired glycaemic control, dyslipidemia and potential muscle atrophy.

1.3.1 Skeletal muscle

In insulin resistant states the ability of insulin to stimulate glucose uptake in skeletal muscle is impaired (DeFronzo, 1988; DeFronzo, 2004; DeFronzo *et al.*, 1989; DeFronzo *et al.*, 1985; Ferrannini *et al.*, 1988; Gerich *et al.*, 1990; Mitrakou *et al.*, 1990; Woerle *et al.*, 2006). As

skeletal muscle is the predominant site of insulin-stimulated glucose uptake this reduces plasma glucose R_d resulting in dysregulations in whole-body glucose metabolism. This is evident during fasting, but is considerably more pertinent during the postprandial period when circulating glucose concentrations are greater. The reduction in insulin-stimulated glucose uptake is due to a combination of defects in insulin signalling (i.e. reduced IRS-1 tyrosine phosphorylation resulting in decreased PI3-K activation), glucose transport (i.e. impaired GLUT4 translocation) and intracellular glucose metabolism (i.e. decreased glucose phosphorylation, reduced glucose oxidation and glycolytic flux and reduced activation of glycogen synthase) (Abdul-Ghani, & DeFronzo, 2010; Bajaj, & Defronzo, 2003; Bouzakri *et al.*, 2005; Cusi *et al.*, 2000; Karlsson, & Zierath, 2007).

Evidence also suggests that relative muscle mass is inversely related to whole-body insulin resistance (Srikanthan, & Karlamangla, 2011), and T2DM is associated with impaired skeletal muscle function and an accelerated loss of lean mass with aging (sarcopenia) (Kim *et al.*, 2010; Park *et al.*, 2009; Park *et al.*, 2006; Park *et al.*, 2007). Taken together these findings suggest that insulin resistance negatively impacts skeletal muscle protein metabolism. Reductions in muscle mass and strength are directly associated with mortality rates in the elderly (McLeod *et al.*, 2016). Furthermore, as skeletal muscle in the primary site for glucose uptake following feeding, reductions in skeletal muscle mass would have a profound effect on whole-body glycaemic control. In humans the maintenance of skeletal muscle mass is dependent on the dynamic equilibrium between MPS and MPB. When investigating the MPS response to carbohydrate and protein ingestion in T2DM patients and healthy controls, Manders *et al.* (2008) demonstrated a 60-70% difference in muscle protein fractional synthesis rate (FSR) between these two cohorts, with the healthy controls displaying a much greater postprandial anabolic response. This would suggest that insulin resistance impairs net muscle protein balance by reducing the MPS response to nutrient ingestion. Similar findings have been observed in obese, insulin-resistant individuals (Chevalier *et al.*, 2005; Guillet *et al.*, 2009; Murton *et al.*, 2015; Pereira *et al.*, 2008). However, there are reports of 'normalised' anabolic responses in individuals with T2DM in response to insulin and amino acid stimulation (Bassil *et al.*, 2011), although Bassil *et al.* (2011) compared leucine kinetics with an historical lean cohort, meaning these results should be interpreted with caution. Many of the above studies have investigated MPS responses in overweight/obese individuals with T2DM and are therefore unable to disentangle the effects of obesity and insulin resistance *per se*, and can merely suggest that one, or both, of these factors impact upon the ability to

stimulate MPS. In an attempt to address this limitation Stephens *et al.* (2015) induced insulin resistance by way of Intralipid infusion in young, healthy males. In that study, MPS responses to insulin and amino acid stimulation were compared during either 10% Intralipid (100 mL/h), or normal saline infusion. Their results display that mixed-muscle fractional synthetic rate (FSR) increased 2.2-fold in response to insulin and amino acid ingestion during the saline trial, whereas lipid infusion completely suppressed this anabolic response. From these findings the authors conclude that lipid-induced insulin resistance blunts the anabolic response to insulin and amino acid stimulation (Stephens *et al.*, 2015). However, the mechanism by which lipid infusions induce insulin resistance may not be representative of human pathogenesis, and it remains to be seen if the same response is observed in a more physiological model of insulin resistance.

1.3.2 Liver

Similar to the response seen in skeletal muscle, insulin resistance in hepatocytes leads to reduced glucose uptake and glycogen synthesis (Carey *et al.*, 2003; Magnusson *et al.*, 1992) reducing plasma glucose R_d. However, hepatic insulin resistance also results in a reduced ability of insulin to suppress EGP (Bell *et al.*, 1989; DeFronzo *et al.*, 1982; Kelley *et al.*, 1994; Mitrakou *et al.*, 1992; Mitrakou *et al.*, 1990; Singhal *et al.*, 2002; Woerle *et al.*, 2006), attributable to both increased gluconeogenesis (Firth *et al.*, 1986; McMahon *et al.*, 1989; Meyer *et al.*, 2004; Woerle *et al.*, 2006) and hepatic glycogen recycling (glycogenolysis) (Woerle *et al.*, 2006). This reduced suppression of EGP augments the glucose perturbation caused by carbohydrate ingestion, leading to a greater plasma glucose R_a. It has been suggested that reductions in pancreatic α-cell insulin sensitivity leading to increased glucagon secretion are partly responsible for this dysfunctional response (Tsuchiyama *et al.*, 2007).

Evidence suggests that hepatic insulin resistance also contributes to the dyslipidemia commonly observed in insulin resistant individuals (Ginsberg *et al.*, 2005). One of the major abnormalities is hepatic overproduction of VLDL (Adiels *et al.*, 2007; Pramfalk *et al.*, 2016). Paradoxically, the action of insulin on the SREBP1-c pathway remains insulin sensitive even in insulin resistant individuals (Brown, & Goldstein, 2008). This would serve to enhance transcription of both gluconeogenic and lipogenic genes resulting in increased DNL. The induction of DNL would also have the additive effect of reducing fatty acid oxidation as malonyl-CoA (a DNL intermediate) is a potent inhibitor of carnitine palmitoyl transferase

(CPT)-1 (McGarry *et al.*, 1977), which may result in a greater secretion of TG in VLDL and hypertriglyceridemia (Boden, 2006; Hodson *et al.*, 2015; Matikainen *et al.*, 2014).

1.3.3 Adipose tissue

The principal defect seen in adipose tissue insulin resistance is the increased hydrolysis of triglycerides from adipocytes due to the failure of insulin to suppress HSL (Groop *et al.*, 1989; Groop *et al.*, 1991), this can lead to an increase in plasma non-esterifed fatty acids (NEFA) (Ferrannini *et al.*, 1983). It has been proposed that increased NEFA may be a contributing factor to the metabolic abnormalities seen in insulin resistance and T2DM (Lewis *et al.*, 2002). There are also reports that obese insulin resistant individuals may demonstrate a delayed clearance of triglyceride-rich lipoproteins during the postprandial period compared to their healthy weight counterparts, possibly contributing to hypertriglyceridemia and atherosclerosis (Larsen *et al.*, 2015). However, whether this is due to a reduced sensitivity of LPL to insulin remains to be determined.

Adipose tissue is now recognised as an important endocrine organ, secreting a number of operationally active proteins (adipokines) which are thought to be involved in the regulation of whole-body metabolism and neuroendocrine control of feeding related behaviours (for review see Booth *et al.* (2016)). It has been reported that adipokine secretion is altered in the insulin resistant state (Andersson *et al.*, 2016), although it is unclear as to whether this is a cause or a consequence of this disease state.

1.3.4 Pancreatic β-cells

Changes in insulin sensitivity are also associated with reciprocal changes in insulin secretion. In healthy β-cells the adaptive response to insulin resistance involves a hyperbolic increase in insulin secretion (mediated through changes in both β-cell mass and function) in order to compensate for reduced insulin action and maintain glycaemic control (Kahn *et al.*, 1993). A failure in this compensatory response is one of the principal defects in T2DM, as evidenced by the delayed and relatively diminished secretory function of β-cells in these individuals compared to their healthy counterparts (Kahn, 2001a; Kahn, 2001b), and the observation that the ~50% reduction in β-cell mass in T2DM does not sufficiently explain the reduction in insulin secretion (Butler *et al.*, 2003; Kahn *et al.*, 2006; Kloppel *et al.*, 1985). Longitudinal data from the Pima Indians further supports this, demonstrating that the transition from normal, to impaired glucose tolerance, to T2DM is characterised by a progressive loss of β-

cell function (Weyer *et al.*, 1999). Similar observations have been made in other ethnic cohorts (Festa *et al.*, 2006).

1.4 Mechanisms of insulin resistance

The exact mechanisms that lead to insulin resistance in these tissues are not fully understood, and multiple hypotheses have been proposed. These include, ectopic lipid accumulation, increased circulating concentrations of proinflammatory cytokines, and the development of endoplasmic reticulum (ER) and oxidative stress, amongst others. An in depth discussion of each of these hypothesised mechanisms and their contribution to whole-body insulin resistance is beyond the scope of this thesis, and the reader is directed to a number of comprehensive review articles covering this topic (Abdul-Ghani, & DeFronzo, 2010; Duque-Guimaraes, & Ozanne, 2013; Samuel *et al.*, 2010; Samuel, & Shulman, 2012; Samuel, & Shulman, 2016). Briefly, regarding ectopic lipid accumulation, it is clear that obesity is associated with an increase in intramyocellular lipid (IMCL) (Goodpaster *et al.*, 2000; Thamer *et al.*, 2003). A number of previous studies have reported that IMCL content is more tightly correlated with insulin resistance than other risk factors, such as fat mass, circulating lipid levels, and fasting blood glucose (Krssak *et al.*, 1999; Perseghin *et al.*, 1999; Virkamaki *et al.*, 2001). However, others have shown that IMCL content and insulin resistance are completely unrelated (Thamer *et al.*, 2003), or even that IMCL content is inversely related to insulin resistance (Goodpaster *et al.*, 2001; Haus *et al.*, 2011). These confusing reports can be partly explained by the 'exercise paradox', in which endurance training can increase both IMCL content and insulin sensitivity (Goodpaster *et al.*, 2001). Therefore, while obesity and endurance training influence insulin sensitivity in opposite directions, they are both associated with IMCL accumulation. However, although IMCL predominantly reflects triglyceride content, evidence suggests that it is not an accumulation of triglycerides themselves that initiate insulin resistance, but an accumulation of specific fatty acid metabolites (e.g. diacylglycerides (DAGs), fatty acyl-COA and ceramides) (Adams *et al.*, 2004; Chaurasia, & Summers, 2015). It is proposed that accumulation of these lipid species impairs proximal insulin signalling, likely through activation of PKC isoforms which phosphorylate IRS-1 serine residues, inhibiting the normal tyrosine kinase cascade through counter-regulatory serine/threonine phosphorylation and reducing PI3-K activity and skeletal muscle glucose uptake (Zick, 2005). This has previously been demonstrated in a number of iv lipid infusion studies in healthy volunteers (Dresner *et al.*, 1999; Itani *et al.*, 2002; Szendroedi *et al.*, 2014). There is evidence to suggest that this same mechanism of action is

apparent in hepatic insulin resistance (i.e. intracellular accumulation of lipids activating PKC isoforms resulting in impaired insulin signalling) (Kumashiro *et al.*, 2011; Samuel *et al.*, 2004; Samuel *et al.*, 2007). However, further research elucidating the contribution of IMCL accumulation to whole-body insulin resistance in humans is required.

A further hypothesis is that insulin resistance is induced by an increase in proinflammatory cytokines. This comes from the observation that obesity and insulin resistance are both characterised by a low-grade state of inflammation (Hotamisligil, 2006). This inflammatory response differs from the classical model of inflammation in that the principal signs of redness, swelling, pain, and increased basal metabolic rate are not apparent, although a similar set of molecules and signalling pathways are activated (Medzhitov, 2008). The link between obesity, inflammation and insulin resistance seems to be mediated by the adipose tissue itself. Along with being the predominant lipid storage organ, adipose tissue is also accepted as being the largest endocrine organ in the human body, responsible for the secretion of adipokines (e.g. adiponectin and resistin), chemokines (e.g. monocyte chemotactic protein 1 (MCP-1), and interleukin 8 (IL-8)) and proinflammatory cytokines (e.g. interleukin 6 (IL-6), and tumour necrosis factor α (TNF-α)) (Trayhurn, 2005). Excessive growth of adipose tissues leads to adipocyte hypertrophy and a disturbance in the adipocyte secretory profile, leading to an increased secretion of proinflammatory cytokines (Wellen, & Hotamisligil, 2005). It has been shown that an increase in these inflammatory signals (IL-6 and TNF-α in particular) induce insulin resistance via two operationally diverse pathways; jun-N terminal kinase-1 (JNK-1), and Iκ kinase β (IKK-β)/nuclear factor kappa β (NF-κB). JNK-1 has been shown to promote insulin resistance through phosphorylation of serine residues in IRS-1. In contrast, IKKβ liberates NF-κB for translocation into the cell nucleus where it promotes the expression of numerous target genes whose products impair insulin signalling (Boden, 2006; Boden *et al.*, 2005; Krogh-Madsen *et al.*, 2006). However, much of our knowledge regarding inflammation, obesity, and insulin resistance is based correlational data in adult subject groups, meaning a direct causal link is not yet established (Adabimohazab *et al.*, 2016). Furthermore, reductions in whole-body and skeletal muscle insulin sensitivity have previously been observed following short-term dietary intervention studies independently of changes in inflammatory markers (Cornford *et al.*, 2013; Knudsen *et al.*, 2012).

The development of ER and oxidative stress has also been implicated in the pathogenesis of insulin resistance. The ER is an intracellular organelle responsible for the synthesis of polypeptides, and post-translational modification and folding of peptides, along with the synthesis of lipids and sterols. It has been seen that in response to cellular stress (e.g. excess NEFA and glucose), ER function becomes impaired triggering a security mechanism known as the "unfolded protein response" (UPR). This has been seen in cultured adipocytes (Guo *et al.*, 2007), liver cells (Wei *et al.*, 2006), and pancreatic β-cells (Karaskov *et al.*, 2006; Kharroubi *et al.*, 2004) . The purpose of the UPR response is to regulate the expression of genes in order to alleviate the stress response. This occurs via activation of three molecular components; inositol-requiring protein 1 (IRE-1), activating transcription factor-6, and double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (Ron, & Walter, 2007). However, the UPR response is also associated with an increase in inflammatory signals including IL-6 and TNF-α, alongside an increase in oxidative stress (Dali-Youcef *et al.*, 2013). Oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS), and antioxidant defences (Betteridge, 2000). It has been proposed that increases in ROS activates NF-κB, and inhibits insulin signalling as described above (Chung *et al.*, 2009). However, the increase of proinflammatory cytokines, ER, and oxidative stress seem to occur in line with enlargements in adiposity, whereas substantial reductions in insulin sensitivity are observed with relatively minor increases in body mass.

The fact at least three distinct hypotheses for the development of insulin resistance exist is evidence in itself of the lack of clarity regarding its pathogenesis, and the literature is awash with conflicting and at times confusing reports. Moreover, it is currently not known if defects in one of the tissues primarily involved in glucose metabolism (i.e. skeletal muscle and liver) precede that of the other. Determining the initial impairments that may promote whole-body insulin resistance (i.e. the time course of changes in tissue specific insulin sensitivity) will allow for more targeted mechanistic investigations and evidence based prevention strategies.

1.5 Studying the development of insulin resistance

A commonly employed method in the investigation of metabolic disease is the use of experimental animal models such as diet-induced obese rodents (Buettner *et al.*, 2007; Heydemann, 2016; Winzell, & Ahren, 2004). However, whilst information from these animal models is insightful, they are at best 'predictive' and not 'representative' of human responses, and as such any results should be treated with caution (Shanks *et al.*, 2009). In humans, due

to ethical considerations, acute intervention studies such as iv lipid-heparin infusions or short-term dietary interventions are often performed. These are intended to mimic the transition towards obesity and reduce insulin sensitivity/impair glycaemic control.

Lipid infusion protocols involve the iv infusion of a triglyceride emulsion in combination with heparin in order to artificially induce intravascular lipolysis (Lee *et al.*, 1988). In healthy subjects, this method rapidly (within 3-4 h) reduces whole-body insulin-stimulated glucose disposal, associated with a sequential pattern of events starting with an increase in circulating triglyceride levels (< 0.5 h), followed by a supraphysiological increase in circulating NEFA levels (~1-1.5 h), a rise in IMCL (~ 2.5 h), and finally a reduction in insulin stimulated glucose disposal (~3-5 h) (Boden, & Jadali, 1991; Roden *et al.*, 1996; Szendroedi *et al.*, 2014). This method was developed based on the longstanding notion that enlarged adipose tissue mass results in elevated plasma NEFA levels, and has thus provided a hypothetical mechanistic link between obesity and insulin resistance (Eckel *et al.*, 2005). However, while it is true that acute elevations in plasma NEFA induce insulin resistance, the link between circulating NEFA concentrations and insulin resistance has been questioned in a recent review of the literature by Karpe *et al.* (2011). The findings of this review suggest that increased circulating NEFA is not simply determined by an increase in fat mass, and insulin resistance can occur without elevations in NEFA. Furthermore, when pooling data from a number of their own studies where arterio-venous measures were obtained, Karpe *et al.* (2011) observed that the relative release of NEFA per kg of adipose tissue actually decreases as adipose tissue mass increases, which in some obese individuals can actually lead to a normalisation of NEFA levels. In addition, no association was observed when NEFA concentrations were examined in relation to fasting insulin concentrations. This evidence casts doubt on the NEFA hypothesis of insulin resistance and the physiological relevance of lipid infusion protocols, and indirectly suggests that other methods, such as high-fat diet and/or overfeeding interventions, may be a more valid representation of the early-phase progression of insulin resistance in humans.

1.6 Metabolic responses to short-term high-fat overfeeding

There is a plenitude of research investigating the impact of high-fat overfeeding in animal models, whereas human data is relatively sparse. Of the available literature it is clear that even short-term (3-28 days) high-fat overfeeding (defined as hypercaloric diets where fat contributes >35% total energy) is associated with negative alterations in glucose metabolism

in healthy, lean subjects, including reductions in whole-body insulin sensitivity and impairments in glycaemic control (Hulston *et al.*, 2015; Samocha-Bonet *et al.*, 2010; Tam *et al.*, 2010; Wulan *et al.*, 2014), increased EGP (Brons *et al.*, 2009) and defects in skeletal muscle insulin signalling (Adochio *et al.*, 2009). However, where impairments in glucose metabolism have been observed it would be satisfying to know the process underpinning these responses; very few of the studies included above provide any mechanistic insight. Information is particularly sparse with regards the tissue-specific responses to short-term high-fat overfeeding, and it remains to be seen if the developmental time-course of insulin resistance differs in a tissue-specific manner.

Evidence from animal studies suggest that alterations in tissue-specific insulin sensitivity in response to high-fat overfeeding are sequential in nature. For instance, Kleemann *et al.* (2010) employed a time-resolved approach whereby mice fed a high-fat diet were assessed for glucose tolerance at baseline and following 1, 6, 9 and 12 weeks of overfeeding, while insulin sensitivity was assessed by hyperinsulinemic-euglycaemic clamps at baseline, and following 6 and 12 weeks of feeding. As expected, animals displayed a gradual increase in body weight, which reached significance at 6 weeks and continued to increase until week 12, with a similar trend observed for adipose tissue mass in all depots (subcutaneous, visceral, and epididymal). Glucose tolerance was impaired after 1 week of high-fat feeding and gradually worsened with continuation of the diet. The use of radioactive glucose tracers during the hyperinsulinemic-euglycaemic clamps allowed the authors to distinguish between the development of insulin resistance in liver, adipose tissue, and muscle. Their findings indicate that high-fat overfeeding reduced hepatic insulin sensitivity at 6-weeks leading to a reduced suppression of EGP under insulin-stimulated conditions, whereas adipose tissue did not display signs of insulin resistance (i.e. reduced glucose uptake) until 12 weeks of feeding in these animals. No differences in skeletal muscle insulin sensitivity were observed in that study. This data demonstrates that, in mice, hepatic insulin resistance is rapidly induced by high-fat overfeeding and has profound consequences for whole-body glycaemic control, whereas complications in adipose tissue and skeletal muscle take longer to develop. This finding is supported by others (Chisholm, & O'Dea, 1987; Kim *et al.*, 2003; Kraegen *et al.*, 1991; Samuel *et al.*, 2004; Samuel *et al.*, 2007).

It is plausible that similar responses to those seen in animals regarding tissue-specific insulin sensitivity would also be observed in humans. However, there are only a few studies to date

which have investigated tissue-specific insulin sensitivity in response to high-fat overfeeding, and the findings of these studies are far from conclusive. Brons *et al.* (2009) subjected lean men to 5-days of high-fat (65% total energy) overfeeding (50% caloric excess). They demonstrated no change in insulin stimulated glucose disposal during a hyperinsulinemic-euglycaemic clamp, but did observe a 26% increase in fasting EGP. This finding would suggest that changes in hepatic insulin sensitivity precede (and possibly mediate) changes at the whole-body level. The authors replicated these findings in 2012 (Brons *et al.*, 2012). In that study the authors report reduced hepatic insulin sensitivity and increased EGP in young males born with a normal-birth weight after 5-days of high-fat overfeeding. Further support for the liver being the predominant site of metabolic dysfunction following high-fat overfeeding comes from Bisschop *et al.* (2001); a high-fat diet led to an a reduction in the suppressive effect of insulin on EGP but no change in insulin mediated glucose disposal (Bisschop *et al.*, 2001). Intriguingly, when comparing their findings in males born with a normal-birth weight to those with a low-birth weight, Brons *et al.* (2012) demonstrated that 5-days of high-fat overfeeding induced whole-body insulin resistance which was attributable to impaired skeletal muscle glucose storage in the low-birth weight cohort (Brons *et al.*, 2012). The finding of defects in skeletal muscle storage are in accordance with those reported by Adochio *et al.* (2009) when overfeeding (40% caloric excess) lean, healthy men and women a high-fat diet (50% total energy) for 5 days. Adochio *et al.* (2009) saw no change in clamp-derived measures of insulin sensitivity when comparisons were made against an isocaloric control diet, but, unlike Brons *et al.* (2009), these authors saw no change in basal EGP either. Despite this, high-fat overfeeding was found to impair skeletal muscle insulin signalling, as evidenced by an increase in serine phosphorylation of IRS-1, and increased total expression of p85α, alterations which are generally associated with skeletal muscle insulin resistance. These discrepant findings highlight the need for further investigation.

Notably, (Hulston *et al.*, 2015) observed a significant increase in the postprandial glucose response to an oral glucose load alongside a non-significant increase in postprandial insulin concentrations following 7-days of high-fat overfeeding. This insufficient compensatory response of insulin potentially indicates that high-fat overfeeding also impairs pancreatic β-cell function. Furthermore, as a negative association appears to exist between increased fasting glucose concentrations and impaired β-cell function, even in those who display glucose levels within the normal range (Utzschneider *et al.*, 2006) the observation of increased fasting plasma glucose levels in Hulston *et al.* (2015), Brons *et al.* (2012) and

Brons *et al.* (2009) further supports this hypothesis. A reduced secretory capacity of pancreatic β-cells has previously been observed in mice subjected to high-fat feeding (Ohtsubo *et al.*, 2011). A potential avenue through which insulin secretion might be impaired is through the reduced sensitivity of β-cells to the incretins hormones. *In vitro* evidence demonstrates that chronic exposure to elevated NEFA disrupts intercellular β-cell communication, impeding the propagation of signals through GLP-1–sensitive pathways (Hodson *et al.*, 2013). It is plausible that insulin resistance of the adipose tissue leads to elevated NEFA that elicit lipotoxic effects on β-cells (Hodson *et al.*, 2013). Patients with T2DM are known to have a diminished meal-induced secretion of GLP-1 (Toft-Nielsen *et al.*, 2001; Vilsboll *et al.*, 2001; Yu *et al.*, 2002) and are resistant to the insulinotropic actions of GIP (Nauck *et al.*, 1986; Nauck *et al.*, 1993; Vilsboll *et al.*, 2002). However, there is very little data concerning the incretin response to high-fat overfeeding in humans. Of the available literature it would appear that the GLP-1 response is well-maintained in response to short-term high-fat overfeeding, whereas fasting GIP is increased (Brons *et al.*, 2012; Brons *et al.*, 2009). This increase in GIP potentially represents an adaptive response in order to compensate for a reduction in β-cell function. However, the author is only aware of two studies which have investigated the incretin response to high-fat overfeeding (Brons *et al.*, 2012; Brons *et al.*, 2009), highlighting the need for further studies.

A further avenue that warrants investigation is the speed at which high-fat overfeeding impairs glucose metabolism; evidence suggests that diet-induced impairments may occur very rapidly. Nowotny *et al.* (2013) reported that oral administration of a single dose of soybean oil (100 mL), which is enriched with polyunsaturated fat (61% polyunsaturated (PUFA), 23% monounsaturated (MUFA), and 16% saturated (SFA)) reduced whole-body insulin sensitivity (assessed by hyperinsulinemic-euglycemic clamp) to a comparable extent and within a similar time-frame (6 h post ingestion/infusion) as an energy- and composition-matched iv lipid-heparin infusion. Insulin sensitivity was assessed 6-8 hours after fat ingestion/infusion (Nowotny *et al.*, 2013), and it is possible that the observed reduction in insulin sensitivity was a transient response related to the ongoing metabolism of fat; it would be of interest to determine if changes persist into the postabsorptive state and occur after consumption of a diet more reflective of Western style eating patterns (i.e. SFA rather than PUFA enriched).

1.7 Conclusions

Short-term (3-28 days) high-fat overfeeding impairs whole-body insulin sensitivity/glycaemic control in healthy, lean individuals prior to substantial gains in body mass/fat (Hulston *et al.*, 2015; Samocha-Bonet *et al.*, 2010; Tam *et al.*, 2010; Wulan *et al.*, 2014). At present, the mechanisms underpinning these processes, and the time-course of development, are not fully understood. In particular, it is currently unclear if impairments in glucose metabolism develop in specific tissues at different rates, and if so which tissues are the first to respond. Based on the majority of studies in animals and humans (Brons *et al.*, 2012; Brons *et al.*, 2009; Chisholm, & O'Dea, 1987; Kim *et al.*, 2003; Kraegen *et al.*, 1991), we would hypothesise that changes in hepatic insulin sensitivity occur prior to, and may mediate, changes in skeletal muscle/whole-body insulin sensitivity. Determining the tissue-specific metabolic alterations to high-fat feeding may reveal important insights regarding the development of insulin resistance and T2DM, and aid the development of future mechanistic investigations and evidence-based prevention strategies. Furthermore, it is currently unclear as to whether the gut hormones play a role in the early metabolic derangements to high-fat overfeeding. Based on the available literature we would postulate that high-fat overfeeding does not influence the gut hormone response to nutrient ingestion, although this hypothesis is based on extremely limited data. Additionally, establishing the speed at which high-fat overfeeding elicits metabolic alterations is important as individuals commonly adopt similar eating strategies during holiday periods or times of celebration. While it has been seen that only 3 days of high-fat overfeeding are required to elicit deleterious alterations in glycaemic control, we would hypothesise that these alterations occur earlier than 3-days and may be apparent after only a single day of excessive fat consumption. Reductions in the MPS response to anabolic stimuli have previously been observed in obese, insulin resistant individuals (Chevalier *et al.*, 2005; Guillet *et al.*, 2009; Murton *et al.*, 2015; Pereira *et al.*, 2008), although it is unclear if this response is driven by insulin resistance, excess adiposity or some hitherto unknown mechanism. Based the findings of (Stephens *et al.*, 2015) in young, healthy, lean individuals we would hypothesise that insulin resistance *per se* is associated with a blunted MPS response; it remains to be determined if similar responses are seen following diet-induced reductions in insulin sensitivity. Any impairments in MPS would have profound consequences for both metabolic health and quality of life in general due to the central role of skeletal muscle in energy balance and glycaemic control, along with mobility and stability (Wolfe, 2006b).

Our laboratory has recently demonstrated that 7-days of high-fat (65% total energy) overfeeding (50% energy excess) led to a significant increase in fasting glucose, and a reduction in whole-body insulin sensitivity (as measured by Matsuda insulin sensitivity index) during an oral glucose tolerance test in young healthy subjects (Hulston *et al.*, 2015). This reduction in insulin sensitivity was observed despite minimal weight gain (0.6 ± 0.2 kg), and with a diet that consisted of realistic experimental meals typical to that seen in Western cultures (i.e. high in saturated fat). We therefore believe that this model represents an ideal platform from which to base future investigations into the early-phase responses involved in the development of insulin resistance.

1.8 Aims and objectives.

1 To corroborate and further characterise a model of short-term, high-fat overfeeding, previously shown by our laboratory to reduce whole-body insulin sensitivity in young, healthy, lean individuals.

2 To investigate tissue-specific insulin sensitivity in response to diet-induced impairments in glycaemic control.

3 To delineate the developmental time-course of diet-induced impairments in whole-body insulin sensitivity.

4 To determine whether diet-induced impairments in glycaemic control impair the anabolic response to nutrient stimulation in young, healthy, lean individuals.

CHAPTER 2

Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal-weight individuals.

[1]This chapter has been accepted for publication (2017)

[1]Parry, S. A., Smith, J. R., Corbett, T. R., Woods, R. M. & Hulston, C. J. (2017). Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal-weight individuals. *Br J Nutr*, **117,** 48-55.

2.1 Abstract

Obesity is undoubtedly caused by a chronic positive energy balance. However, the early metabolic and hormonal responses to overnutrition are poorly described. This study determined glycaemic control and selected gut hormone responses to nutrient intake before and after seven days of high-fat overfeeding. Nine healthy individuals (5 males, 4 females) performed a mixed meal tolerance test (MTT) before and after consuming a high-fat (65%) high-energy (+50%) diet for seven days. Measurements of plasma glucose, NEFA, acylated ghrelin, GLP-1, GIP and serum insulin were taken before (fasting) and at 30 minutes intervals throughout the 180 min MTT (postprandial). Body mass increased by 0.79 ± 0.14 kg after high-fat overfeeding ($p < 0.0001$), and BMI increased by 0.27 ± 0.05 kg/m^2 ($p = 0.002$). High-fat overfeeding also resulted in an 11.6% increase in postprandial glucose AUC ($p = 0.007$) and a 25.9% increase in postprandial insulin AUC ($p = 0.005$). Acylated ghrelin, GLP-1 and GIP responses to the MTT were all unaffected by the high-fat, high-energy diet. These findings demonstrate that even brief periods of high-fat food intake are sufficient to disrupt glycaemic control. However, as the postprandial orexigenic (ghrelin) and anorexigenic/insulintropic (GLP-1 and GIP) hormone responses were unaffected by the diet intervention, it appears that these hormones are resistant to short-term changes in energy balance, and that they do not play a role in the rapid reduction in glycaemic control.

2.2 Introduction

Changes in human behaviour, such as excessive food intake and/or insufficient physical activity, have made obesity a worldwide epidemic (Zimmet *et al.*, 2001). Furthermore, obesity is a significant risk factor for the development of insulin resistance and type 2 diabetes mellitus (T2DM). However, despite the well-known association between obesity and insulin resistance, obesity may not trigger early metabolic dysfunction as negative alterations in glucose metabolism are often reported before substantial gains in body mass are observed. For example, recent human studies report that even brief periods (5-14 days) of high-fat food intake can impair skeletal muscle insulin signalling (Adochio *et al.*, 2009), and reduce both hepatic (Brons *et al.*, 2009) and whole-body insulin sensitivity (Cornford *et al.*, 2013; Hulston *et al.*, 2015). In each of these studies the experimental diets provided an excess of energy as well as a high proportion of fat, and it is not yet clear if the observed impairments in glycaemic control are a result of the additional energy, the high fat content of the diets provided, or a combination of the two. Likewise, the effect of overfeeding with mixed composition diets remains unknown. However, an overconsumption of carbohydrate-rich foods (5 days; +40% energy intake; 60% of energy from carbohydrate) has been reported to enhance skeletal muscle insulin signalling, evidenced by increased tyrosine phosphorylation of insulin receptor-1 (IRS-1) as well as increased IRS-1-associated phosphatidylinositol 3 (PI 3)-kinase activity, whereas high-fat overfeeding (5 days; +40% energy intake; 50% of energy from fat) in the same subjects was found to increase serine phosphorylation of IRS-1 and total expression of p85α (Adochio *et al.*, 2009). Hence it would seem that a lipid overload explains the reduction in insulin sensitivity, rather than a positive energy balance alone. This also fits with the hypothesis that it is an accumulation of reactive intramyocellular lipid species, such as ceramide and diacylglycerol, that inhibits skeletal muscle insulin signalling and impairs GLUT4 translocation (Samuel, & Shulman, 2012; Yu *et al.*, 2002).

Of the previous literature, there has been considerable interest in identifying the molecular mechanisms for peripheral (skeletal muscle) insulin resistance. However, whole-body glycaemic control is coordinated by a variety of integrated physiological processes, involving multiple hormones and their target tissues, and the effects of high-fat food intake on these hormonal responses have received relatively little attention to date. Of particular interest are the two primary incretin hormones: glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). These two hormones are secreted from the intestines in response to nutrient ingestion and it is suggested that they act to control blood glucose levels by

enhancing insulin secretion, suppressing glucagon release and slowing gastric emptying (DeMarco, & Sowers, 2015). Patients with T2DM are known to have a diminished meal-induced secretion of GLP-1 (Toft-Nielsen *et al.*, 2001; Vilsboll *et al.*, 2001; Yu *et al.*, 2002). Not only this, but they can also become resistant to the insulinotropic actions of GIP (Nauck *et al.*, 1986; Nauck *et al.*, 1993; Vilsboll *et al.*, 2002). This loss of an incretin effect may be an important contributor to postprandial hyperglycaemia in T2DM (Holst *et al.*, 2011). Evidence for this also comes from the effective use of GLP-1 receptor agonists and dipeptidyl peptidase (DPP)-IV inhibitors in the treatment of hyperglycaemia (Drucker, 2003; Kountz, 2013).

Another gut hormone of interest is ghrelin, which is primarily secreted by the P/D1 cells lining the fundus of the stomach, and is thought to stimulate hunger via the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurones of the hypothalamus (Murphy, & Bloom, 2006). Ghrelin levels are elevated during fasting and reduced following feeding (Cummings *et al.*, 2001), and ghrelin infusion has been shown to stimulate food intake in both animals (Wren *et al.*, 2001b) and humans (Wren *et al.*, 2001a) alike. In healthy, normal weight individuals, ghrelin levels decrease in proportion to the energy content of the meal (Callahan *et al.*, 2004), whereas obese individuals exhibit both lower fasting levels (Cummings *et al.*, 2002; le Roux *et al.*, 2005; Tschop *et al.*, 2001) and reduced suppression following food intake (English *et al.*, 2002; le Roux *et al.*, 2005).

While the derangements in ghrelin and GLP-1 secretion have been reported in situations of chronic positive energy balance (i.e. obesity) and metabolic disease (i.e. insulin resistance), it is not yet clear whether the reported changes contribute to the development of obesity and insulin resistance, or are consequent of the disease state itself. Therefore, the primary purpose of this study was to determine whether short-term, high-fat overfeeding, an experimental model which impairs whole-body insulin sensitivity, influences gut hormone responses to fasting and feeding. High-fat foods were chosen for the overfeeding intervention due to the frequent use of this model in both animal and human studies of metabolic disease.

2.3 Materials and Methods

2.3.1 Subjects

Nine healthy individuals (5 males and 4 females; their physical characteristics can be seen in Table 2.1) volunteered to participate in this study. The sample size was based on pilot data from our laboratory in which the effect size (Cohens' *d*) of high-fat overfeeding on glycaemic control was calculated as 0.9 (i.e. a large effect). Assuming a similar effect size in this study, α error probability of 0.05 and statistical power of 0.8, a sample size of at least 5 participants was required. The inclusion criteria required subjects to be physically active (performing moderate to vigorous intensity exercise at least 3 times per week for more than 30 minutes at a time), non-smokers, free from cardiovascular and metabolic disease, not taking any medication, weight stable for at least 6 months, and with a body mass index (BMI) between 19-25 kg/m^2). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Loughborough University Ethical Subcommittee for human participants. The experimental procedures and possible risks were fully explained to the subjects before their written informed consent was given.

Table 2.1 Subject characteristics before and after 7 days of high-fat overfeeding

Characteristics	**Before overfeeding**	**After overfeeding**
Age (years)	23 ± 1	-
Height (cm)	171.6 ± 2.0	-
Body mass (kg)	65.6 ± 2.1	66.3 ± 2.0 *
BMI (kg/m^2)	22.3 ± 0.6	22.5 ± 0.6 *

Data presented are means ± SEM (n = 9). * denotes significant change following the dietary intervention ($p < 0.05$).

2.3.2 Pre-testing

Prior to the start of the study, subjects attended the laboratory for an initial assessment of their baseline anthropometric characteristics (height, weight and BMI). This information was then used to estimate their resting energy expenditure (REE) according to the calculations described by Mifflin *et al.* (1990). A standard correction for physical activity level (1.6 and 1.7 times REE for females and males, respectively) was applied in order to estimate total

daily energy requirements. This information was then used to determine individual energy intakes for the week-long overfeeding period (diet details described later).

2.3.3 Experimental design

After the initial pre-testing visit, subjects attended the laboratory for a mixed meal tolerance test (MTT) (details of which can be seen in the experimental protocol below). Subjects were then provided with all food to be consumed for the following 7 days. The experimental diet was designed to be high in fat (65% total energy) and provide a severe energy excess (+50% kJ). Individual diet plans were designed using NetWISP nutrition software (Tinuviel Software Ltd, UK). All foods were purchased and prepared by the research team and subjects were instructed to consume all food provided and to avoid consuming additional food or nutritive beverages. Food intake followed a normal daily feeding pattern (i.e., breakfast, lunch, dinner and snacks) and water intake was allowed ad libitum throughout the dietary intervention. Foods such as processed meats, dairy products, and pastries were used extensively throughout the diet intervention, and cooking instructions required subjects to fry foods where possible and to avoid wasting any fat left over from the cooking process. Mean energy and macronutrient intake during the intervention period can be seen in Table 2.2 and a detailed example of typical daily food intake can be seen in Table 2.3. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fats made up 46 ± 0.9%, 37 ± 0.6%, and 9 ± 0.4% of the fat intake, respectively. Upon completion of the 7-day overfeeding period, subjects returned to the laboratory for a second MTT.

Table 2.2 Estimated daily energy requirement and actual energy and macronutrient intake during the high-fat overfeeding period

	Estimated energy requirement	**Self-reported habitual intake**	**Experimental energy intake**
Energy (kJ)	10717 ± 481	8593 ± 749	16075 ± 722 *†
Fat (g)	-	74 ± 10	277 ± 12 †
Carbohydrate (g)	-	263 ± 23	211 ± 9 †
Protein (g)	-	100 ± 12	125 ± 6 †

Data presented are means ± SEM (n = 9). * denotes significantly different to estimated energy requirement ($p < 0.05$). † denotes significantly different to reported intake ($p < 0.05$)

2.3.4 Diet records, physical activity and compliance during high-fat overfeeding

During the pre-testing visit, subjects were provided with standardised forms and digital kitchen scales for the purpose of recording weighed food intake for 3-5 days prior to the first main trial. Subjects also received detailed written and verbal instructions on how best to complete these records. However, due to the well-known issues with self-reporting of energy intake (Dhurandhar *et al.*, 2015), especially underreporting of food intake (Goris *et al.*, 2000; Macdiarmid, & Blundell, 1998; Salle *et al.*, 2006), even amongst lean and very well-motivated subjects (Goris, & Westerterp, 1999), it was decided that estimated energy requirements would provide a better overall baseline from which to design and implement the overfeeding intervention.

Subjects were expected to eat all of the food provided, and the importance of this was made explicitly clear to them during initial consultation and recruitment, but were told to report and return any uneaten foods so that our calculations could be adjusted if need be. In order to improve diet compliance, subjects were asked to complete a food preferences checklist to ensure that they only received foods that they were willing to eat; thereby increasing the palatability of the diet. Subjects were also given a copy of their diet plans and asked to tick off individual foods/meals as they were consumed. Adherence to the diet was assessed by daily interviews that were conducted when subjects collected their food bundles. Only one subject reported any issues with the diet, and they returned part of an uneaten steak and ale pie from one of the meals. Other than this we are confident that the diet was followed; as evidenced by a consistent weight gain in all subjects.

All subjects participated in physical activity on a regular basis and were required to continue this throughout the overfeeding period. The written information and verbal instructions stated that subjects should expect to gain a small amount of weight and that they should not attempt to offset the additional energy intake by exercising longer, harder or more frequently.

Table 2.3 Example food intake for 1 day of high-fat overfeeding

Breakfast	
Foods	3 large pork sausages (175 g), 4 rashers of streaky bacon (80 g), 2 large fried eggs (120 g), 1 medium slice of fried white bread (36 g), whole milk (300 mL)
Protein (g)	61
Carbohydrate (g)	47
Fat (g)	93
Energy (kJ)	5277
% of the days intake	31
Lunch	
Foods	2 slices of medium white bread (72 g), butter (15 g), cheddar cheese (70 g), mayonnaise (15 g)
Protein (g)	27
Carbohydrate (g)	36
Fat (g)	47
Energy (kJ)	2810
% of the days intake	16
Snack	
Foods	Potato crisps (50 g), milk chocolate bar (49 g)
Protein (g)	7
Carbohydrate (g)	55
Fat (g)	32
Energy (kJ)	2238
% of the days intake	13
Dinner	
Foods	2 beef burgers (200 g), 4 rashers of streaky bacon (80 g), cheddar cheese (60 g), coleslaw (100 g)
Protein (g)	63
Carbohydrate (g)	5
Fat (g)	115
Energy (kJ)	5411

% of the days intake	31
Dessert	
Foods	Chocolate sundae (140 g)
Protein (g)	4
Carbohydrate (g)	37
Fat (g)	21
Energy (kJ)	1474
% of the days intake	9
Total intake	
Protein (g)	162
Carbohydrate (g)	180
Fat (g)	308
Energy (kJ)	17210

Reported values are from a single subjects' food intake on 1 day of the overfeeding intervention. Water intake was allowed ad libitum.

2.3.5 Experimental protocol

On the experimental days (before and after overfeeding), subjects reported to the laboratory between 07.00 and 09.00 h after an overnight fast of at least 10 h and having refrained from physical activity for 48 h. After voiding and being weighed, a 20 gauge Teflon catheter (Venflon, Becton, Dickinson, Plymouth, UK) was inserted into an antecubital vein of one arm to allow for repeated blood sampling during the 3 h MTT. A baseline, fasting blood sample (12.5 mL) was obtained before consumption of a standardised breakfast test meal (MTT). The MTT consisted of 45 g Rice Krispies, 72 g white bread (toasted), 20 g butter, 30 g strawberry jam and 300 mL whole milk. The energy intake and macronutrient composition of the test meal was 3227 kJ; 30 g fat, 112 g carbohydrate, and 19 g protein. Upon finishing the meal, further blood samples of 12.5 mL were obtained at 30, 60, 90, 120, 150 and 180 min.

2.3.6 Blood sampling

For analysis of glucose, non-esterified fatty acids (NEFA), triglyceride (TG), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), GLP-1 and GIP, whole blood samples were collected in 4.9 mL ethylenediaminetetraacetic acid (EDTA; 1.75

mg/mL) treated tubes (Sarstedt, Leicester, UK) and spun at 1,750 *g* in a refrigerated centrifuge (4°C) for 10 min. The resulting plasma was aliquoted into 1.5 mL Eppendorfs before being stored at -20°C until analysis. For analysis of insulin, whole blood was collected in 4.5 mL tubes containing a clotting catalyst (Sarstedt, Leicester, UK). Samples were left at room temperature until complete clotting had occurred; after which they were centrifuged at 1,750 *g* for 10 min. The resulting serum was then aliquoted into 1.5 mL Eppendorfs and stored at -20°C until analysis. Finally, to prevent the degradation of acylated ghrelin, a 25 µL solution containing potassium phosphate buffer (PBS), p-hydroxymercuribenzoic acid (PHMB) and sodium hydroxide (NaOH) was mixed thoroughly with 2.5 mL of whole blood in 2.5 mL EDTA treated tubes. Samples were then centrifuged at 1,750 *g* for 10 min after which 500 µL of the resulting supernatant was removed and added to 50 µL of 1 M hydrochloric acid. Acidified samples were centrifuged for a further 5 min at 1,750 *g* before being stored at -20°C until analysis.

2.3.7 Analytical procedures

Plasma samples were analysed using commercially available spectrophotometric assays for glucose, triglyceride, HDL, LDL, total cholesterol (Horiba Medical, Northampton, UK) and NEFA (Randox, County Antrim, UK) concentrations using a semi-automatic analyser (Pentra 400; Horiba Medical, Northampton, UK). The coefficient of variation (CV) for plasma glucose, triglyceride, HDL, LDL, total cholesterol and NEFA was 0.5, 3.0, 1.6, 0.5, 0.3 and 4.1%, respectively. Serum insulin concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA: EIA-2935, DRG instruments GmBH, Germany) and the CV was 2%. Acylated ghrelin concentrations were determined using an ELISA (EIA-A05106, SPI BIO, France) and the CV was 16%. Total plasma GLP-1 and GIP concentrations were also determined via ELISA (EZGLP1T-36K and EZHGIP-54K, respectively; Merck Millipore, Darmstadt, Germany). The CV was 7% for GLP-1 and 5% for GIP. To eliminate inter-assay variation, samples from each participant were analysed in the same run.

2.3.8 Calculations and statistics

Area under the curve (AUC) for glucose and insulin was calculated using the trapezoidal rule with zero as the baseline. Data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS (V21.0) for windows (SPSS Inc, Chicago, IL). Paired t-tests were used to compare differences in body mass, BMI, and fasting metabolic responses before and after overfeeding, whereas the dynamic hormonal and metabolic

responses to the MTT were compared using a two-way (trial x time) repeated measures analysis of variance (ANOVA) and Bonferroni *post hoc* analysis where appropriate. Statistical significance was set at $p < 0.05$.

2.4 Results

2.4.1 Weight gain and BMI

All nine subjects gained body mass following 7 days of high-fat overfeeding (increasing by 0.79 ± 0.14 kg; $p < 0.0001$, Table 2.1), and their BMI increased by 0.27 ± 0.05 kg/m^2 ($p = 0.002$; Table 2.1).

2.4.2 Fasting plasma metabolites

Fasting substrate, hormone and lipoprotein concentrations before and after high-fat overfeeding are presented in table 2.4. Fasting plasma glucose, HDL and GIP increased following overfeeding ($p = 0.025$, $p = 0.012$ and $p = 0.017$, respectively), while fasting plasma TG and NEFA decreased ($p = 0.039$ and $p = 0.023$, respectively). Fasting serum insulin, plasma acylated ghrelin, LDL, total cholesterol, and GLP-1 were all unaffected by high-fat overfeeding.

Table 2.4 Fasting plasma substrate and hormone concentrations before and after 7-days of high-fat overfeeding

	Before overfeeding	**After overfeeding**
Glucose (mmol/L)	5.5 ± 0.1	5.8 ± 0.1 *
Insulin (pmol/L)	67 ± 8	79 ± 9
NEFA (mmol/L)	0.60 ± 0.05	0.40 ± 0.06 *
Triglyceride (mmol/L)	1.0 ± 0.1	0.7 ± 0.1 *
Total cholesterol (mmol/L)	4.0 ± 0.2	4.0 ± 0.2
HDL (mmol/L)	1.3 ± 0.1	1.5 ± 0.1 *
LDL (mmol/L)	1.8 ± 0.2	1.8 ± 0.1
Acylated ghrelin (pmol/L)	318 ± 57	268 ± 39
GLP-1 (pmol/L)	31 ± 4	31 ± 4
GIP (pmol/L)	22 ± 2	36 ± 6 *

Data presented are mean ± SEM (n = 9). * denotes significant change following the dietary intervention ($p < 0.05$)

2.4.3 Mixed meal tolerance test

Substrate and hormone responses to the 3 hour MTT are presented in figure 2.1. Plasma glucose and serum insulin concentrations increased in response to the MTT, peaking 30 min

after meal ingestion. Seven days of high-fat overfeeding increased plasma glucose AUC by 11.6% (from 1020 ± 74 mmol/L per 180 min before overfeeding to 1138 ± 56 mmol/L per 180 min after overfeeding; $p = 0.007$, figure 2.1a) and serum insulin AUC by 25.9% relative to baseline (from 53267 ± 6375 pmol/L per 180 min before overfeeding to 67046 ± 6849 pmol/L per 180 min after overfeeding; $p = 0.005$, figure 2.1b). Plasma NEFA concentrations decreased following food consumption. However, there was a more pronounced meal-induced suppression of plasma NEFA before high-fat overfeeding than afterwards ($p < 0.0001$, figure 2.1c). Plasma acylated ghrelin concentrations decreased rapidly following food consumption ($p < 0.0001$, figure 2.1d), reaching a nadir at the 60 min sample point and remaining supressed throughout the entire postprandial measurement period. This response was not influenced by high-fat overfeeding. Plasma GLP-1 concentrations peaked 30 min after food ingestion, returning to fasting levels thereafter, with no difference before and after high-fat overfeeding (figure 2.1e). Plasma GIP concentrations increased approximately 3-fold immediately following food consumption and remained elevated throughout the 3 h MTT ($p < 0.0001$), but again this response was not influenced by adherence to the high-fat, high-energy diet (figure 2.1f).

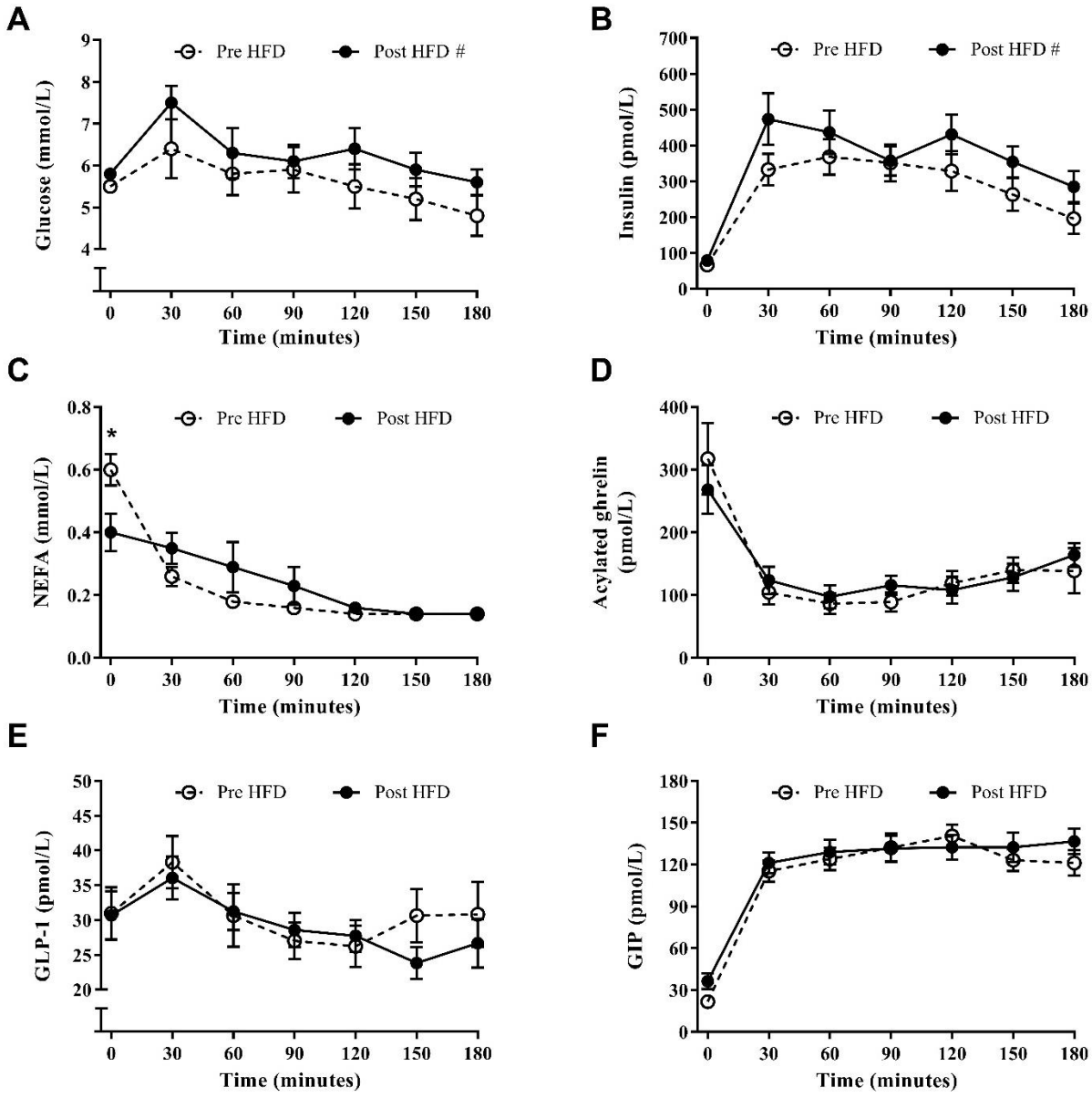

Figure 2.1 Plasma glucose (A), serum insulin (B), plasma NEFA (C), plasma acylated ghrelin (D), total plasma GLP-1 (E), and total plasma GIP (F) concentrations during a 3 hour meal tolerance test conducted before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Data presented are mean ± SEM (n = 9). # denotes significant main effect of trial/high-fat overfeeding ($p < 0.05$). * denotes significant difference between trials at the annotated time point ($p < 0.05$).

2.5 Discussion

The main finding of the present study was that postprandial responses of selected gut hormones (acylated ghrelin, GLP-1 and GIP) were unaffected by short-term, high-fat overfeeding, and that only fasting levels of GIP were altered (increased) as a result of the dietary intervention. A secondary finding was that excessive consumption of high-fat foods impaired glycaemic control, as evidenced by a significant increase in postprandial glucose and insulin AUC.

The incretin hormones, GLP-1 and GIP, are thought to be responsible for the augmentation of insulin secretion that occurs after food intake compared with intravenous nutrient administration. We chose to investigate the impact of short-term, high-fat overfeeding on meal-induced GLP-1 and GIP responses as patients with T2DM exhibit a reduced GLP-1 secretion following nutrient ingestion (Toft-Nielsen *et al.*, 2001; Vilsboll *et al.*, 2001) and may become resistant to the insulinotropic actions of GIP (Nauck *et al.*, 1986; Nauck *et al.*, 1993; Vilsboll *et al.*, 2002), suggesting that a diminished incretin effect might be partly responsible for the development of postprandial hyperglycaemia. In the present study, however, we report elevated postprandial glucose and insulin concentrations following 7 days of high-fat overfeeding without any changes in GLP-1 or GIP. In this regard, elevated insulin concentrations are most probably a simple compensatory mechanism for reduced insulin sensitivity (hepatic and/or peripheral tissues) and elevated glucose concentrations. Thus, an altered incretin effect does not appear to play a role in the early adaptive response to overnutrition or the observed impairment in glycaemic control. Whilst we did observe a small, but significant, increase in fasting GIP concentrations, the physiological relevance of this remains unclear as fasting insulin concentrations were seemingly unaffected.

As mentioned previously, ghrelin concentrations are known to increase during fasting and decrease following food intake (Cummings *et al.*, 2001). This, combined with the observation that ghrelin administration stimulates appetite and food intake (Lawrence *et al.*, 2002; Wren *et al.*, 2001a; Wren *et al.*, 2001b), has led to the suggestion that ghrelin is an appetite-regulating hormone that is responsible (at least partially) for eating behaviour. Thus, reduced ghrelin levels reported in obese (Cummings *et al.*, 2002; le Roux *et al.*, 2005; Tschop *et al.*, 2001) and insulin resistant (McLaughlin *et al.*, 2004; Stepien *et al.*, 2011) individuals might represent a feedback loop by which the body attempts to reduce food intake within individuals that have been exposed to a chronic positive energy balance. Ghrelin is also

known to inhibit insulin secretion (Broglio *et al.*, 2001; Dezaki *et al.*, 2004; Dezaki *et al.*, 2006; Reimer *et al.*, 2003; Tong *et al.*, 2010), and may, therefore, play a role in glucose homeostasis. Indeed, ghrelin knock-out mice exhibit elevated basal insulin concentrations, enhanced glucose-stimulated insulin secretion, and improved peripheral insulin sensitivity when compared to wild-type controls (Sun *et al.*, 2006). With this in mind, reduced ghrelin levels might also be an attempt to lower glucose concentrations within hyperglycaemic obese and insulin resistant populations. Given the discussion points above, we might have expected to see a high-fat diet-induced decrease in fasting and/or postprandial acylated ghrelin concentrations, especially as we observed significant gains in body mass (presumably body fat) and increases in both fasting and postprandial glucose concentrations, but this was clearly not the case (Figure 2.1d). However, our results are in accordance with other overfeeding studies ranging in duration from 3-100 days (Brons *et al.*, 2009; Hagobian *et al.*, 2008; Ravussin *et al.*, 2001; Votruba *et al.*, 2009). Thus, it would seem that changes in circulating ghrelin concentrations occur secondary to the development of obesity and/or insulin resistance rather than in response to relatively short-term positive energy balance or modest increases in blood glucose concentrations.

Whilst the selected gut hormones demonstrated little response to the dietary intervention, high-fat overfeeding resulted in a significant increase in fasting glucose and postprandial glucose and insulin concentrations (Figures 2.1a and 2.1b), which is consistent with a number of previous human studies (Cornford *et al.*, 2013; Hulston *et al.*, 2015; Numao *et al.*, 2012; Pehleman *et al.*, 2005; Sparti, & Decombaz, 1992). Notably, our findings suggest some degree of impaired pancreatic β-cell dysfunction as, in health, the adaptive response to changes in insulin sensitivity typically results in a reciprocal increase or decrease in insulin release to maintain glucose homeostasis (Kahn *et al.*, 2006). For example, when experimentally inducing insulin resistance through administration of nicotinic acid, (Kahn *et al.*, 1989) observed an increase in insulin secretion that was sufficient to maintain glucose tolerance in young healthy males. Furthermore, a negative association appears to exist between increased fasting glucose concentrations and impaired β-cell function, even in those who display glucose levels within the normal range (Utzschneider *et al.*, 2006). Reductions in β-cell function have previously been reported to be one of the key mediators in the onset of T2DM (Kahn, 2001b). Others have reported impairments in skeletal muscle insulin signalling without (possibly before) a corresponding decrease in whole-body insulin sensitivity (Adochio *et al.*, 2009), or reduced hepatic insulin sensitivity without changes in peripheral

glucose uptake (Brons *et al.*, 2009). The lack of mechanistic agreement between some of these studies is most likely explained by differences in the duration of overfeeding, the varying energy content and/or macronutrient composition of the diets administered, or the particular method used for assessing insulin action and glycaemic control (oral glucose tolerance test *vs*. hyperinsulinaemic euglycaemic clamp *vs*. MTT). Where impairments in postprandial glycaemic control have been observed, it would be useful to know the processes responsible for such an effect. Blood glucose concentrations are governed by the balance between the rate of appearance of glucose from the gut, endogenous glucose production (primarily from the liver), and peripheral glucose uptake (mainly skeletal muscle). Therefore, the high-fat diet-induced increase in postprandial glucose concentration could be due to a defect in one, or a number, of these processes, which warrants further investigation.

In addition to changes in glucose and insulin concentrations, we also observed a significant decrease in fasting plasma triglyceride and NEFA concentrations after 7 days of high-fat overfeeding. This is consistent with previous work by us (Hulston *et al.*, 2015) and others (Adochio *et al.*, 2009; Lagerpusch *et al.*, 2012; Wulan *et al.*, 2014) and most likely reflects a decrease in endogenous triglyceride production as a result of increased fat consumption (Hellerstein, 2002) and suppression of adipose tissue lipolysis as a result of consuming larger and/or more frequent meals. It has been suggested that elevated NEFA concentrations might be responsible for the development of insulin resistance and T2DM (Eckel *et al.*, 2005). This notion has been fuelled by classical reports of elevated NEFA concentrations in obesity (Opie, & Walfish, 1963) as well as acute studies in which NEFA have been elevated by means of intravenous lipid-heparin infusion (Boden *et al.*, 1994). The later approach elevates NEFA by activating lipoprotein lipase (LPL) located in the vascular endothelium and supplying a lipid-based substrate for hydrolysis. More recently, however, the NEFA hypothesis of insulin resistance has been questioned as NEFA release per kilogram of adipose tissue is reduced as adipose tissue mass increases, and lipid-heparin infusion trials often elicit NEFA concentrations far in excess of the disease state that they aim to mimic (Karpe *et al.*, 2011). Whilst our data tend to support this change in consensus, in that we observed impaired glycaemic control at a time when fasting NEFA levels were reduced, we should also point out that frequent consumption of high-fat foods throughout the week-long diet intervention could have led to a considerable "spill-over" effect, whereby the hydrolysis of diet-derived circulating triglycerides could have driven regular postprandial increases in plasma NEFA.

It is also interesting to note that the high-fat-diet did not affect total cholesterol or LDL concentrations as one might have expected, whereas HDL levels actually increased following the dietary intervention. In general, saturated fats (that were highly prevalent in the present study) raise total cholesterol and LDL whereas polyunsaturated fats lower total cholesterol and LDL, and both types of fat increase HDL (Kris-Etherton, & Yu, 1997; Samaha, 2005). It is likely that our study did not affect total or LDL cholesterol levels due to the short duration of the diet intervention. Large scale population studies have demonstrated a strong association between low levels of HDL and cardiovascular disease risk (Gordon *et al.*, 1977; Jenkins *et al.*, 1978; Wilson *et al.*, 1988); a risk that is progressively reduced with increasing levels of HDL (Gordon *et al.*, 1989). This has been attributed to the potent anti-atherosclerotic properties of HDL (Mahdy Ali *et al.*, 2012). Therefore, the increase in HDL observed in our study may represent a short-term protective response against the atherosclerotic properties of our dietary intervention, although this is largely speculative.

As a last point for consideration, our subjects were all healthy, young, lean and physically active, and yet they still exhibited a rapid reduction in glycaemic control as a result of excessive consumption of high-fat foods. Whilst there is a paucity of information regarding the metabolic responses to overnutrition in humans, especially within at risk populations, one might expect even greater deleterious responses in those who are already overweight, sedentary or elderly.

In conclusion, in this study we have provided further evidence that short-term, high-fat overfeeding leads to impairments in glycaemic control, as indicated by a significant increase in meal-induced glucose and insulin responses. Furthermore, the postprandial responses of GLP-1, GIP and acylated ghrelin were not affected by the dietary intervention, suggesting that these selected gut hormones are not responsive to brief periods of positive energy balance and/or severe lipid overload. Therefore, the incretin hormones, and the gut peptide ghrelin, are not major regulators of the early adaptive responses to overnutrition.

CHAPTER 3

Short-term, high-fat overfeeding impairs glycaemic control in young, healthy, lean individuals by altering the coordinated processes regulating plasma glucose flux.

3.1 Abstract

Short-term (4-14 days) high-fat overfeeding is known to impair glycaemic control and reduce insulin sensitivity. However, the mechanisms underpinning these observed alterations in glucose metabolism are unclear. Thus, the aim of this study was to investigate the individual processes contributing to the early diet-induced impairments in glycaemic control in young, healthy, non-obese individuals. Fourteen individuals (12 males and 2 females) underwent two experimental trials (before and after consumption of a 7-day high-fat [65%] high-energy [+50%] diet) during which glycaemic control and systemic glucose kinetics were analysed by way of a dual-glucose tracer technique. Briefly, after fasting blood samples had been obtained, a primed, continuous infusion of [6,6-$^{2}H_{2}$]glucose was initiated and continued for the duration of the experiment. After 120 minutes of infusion, subjects then ingested a mixed carbohydrate and protein beverage which contained 50 g glucose (1.6 g [U-$^{13}C_{6}$]glucose) and 15 g whey protein. Measurements of plasma glucose, NEFA and serum insulin were obtained before, and 15, 30, 45, 60, 90 and 120 minutes post-ingestion. Postprandial plasma glucose AUC was increased by 11.3% (p = 0.006) after overfeeding, and postprandial serum insulin AUC was increased by 17% (p = 0.068). Analysis of glucose kinetics revealed the increased postprandial glucose response was not attributable to tissue-specific alterations in glucose metabolism (i.e. endogenous glucose production or glucose disposal), but rather an imbalance in the relationship between plasma glucose rate of appearance and disappearance. This imbalance resulted in a 2.5-fold increase in net glucose influx at 30 minutes post-ingestion, and a ~28% reduction in glucose efflux at 45 minutes. In conclusion, 7-days high-fat overfeeding impairs glycaemic control in young, healthy, non-obese individuals by influencing the coordinated processes regulating plasma glucose flux.

Acknowledgement: Stable isotope tracer analysis presented in this chapter was conducted by Professor Gerrit van Hall and his team at The Clinical Metabolomics Core Facility, Righospitalet.

3.2 Introduction

Type 2 diabetes mellitus (T2DM) represents one of the major causes of morbidity and mortality worldwide (Naghavi *et al.*, 2015). Alongside β-cell failure, one of the principal defects in T2DM is a state of insulin resistance; defined as a reduced responsiveness of target tissues to the physiological actions of insulin (Savage *et al.*, 2005). Insulin resistance impairs a broad array of metabolic processes in numerous tissues, however, the most profound effects are seen in the dysregulation of glucose homeostasis. This dysregulation is mediated by two distinct defects; a reduction in peripheral (i.e. predominantly skeletal muscle, but also adipose tissue) glucose uptake, and increased endogenous glucose production (EGP), predominantly driven by changes in hepatic glucose output (Samuel, & Shulman, 2016). Elevations in glucose concentration are associated with a concomitant increase in insulin secretion, and eventual hyperinsulinemia. The prevailing theory regarding the development of T2DM is that hyperinsulinemia, caused by chronic hyperglycaemia, effectively exhausts the secretory capacity of β-cell, resulting in a gradual decline in function and ultimate failure (Cerf, 2013).

At present, the development of insulin resistance is not fully understood. Obesity is considered a significant risk factor. However, there is substantial evidence that brief periods of positive energy balance can elicit negative alterations in glucose metabolism before substantial increases in body mass/fat. For instance, our laboratory has previously observed reductions in whole-body insulin sensitivity of approximately 25% following a 7-day high-fat, high-energy diet (Hulston *et al.*, 2015). A finding which is supported by other short-term (4-14 days) overfeeding studies (Cornford *et al.*, 2013; Lagerpusch *et al.*, 2012; Parry *et al.*, 2017; Wulan *et al.*, 2014). However, none of the above mentioned studies are able to provide any information regarding the mechanisms underpinning these whole-body impairments. Therefore, very little is known regarding the pathological time-course of events, and tissue-specific responses, which are associated with impaired glycaemic control and the development of whole-body insulin resistance. When investigating the time-course of impairments in insulin sensitivity using a model of combined overfeeding and reduced physical activity, Knudsen *et al.* (2012) demonstrated reductions in oral glucose tolerance derived estimates of whole-body insulin sensitivity after only 3-days. This marker of insulin sensitivity was further reduced at 7-days and was associated with a compensatory increase in postprandial insulin levels during the oral glucose tolerance test which were sufficient to maintain plasma glucose homeostasis. At 14-days of intervention clamp derived measures of

insulin sensitivity were reduced, attributable to a fall in insulin stimulated glucose uptake whereas insulins ability to suppress EGP was maintained (Knudsen *et al.*, 2012).

The findings of Knudsen *et al.* (2012) are supported by Adochio *et al.* (2009) who observed defects in skeletal muscle insulin signalling in response to a 5-day high-fat high-energy diet. These signalling defects occurred without concurrent changes in clamp-derived measures of whole-body insulin sensitivity, suggesting that changes at the level of skeletal muscle develop ahead of (and possibly mediate) whole-body impairments. However, in direct opposition to this, Brons *et al.* (2009) and (Brons *et al.*, 2012) observed a reduction in hepatic insulin sensitivity and increased fasting EGP in lean, healthy men subjected to 5-days of high-fat overfeeding, but no change in insulin-mediated glucose uptake during a hyperinsulinemic-euglycaemic clamp. A finding which is similar to that seen in lean women after 3 days of overfeeding (Cornier *et al.*, 2006). This suggests that it is indeed the liver which is the primary site of metabolic dysfunction. These discrepant findings highlight the need for further investigations in this area. Therefore, the aims of this study are to provide a greater insight into the early-phase impairments in glycaemic control. To this end, we herein utilised a model of short-term high-fat overfeeding previously shown by our laboratory to reduce whole-body insulin sensitivity and impair glycaemic control (Hulston *et al.*, 2015; Parry *et al.*, 2017), in combination with dual-isotopic tracers in order to investigate systemic glucose kinetics in response to diet-induced impairments in glycaemic control in young, healthy, lean individuals. Using this approach it is possible to determine EGP, the absorption of digested glucose from the gut, and whole-body glucose uptake.

3.3 Methods

3.3.1 Subjects

Seventeen healthy individuals were initially recruited to participate in this study but two were excluded due to experimenter concerns regarding dietary compliance. Furthermore, a full data set is unavailable for one participant due to experimental error with the preparation of the tracer infusate. Therefore, data is presented for 14 subjects (12 males and 2 females; their physical characteristics can be seen in Table 3.1). The inclusion criteria required subjects to be physically active (exercising at least 3 times per week for more than 30 minutes at a time), non-smokers, free from cardiovascular and metabolic disease, not taking any medication, weight stable for at least 6 months, and with a body mass index (BMI) below 30 kg/m^2. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Loughborough University Ethical subcommittee for human participants. The experimental procedures and possible risks were fully explained to the subjects before their written informed consent was given.

Table 3.1 Subject characteristics before and after 7 days of high-fat overfeeding.

Characteristics	**Before overfeeding**	**After overfeeding**
Age (years)	24.9 ± 1.0	-
Height (cm)	176.5 ± 2.2	-
Body mass (kg)	77.4 ± 3.2	78.8 ± 3.3 *
BMI (kg/m^2)	24.7 ± 0.6	25.1 ± 0.7 *

Data presented are means ± SEM (n = 14). * denotes significant change following the dietary intervention ($p < 0.05$).

3.3.2 Pre-testing

Prior to the start of the study, subjects attended the laboratory for an initial assessment of their baseline anthropometric characteristics (height, weight and BMI). This information was then used to estimate their resting energy expenditure (REE) according to the calculations described by Mifflin *et al.* (1990). A standard correction for physical activity level (1.6 and 1.7 times REE for females and males, respectively) was applied in order to estimate total daily energy requirements. Diet-induced thermogenesis (DIT) was estimated pre-intervention

as 10% of energy intake (Westerterp, 2004). This information was then used to determine individual energy intakes for the week-long overfeeding period (diet details described later).

3.3.3 Experimental design

Approximately 1-week after the initial pre-testing visit, subjects returned to the laboratory for two experimental trials; one immediately before, and one immediately after, a 7-day, high-energy, high-fat dietary intervention (described below). Briefly, during the experimental trials, circulating substrate and hormone concentrations were determined before, and for 2-h after the ingestion of a mixed carbohydrate and protein beverage (described in detail below). A dual-glucose tracer technique (described in detail below) was also utilised at this time to determine systemic glucose kinetics (i.e. total glucose rate of appearance [$R_{a\ total}$], glucose rate of disappearance [R_d], oral glucose rate of appearance [$R_{a\ oral}$], and endogenous glucose rate of appearance [$R_{a\ endo}$]). The dietary intervention was designed to be high in fat (65% total energy) and provide a severe energy excess (+ 50% kJ). Mean energy intake throughout the dietary intervention was 20181 ± 734 kJ, with 13117 ± 477 kJ provided as fat (more detailed information regarding the dietary intervention can be viewed in chapter 2). All foods were purchased and prepared by the research team. Subjects were instructed to eat all of the food provided, and to maintain normal, habitual physical activity levels during the intervention period.

3.3.4 Experimental protocol

On the experimental days (before and after overfeeding) subjects reported to the laboratory between 07.00 and 09.00 h after an overnight fast of at least 10 h and having refrained from physical activity for 48 h. After voiding and being weighed, a 20 gauge Teflon catheter (Venflon, Becton, Dickinson, Plymouth, UK) was inserted into an antecubital vein on each arm to allow for repeated blood sampling and infusion of stable isotope tracers. A baseline, fasting venous blood sample (10 mL) was obtained to determine fasting metabolite concentrations and background isotopic enrichment before a primed constant infusion of [6,6-$^2H_2$]glucose (0.35 µmol/kg/min, prime 14 µmol/kg) was initiated and continued for the duration of the experiment. Blood samples (10 mL) were obtained 90, 105 and 120 min into the infusion period to ascertain isotopic steady state, after subjects ingested a mixed carbohydrate and protein beverage (CHO + PRO; described in detail below). Further venous blood samples (10 mL) were obtained at 15, 30, 45, 60, 90 and 120 min post-ingestion, and additional muscle biopsies were obtained at 30 and 120 min post-ingestion. Stable isotopes

were purchased from Cambridge Isotope Laboratories (Andover, MA). A schematic of the experimental protocol can be viewed in Figure 3.1.

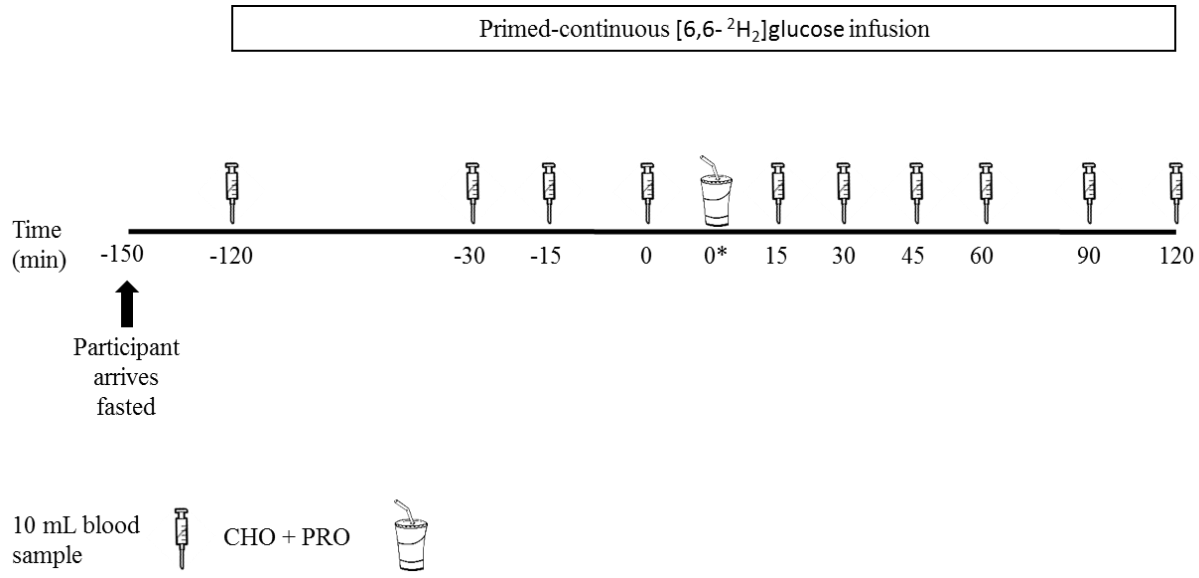

Figure 3.1. Schematic of experimental protocol. CHO + PRO = 50 g glucose (48.4 g unlabelled and 1.6 g [U-^{13}C]glucose) and 15 g whey protein. * denotes an approximate 10 minute delay between sampling and ingesting the CHO + PRO beverage.

3.3.5 Beverages

The CHO + PRO beverage was a 12.5% glucose solution (48.4 g unlabelled and 1.6 g [U-^{13}C]glucose dissolved in 400 mL of water) with the addition of 15 g whey protein in the form of a commercially available protein supplement (Volac; UltraWhey 90, Hertfordshire, UK). The amino acid content of the protein was (in percent content wt:wt): Alanine, 5; Arginine, 2.1; Aspartic acid, 11; Cysteine, 2.2; Glutamic acid, 18.1; Glycine, 1.4; Histidine, 1.7; Isoleucine, 6.4; Leucine, 10.6; Lysine, 9.6; Methionine, 2.2; Phenylalanine, 3; Proline, 5.5; Serine, 4.6; Threonine, 6.7; Tryptophan, 1.4; Tyrosine, 2.6, and Valine, 5.9.

3.3.6 Blood sampling

For analysis of glucose, non-esterified fatty acids (NEFA), triglyceride (TG), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein LDL, and tracer concentrations and enrichments, whole blood samples were collected in 4.9 mL ethylenediaminetetraacetic acid (EDTA; 1.75 mg/mL) treated tubes (Sarstedt, Leicester, UK) and spun for 10 min at 1,750 *g* in a refrigerated centrifuge (4°C). The resulting plasma was

aliquoted into 1.5 mL Eppendorfs and stored at -20°C until analysis. For analysis of insulin, whole blood was collected in 4.5 mL tubes containing a clotting catalyst (Sarstedt, Leicester, UK). Samples were left at room temperature until complete clotting had occurred, after which they were also spun for 10 min at 1,750 *g* in a refrigerated centrifuge (4°C). The resulting serum was aliquoted into 1.5 mL Eppendorfs and stored at -20°C until analysis.

3.3.7 Analytical procedures

Plasma samples were analysed using commercially available spectrophotometric assays for TG, total cholesterol, HDL, LDL, (Horiba Medical, Northampton, UK) and NEFA (Randox, County Antrim, UK) concentrations using a semi-automatic analyser (Pentra 400; Horiba Medical, Northampton, UK). Serum insulin concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA: EIA-2935, DRG instruments GmBH, Germany). To eliminate inter-assay variation, samples from each participant were analysed in the same run.

3.3.8 Plasma glucose concentration and enrichment analysis

Prior to derivatisation, 50 µL of plasma was mixed with 50 µL internal standard ([U-^{13}C,$^{2}H_7$]glucose), and 100 µL ethyl acetate (LiChroSolv ≥99.8%, Merck KGaA, Germany). Samples were then vortexed for 5 minutes (2000 rpm) using an Eppendorf MixMate (Eppendorf AG, Hamburg, Germany), before being centrifuged at 15,700 relative centrifugal force (rcf) for 5 minutes (Sigma 1K15 centrifuge, Osterode am Harz, Germany). After centrifugation, the upper organic phase was disposed, and derivatisation was performed by adding 20 µL of 1 M K_2HPO_4, 20 µL of 8 M NaOH, and 10 µL benzoyl chloride to the remaining sample. The benzoylated derivatives were then immediately vortexed for 5 minutes, before being neutralised with 10 µL 1.4 M H_3PO_4 and extracted with 500 µL ethylacetate. Samples were then vortexed for 2-3 minutes (2000 rpm), before being centrifuged at 15,700 rcf for 5 minutes. Hereafter, 100 µL of the ethylacetate phase was aliquoted into a new Eppendorf. The extraction procedure was repeated once more and a further 100 µL ethylacetate was added to the first; resulting in a final volume of 200 µL. This sample was then evaporated to dryness under a stream of nitrogen for 5-10 minutes. The precipitate was then re-dissolved in 200 µL injection buffer (10 mM NH_4AC in 75:25 [v/v %] acetonitrile/water) and vortexed for 1 minute prior to being filtered through a 0.45 µm 96-well filter plate within a vacuum manifold (Pall Corporation, Ann Arbor, MI, USA) and directionally transferred to high performance liquid chromatography (HPLC) vials for

analysis. The enrichment of [6,6-$^{2}H_2$]glucose and [U-^{13}C]glucose was then determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The glucose derivatives were quantified by selected ion monitoring at mass-to-charge (m/z) of 231, 233, 237, and 243 for glucose, [6,6-$^{2}H_2$]glucose (M+2), [U-^{13}C]glucose (M+6), and [U-^{13}C,$^{2}H_7$]glucose (the M+12 peak was utilised due to the loss of one of the seven deuterium labels during fragmentation by MS/MS).

3.3.9 Systemic glucose kinetics

$R_{a\ total}$ (equation 1) and R_d (equation 2) of glucose was determined through analysis of [6,6-$^{2}H_2$]glucose in combination with the single-pool, non-steady state equations of (Steele, 1959), modified for use with stable isotopes (Radziuk *et al.*, 1978). $R_{a\ total}$ represents the combined systemic appearance of glucose from $R_{a\ endo}$ (primarily from the liver and a possible minor contribution from the kidneys), and the digestion and absorption of glucose from ingested carbohydrate ($R_{a\ oral}$).

1) $R_{a\ total} = F - V \cdot ([C_2 + C_1]/2) \times [E_{p2} - E_{p1}]/t_2\text{-}t_1)/([E_{p2} + E_{p1}]/2)$

2) $R_d = R_{a\ total} - V \times (C_2 + C_1)/(t_2 - t_1)$

Where F represents the glucose infusion rate; V is volume for distribution (40 mL/kg); C_1 and C_2 are total glucose concentrations (i.e. the sum of endogenous unlabelled, infused [6,6-$^{2}H_2$]glucose, and ingested [U-^{13}C]glucose) at time-points (t) t_1 and t_2 respectively; and E_{p1} and E_{p2} are the [6,6-$^{2}H_2$]glucose enrichments in plasma at t_1 and t_2 respectively. $R_{a\ endo}$ was determined via modification of equation 1; where, C_1 and C_2 are $^{2}H_2$ glucose concentrations at time-points t_1 and t_2 respectively. $R_{a\ oral} = R_{a\ total} - R_{a\ endo}$.

3.3.10 Calculations and statistics

Area under the curve (AUC) for glucose, insulin and NEFA was calculated using the trapezoidal rule with zero as the baseline. Data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS (V21.0) for windows (SPSS Inc, Chicago, IL). Paired t-tests were used to compare differences in body mass, BMI, and fasting metabolic responses before and after overfeeding, whereas the dynamic hormonal and metabolic responses to carbohydrate and protein ingestion were compared using a two-way (trial x time) repeated measures analysis of variance (ANOVA) and Bonferroni *post hoc*

analysis where appropriate. Statistical significance was set at $p < 0.05$. Prior to analysis, all data were tested for normality according to the Shapiro-Wilk test. Due to demonstrating a skewed distribution, and thus violating the assumptions of normality, logarithmic transformation of $R_{a\ total}$, R_d, $R_{a\ oral}$ and $R_{a\ endo}$ data was performed prior to statistical analysis. However, in order to maintain physiological relevance, all data are maintained as raw values for the purpose of graphical presentation.

3.4 Results

3.4.1 Weight gain and BMI

All 14 subjects gained body mass following the overfeeding intervention (increasing by 1.3 ± 0.3 kg) ($p < 0.0001$, Table 3.1), leading to an increase in BMI of 0.44 ± 0.08 kg/m^2 ($p < 0.0001$, Table 3.1).

3.4.2 Fasting metabolic responses

Fasting substrate, hormone and lipoprotein concentrations before and after overfeeding are presented in table 3.2. Fasting plasma glucose, HDL, total cholesterol and serum insulin all increased after overfeeding ($p = 0.05$, $p < 0.0001$, $p = 0.011$, and $p = 0.04$, respectively). Whereas, plasma TG and serum NEFA decreased ($p < 0.0001$, and $p = 0.005$, respectively). Fasting plasma LDL was unaffected by high-fat overfeeding ($p = 0.182$).

Table 3.2 Fasting substrate, hormone, and lipoprotein concentrations before and after 7-days of high-fat overfeeding.

	Before overfeeding	**After overfeeding**
Glucose (mmol/L)	4.78 ± 0.08	5.08 ± 0.08 *
Insulin (pmol/L)	65 ± 6	76 ± 5 *
TG (mmol/L)	0.82 ± 0.07	0.59 ± 0.06 *
NEFA (mmol/L)	0.66 ± 0.09	0.41 ± 0.05 *
LDL (mmol/L)	2.17 ± 0.14	2.08 ± 0.14
HDL (mmol/L)	1.32 ± 0.08	1.57 ± 0.08 *
Total cholesterol (mmol/L)	3.75 ± 0.16	3.90 ± 0.12 *

Data presented are means ± SEM (n = 14). Fasting plasma glucose, NEFA and serum insulin represent mean values across the -30 - 0 min period before CHO + PRO ingestion. All other measures were performed on the single, -30 min sample. * denotes significant change following the dietary intervention ($p < 0.05$).

3.4.3 Substrate and hormone responses to carbohydrate and protein ingestion

Substrate and hormone responses to CHO + PRO before and after overfeeding are presented in Figure 3.2. Plasma glucose and serum insulin increased in response to CHO + PRO ingestion, peaking at 30-45 min (figure 3.2a and 3.2c, respectively). There was a significant trial x time interaction evident for plasma glucose ($p = 0.002$, figure 3.2a). Plasma glucose

AUC was increased by 11.3% after overfeeding (from 596 ± 24 mmol/L per 120 min before overfeeding to 663 ± 20 mmol/L per 120 min after overfeeding; $p = 0.006$, figure 3.2b). A significant trial x time interaction was also evident for serum insulin ($p = 0.002$, figure 3.2c). Serum insulin AUC increased by 17% following overfeeding (from 34433 ± 3958 pmol/L per 120 min before overfeeding to 40210 ± 3332 pmol/L per 120 min after overfeeding), but this failed to reach significance ($p = 0.068$, figure 3.2d). Plasma NEFA concentrations decreased in response to CHO + PRO ingestion, reaching a nadir at 90 minutes post-ingestion in both trials (figure 3.2e). Plasma NEFA also displayed a significant time x trial interaction ($p < 0.0001$, figure 3.2e). Plasma NEFA AUC was reduced by 17% after overfeeding (from 26.4 ± 2.4 mmol/L per 120 min before overfeeding, to 21.9 ± 1.7 mmol/L per 120 min after overfeeding; $p = 0.045$, figure 3.2f).

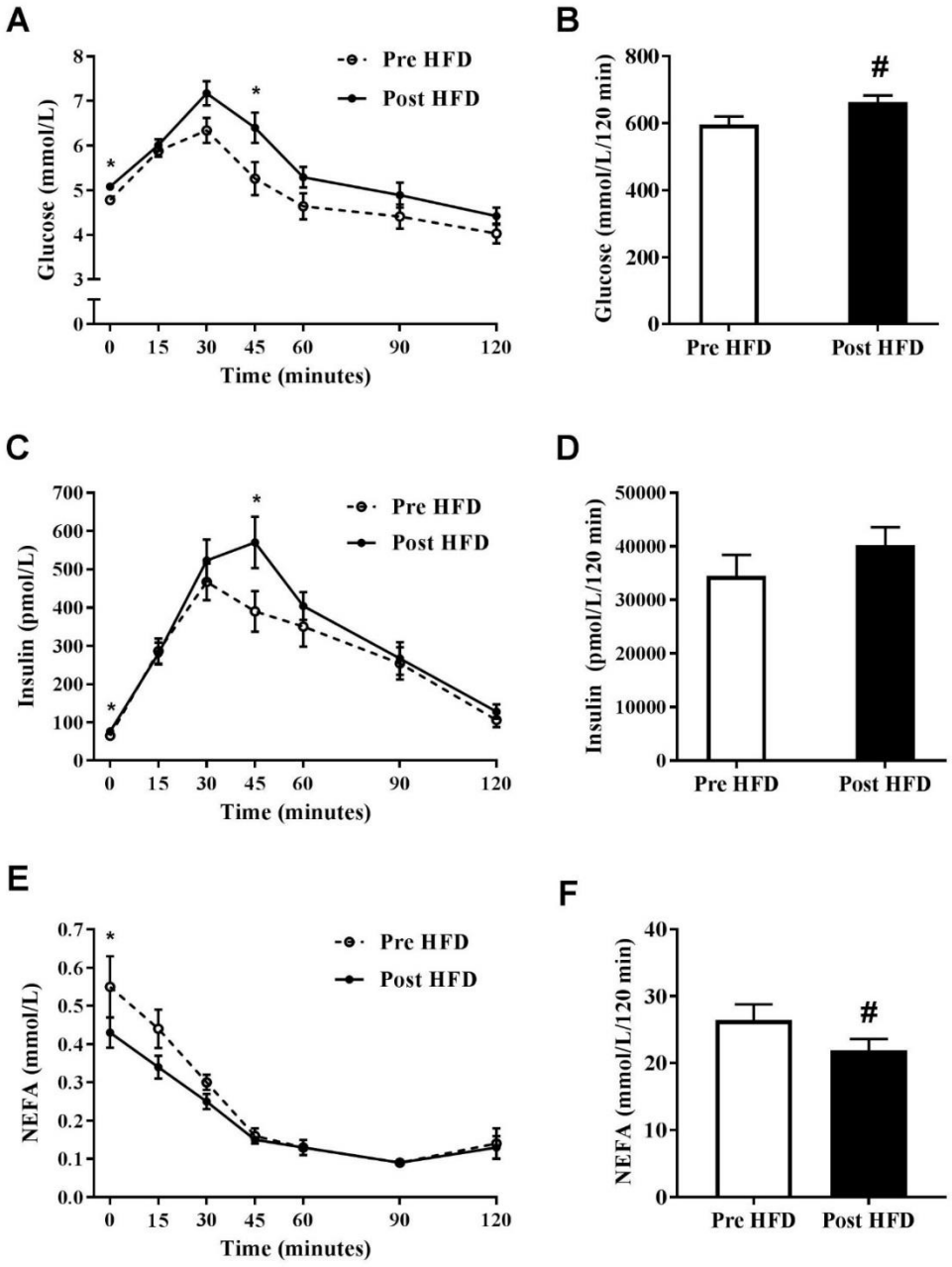

Figure 3.2 Fasting and postprandial plasma glucose (A), serum insulin (C), and plasma NEFA (E) concentrations, and plasma glucose (B), serum insulin (D), and plasma NEFA AUC (F), before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Time point 0 represents mean (-30-0 minutes) fasting values. Data presented are means ± SEM (n = 14). * denotes significant difference between trials at the annotated time point ($p < 0.05$). # denotes significant main effect of trial/high-fat overfeeding ($p < 0.05$).

3.4.4 Systemic glucose kinetics

Plasma enrichment of the infused [6,6-$^{2}H_2$]glucose and ingested [U-^{13}C]glucose are presented in figure 3.3. Plasma [6,6-$^{2}H_2$]glucose enrichment decreased following CHO + PRO

ingestion, reaching a nadir at 90 minutes post-ingestion, whereas [U-^{13}C]glucose enrichment displayed an inverse response, increasing after CHO + PRO ingestion and peaking at 90 minutes. No difference in plasma [6,6-$^{2}H_2$]glucose or [U-^{13}C]glucose enrichments were evident between trials (p = 0.330, and p = 0.066, respectively). Plasma $R_{a\ total}$, R_d, $R_{a\ oral}$, and $R_{a\ endo}$ are presented in figure 3.4a-d, respectively. As expected, plasma $R_{a\ total}$ increased in response to CHO + PRO ingestion, peaking at 90 minutes post ingestion (figure 3.4a). $R_{a\ oral}$ also increased across time, peaking at 90 minutes (figure 3.4c). $R_{a\ endo}$ displayed an inverse response, gradually decreasing across time and reaching a nadir at 120 minutes (figure 3.4d). Plasma glucose R_d gradually increased from 15 minutes post-ingestion onwards, reaching a peak at 60-90 minutes (figure 3.4b). Overfeeding did not influence $R_{a\ total}$ (p = 0.168), R_d (p = 0.098), $R_{a\ oral}$ (p = 0.148), or $R_{a\ endo}$ (p = 0.217). Nonetheless, while neither $R_{a\ total}$ nor R_d displayed significant differences between trials, a significant trial x time interaction was observed when calculating $R_{a\ total}$ - R_d (p = 0.023, figure 3.5). This interaction affect is attributable to larger differences between $R_{a\ total}$ and R_d at 30, 45 and 60 minutes post CHO + PRO ingestion when comparisons are made across trials. To elaborate, during both trials there was an imbalance in favour of $R_{a\ total}$ evident at 30 minutes post ingestion. However, the magnitude of this imbalance was considerably greater after overfeeding, resulting in a 2.5-fold greater net glucose influx at this time. Furthermore, while both trials displayed a shift in balance in favour of R_d at 45 minutes post-ingestion, net efflux was 28% lower after overfeeding. These divergent responses (i.e. greater net glucose influx at 30 minutes, and reduced net glucose efflux at 45 minutes) are responsible for the higher postprandial plasma glucose concentrations seen after overfeeding (figure 3.2a). Net glucose efflux at 60 minutes post ingestion was 80% greater after overfeeding (figure 3.5). However, this compensatory response was insufficient to normalise plasma glucose concentrations which remained higher than pre-overfeeding levels throughout the postprandial period (figure 3.2a).

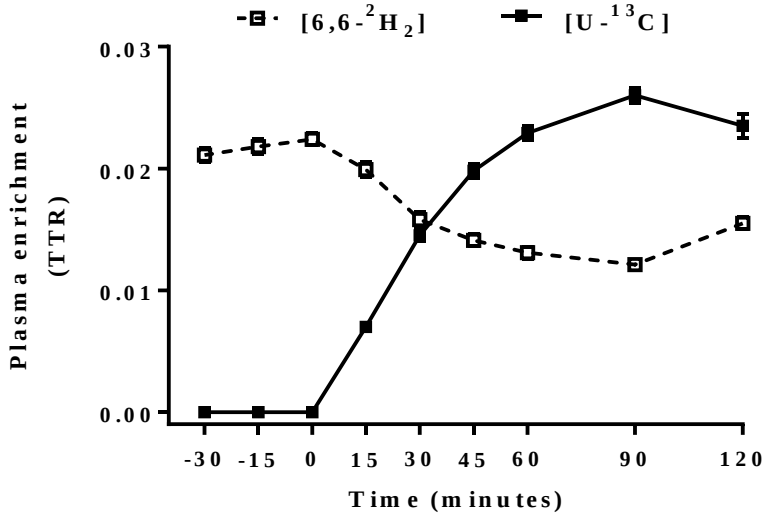

Figure 3.3 Plasma [6,6-$^{2}H_2$] and [U-^{13}C] glucose enrichments. Time points -30 – 0 min represent the final 30 min of the initial 2-h infusion period. All subsequent time points are following the ingestion of CHO + PRO. Data presented are means ± SEM (n = 28). As no significant differences were evident between trials (i.e. before and after overfeeding) data were grouped for graphical presentation.

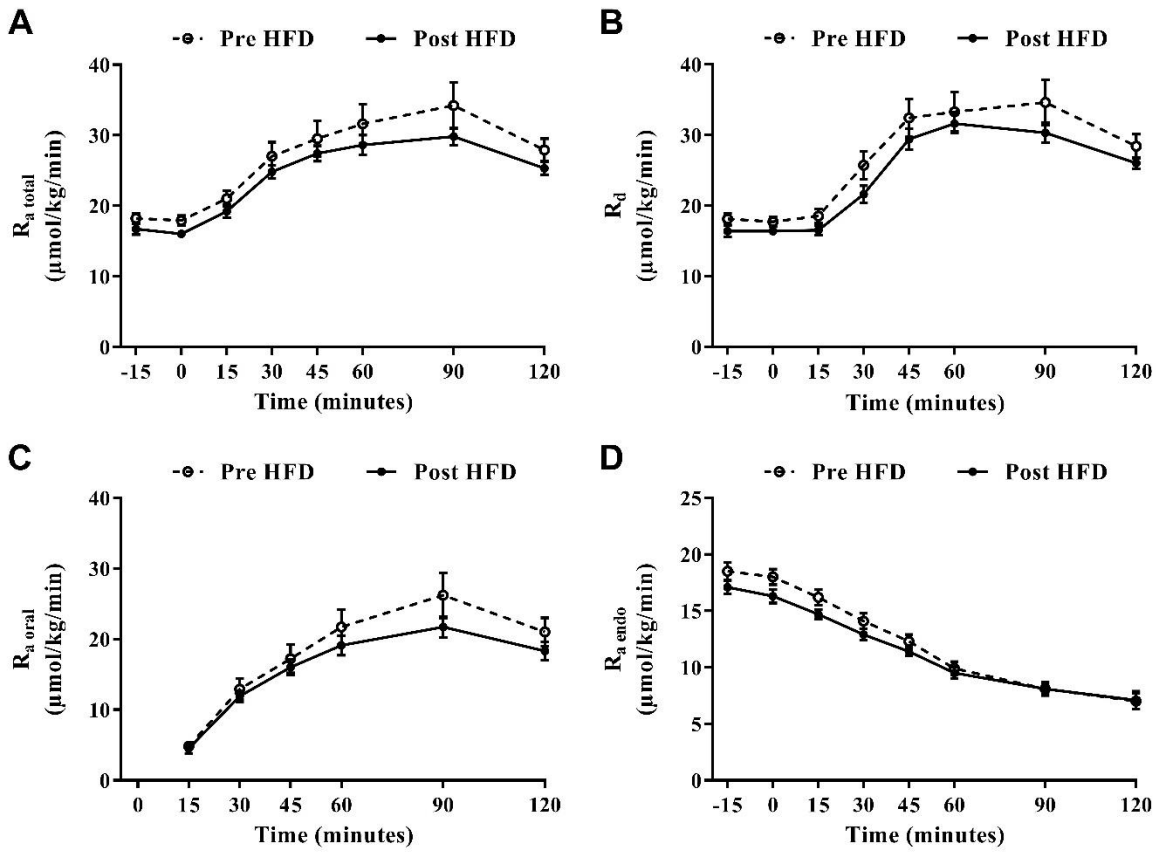

Figure 3.4 Plasma glucose $R_{a\ total}$ (A), R_d (B), $R_{a\ oral}$ (C), and $R_{a\ endo}$ (D) before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Time points -15 – 0 min represent the final 15 min of the initial 2-h infusion period. All subsequent time points are following the ingestion of CHO + PRO. Data presented are means ± SEM (n = 14).

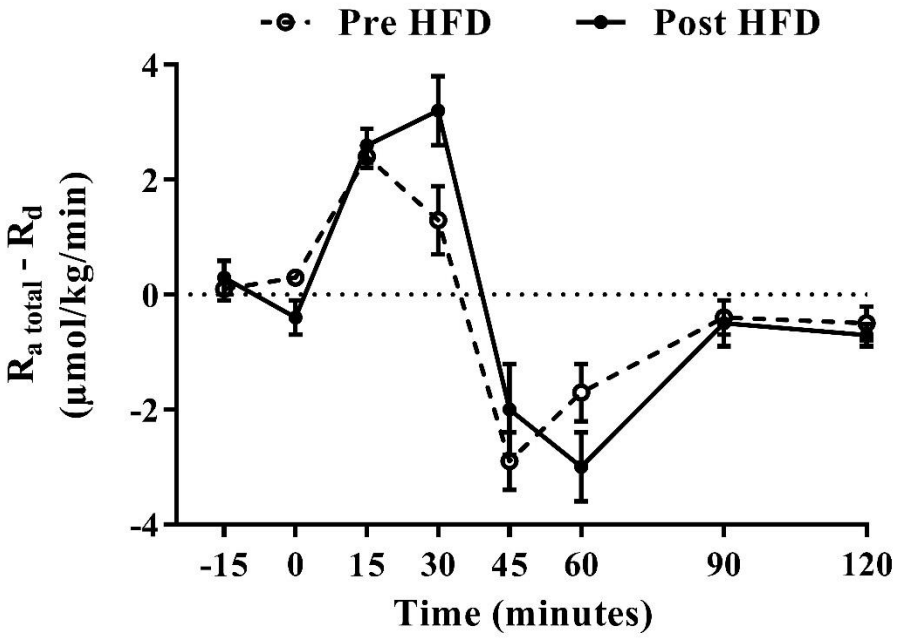

Figure 3.5 Fasting and postprandial $R_{a\ total}$ - R_d before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Time points -15 – 0 min represent the final 15 min of the initial 2-h infusion period. All subsequent time points are following the ingestion of CHO + PRO. Data presented are means ± SEM (n = 14).

3.5 Discussion

The main finding of the present study was that 7-days of high-fat overfeeding impaired glycaemic control in young, healthy, non-obese individuals, as evidenced by a significant increase in postprandial glucose AUC in the presence of similar, or somewhat elevated, insulin levels (17% non-significant increase). Our data indicates that this impairment is underpinned by a subtle change in the relationship between glucose appearance and disappearance that would favour an accretion of plasma glucose over time, rather than overt tissue-specific alterations in glucose metabolism such as increased EGP or reduced peripheral glucose uptake.

Negative alterations in glucose metabolism following short-term (4-14 days) overfeeding protocols, including impaired glycaemic control and reductions in whole-body insulin sensitivity, have previously been observed by our laboratory and others (Cornford *et al.*, 2013; Hulston *et al.*, 2015; Lagerpusch *et al.*, 2012; Parry *et al.*, 2017; Wulan *et al.*, 2014). However, while these studies highlight the rapidity with which overnutrition can negatively impact glucose metabolism, they provide no information as to the mechanisms underpinning these whole-body impairments. The utilisation of a dual-glucose tracer technique in this study allowed us to examine the individual processes which contribute to whole-body glycaemic control (i.e. intestinal absorption of glucose following carbohydrate ingestion [$R_{a\ oral}$], EGP [$R_{a\ endo}$], or whole-body glucose uptake [R_d]), and thus gain a more complete understanding of the early metabolic responses to overnutrition. Intriguingly, in the present study postprandial glucose AUC was increased by 11.3% after overfeeding, however, this increase occurred without any observable, diet-induced changes in $R_{a\ total}$, R_d, $R_{a\ oral}$ or $R_{a\ endo}$. Nonetheless, when comparing plasma glucose flux ($R_{a\ total}$ - R_d) between trials it is evident that overfeeding induces an imbalance between glucose appearance and disappearance which would favour the accretion of plasma glucose. These imbalances manifest as a 2.5-fold increase in net glucose influx at 30 minutes post-ingestion, and a ~28% reduction in net glucose efflux at 45 minutes. Therefore, the findings of our study would suggest that it is not overt, tissue-specific alterations in glucose metabolism *per se* (e.g. increased EGP or reduced peripheral glucose uptake), that underpin the early impairments in glycaemic control, but rather, subtle changes in the coordinated responses which regulate glucose flux.

There is limited available literature providing any mechanistic insight into the early-phase responses to overnutrition in humans. Furthermore, of the literature that is available there

appears considerable inconsistencies; with some reports suggesting the early impairments in whole-body glucose metabolism are attributable to changes in hepatic insulin sensitivity and increased EGP (Brons *et al.*, 2009; Cornier *et al.*, 2006), whereas others suggest that skeletal muscle is the primary site of metabolic dysfunction (Adochio *et al.*, 2009; Knudsen *et al.*, 2012). It is likely that these divergent responses are attributable to methodological differences such as the duration and magnitude of overfeeding, the macronutrient composition of the experimental diets, and the physical activity levels of participants during the intervention. These methodological differences make direct comparisons with the present study difficult. Furthermore, all of the above mentioned studies assessed insulin sensitivity by way of a hyperinsulinemic-euglycaemic clamp. While the hyperinsulinemic-euglycaemic clamp is considered the "gold-standard" method for determining insulin sensitivity in humans (Kim, 2009), it is a steady-state (static) measure and therefore does not accurately reflect the dynamic glucose and insulin responses seen in humans during the postprandial period. The use of a dual-glucose tracer technique can therefore be considered a major strength of the current study as it allows for determination of the integrated physiological responses seen in humans after feeding. However, it is pertinent to note that unlike the hyperinsulinemic-euglycaemic clamp our method measures oral glucose tolerance and not insulin sensitivity, and it is therefore influenced by a variety of factors including insulin sensitivity, insulin secretion, incretin hormone response, and neural inputs (Bartoli *et al.*, 2011; Stumvoll *et al.*, 2001). Therefore it is possible that this method is not sensitive enough to detect small differences in tissue-specific insulin sensitivity. The sensitivity of the dual-glucose tracer technique has previously been questioned, and a triple-tracer method proposed (Basu *et al.*, 2003; Toffolo *et al.*, 2006; Toffolo *et al.*, 2008). However, whilst the triple tracer approach may be more sensitive, it requires a substantial degree of expertise due to the experimental and analytical complexity of the method. Expertise that is currently unavailable to our laboratory. Conversely, the dual-glucose method has previously been reported to be accurate and reliable in postprandial conditions (Haidar *et al.*, 2012). It would be of interest to combine the dual-glucose tracer method with arterio-venous difference measures across skeletal muscle and adipose tissue in an attempt to improve sensitivity; it would not be possible to assess hepatic insulin sensitivity in this manner. Additionally, while we failed to see significant changes in factors such as EGP or peripheral glucose disposal in the current study, it is possible that significant differences in these processes may have been apparent under more controlled levels of insulinaemia, such as that achieved during hyperinsulinemic-euglycaemic clamps. This premise can also be extended to explain the apparent discrepancies

regarding tissue-specific responses reported in the above studies (Adochio *et al.*, 2009; Brons *et al.*, 2009; Cornier *et al.*, 2006; Knudsen *et al.*, 2012), as each of these studies assessed insulin sensitivity under different insulin infusion rates.

Alongside augmented glucose and insulin responses, we also observed a significant reduction in fasting plasma NEFA and TG after the 7-day overfeeding intervention. Reductions in fasting NEFA and TG seem to be a salient feature of short-term, high-fat, overfeeding studies, (Adochio *et al.*, 2009; Brons *et al.*, 2009; Parry *et al.*, 2017; Wulan *et al.*, 2014). However, the underlying cause of these responses are yet to be clarified. Possible explanations include a reduction in endogenous TG production due to a relative reduction in carbohydrate consumption throughout the dietary interventions (Hellerstein, 2002), and an increase in the suppression of adipose tissue lipolysis resulting from increased insulin concentrations. Nevertheless, the reduction in plasma TG and NEFA could also be due to increased uptake/storage in ectopic depots such as the liver and skeletal muscle. Indeed, substantial increases in intramyocellular (Adochio *et al.*, 2009; Bachmann *et al.*, 2001; Schrauwen-Hinderling *et al.*, 2005), and hepatic (Rosqvist *et al.*, 2014; Sobrecases *et al.*, 2010; van der Meer *et al.*, 2008) lipid concentrations have previously been reported after short-term high-fat overfeeding. Previous research investigating the correlation between insulin sensitivity and intramyocellular lipid (IMCL) concentrations have demonstrated equivocal findings, with positive (Goodpaster *et al.*, 2001; Haus *et al.*, 2011), negative (Krssak *et al.*, 1999; Pan *et al.*, 1997), and no relationship (Thamer *et al.*, 2003) all reported. Conversely, there is evidence to suggest that increases in hepatic lipid content may be more closely related to insulin resistance and other features of the metabolic syndrome than IMCL accumulation (Kotronen *et al.*, 2008). This is interesting as both the macronutrient and fatty acid composition of diets appears to differently modulate hepatic fat content, even in the context of overfeeding; with diets which are high in saturated fat increasing liver fat to a greater extent than diets high in carbohydrate, or monounsaturated and polyunsaturated fat (Yki-Jarvinen, 2015). Thus, some of the inconsistencies between overfeeding studies may be explained by differences in the site of lipid deposition attributable to different intervention diets. However, further research focusing on in depth measures of fatty metabolism are required in order to elucidate this.

The experimental cohort chosen in the current study were young, healthy, non-obese, males and females who were recreationally active. These individuals were chosen in order to avoid

the potentially confounding factors associated with excess adiposity, thus ensuring any observed responses were a result of the intervention and not due to pre-existing metabolic complications or chronic inactivity. However, it is likely that these individuals are relatively metabolically flexible, and are thus able to somewhat adapt to the dietary intervention. Indeed, while we saw significant increases in the postprandial glucose response after overfeeding, circulating glucose levels at 2 h post-glucose load were considerably lower than the diagnostic values of impaired glucose tolerance (i.e. 7.7-11 mmol/L), although, it must be noted that in this study participants ingested 50g of glucose rather than the standard 75g used in clinical settings (Nathan *et al.,* 2007). It is therefore possible that the dietary intervention employed in the current study may produce a more dramatic effect, and elicit different responses in populations at risk of developing T2DM (e.g. middle aged, sedentary, overweight males [Lee *et al*., 2013]).

In conclusion, the findings of our study demonstrate that 7-days high-fat overfeeding impairs glycaemic control in young, healthy, non-obese individuals. These impairments are seemingly underpinned, by subtle differences in the coordinated processes that regulate plasma glucose flux rather than tissue-specific alterations in glucose metabolism. It is possible that these findings may be exacerbated in populations at risk of developing T2DM.

CHAPTER 4

A single day of excessive dietary fat intake impairs whole-body insulin sensitivity in healthy, non-obese, young men.

4.1 Abstract

Reductions in insulin sensitivity can be observed in healthy, lean individuals within just a few hours of intravenous lipid-heparin infusion. Short-term (4-14 days) high-fat overfeeding has also been shown to reduce insulin sensitivity and impair glycaemic control in healthy individuals. However, the time-course of these diet-induced impairments remains unclear. Therefore, the aim of this study was to determine if one day of high-energy, high-fat feeding impairs whole-body insulin sensitivity in young, healthy, non-obese males. Ten males underwent an oral glucose tolerance test (OGTT) before and after consuming a high-fat (73%), high-energy (+80%) diet for 1 day. Measurements of plasma glucose, NEFA and serum insulin were measured at baseline (fasting) and during the 2 h postprandial period after glucose ingestion. One day of high-fat overfeeding increased postprandial glucose AUC by 16.2% (p = 0.008), and postprandial insulin AUC by 14.1% (p = 0.085). Whole-body insulin sensitivity, calculated by the Matsuda insulin sensitivity index, was reduced by 24% after overfeeding (p = 0.021). In conclusion, in this study we demonstrate that only a single day of excessive high-fat food intake is sufficient to impair glycaemic control in young, healthy, non-obese males.

4.2 Introduction

Whilst chronic obesity is a major risk factor for the development of insulin resistance and type 2 diabetes mellitus (T2DM), insulin resistance can be induced very rapidly (within just a few hours) in healthy, lean individuals by means of an intravenous (iv) lipid-heparin infusion (Boden *et al.*, 1994; Boden *et al.*, 1991). This model increases plasma non-esterified fatty acids (NEFA) by providing an exogenous source of triglycerides as well as activating endothelial lipoprotein lipase (LPL; an enzyme responsible for the hydrolysis of circulating triglycerides). This, combined with early observations that NEFA are elevated in obese individuals (Opie, & Walfish, 1963) has led to the hypothesis that elevated circulating fatty acids are a major regulator of insulin resistance (Eckel *et al.*, 2005). An alternative approach to study the early onset of insulin resistance and T2DM is through high-fat food consumption (i.e., the high-fat diet model). Using this model, human studies have reported negative alterations in glucose metabolism, including impaired glycaemic control (Cornford *et al.*, 2013; Parry *et al.*, 2017; Wulan *et al.*, 2014), and reductions in whole-body (Hulston *et al.*, 2015), and hepatic (Brons *et al.*, 2009) insulin sensitivity within just a few (4-14) days. Paradoxically, these effects occur despite only small increases in body mass (body fat) and with unchanged or even reduced plasma NEFA concentrations; thereby casting some doubt on the NEFA hypothesis of insulin resistance.

Currently, very little is known regarding the time-course of the observed diet-induced impairments in glucose metabolism. One recent study reported that oral administration of a single dose of soybean oil (100 mL) reduced whole-body insulin sensitivity (assessed by hyperinsulinaemic-euglycaemic clamp) to a level comparable to that observed during an energy- and composition-matched iv lipid-heparin infusion (Nowotny *et al.*, 2013). Intriguingly, this response occurred independently of NEFA levels, which were elevated during fat infusion but unchanged following fat ingestion, and may suggest that insulin resistance occurs via different mechanistic pathways depending on the method of lipid administration. A practical criticism of the study by Nowotny *et al.* (2013) would be the use of a single fat supplement (i.e. soybean oil), as opposed to realistic meal options, and the fact this supplement was particularly high in polyunsaturated fat (61% polyunsaturated, 23% monounsaturated, and 16% saturated), whereas a Westernised diet is typically far higher in saturated fat (van Dam *et al.*, 2002). Additionally, insulin sensitivity was assessed 6 hours post-ingestion (or commencement of continuous lipid-heparin infusion) and, as the responses

associated with the digestion and metabolism of dietary fat typically last between 6 to 8 h (Pramfalk *et al.*, 2015; Pramfalk *et al.*, 2016), it is likely that the subjects were still in a postprandial state upon commencement of the clamp. Elevated levels of both chylomicron triglycerides and incretin hormones (glucagon-like peptide-1 [GLP-1] and gastric inhibitory polypeptide [GIP]) at 6 h support this notion. Thus, the observed decrease in insulin sensitivity may have been a transient response to fat ingestion/infusion, and it remains to be seen whether this response would persist into the postabsorptive state. To address these issues, we determined the effect of a single day of high-fat food intake on whole-body insulin sensitivity. Food intake followed a normal daily feeding pattern (i.e., breakfast, lunch, dinner and snacks), was high in saturated fat, and the assessment of insulin sensitivity took place after an overnight fast of at least 10 h.

4.3 Methods

4.3.1 Subjects

Ten healthy males (their physical characteristics can be seen in Table 4.1) volunteered to participate in this study. The inclusion criteria required subjects to be physically active (exercising at least 3 times per week for more than 30 minutes at a time), non-smokers, free from cardiovascular and metabolic disease, not taking any medication, weight stable for at least 6 months, and with a body mass index (BMI) below 30 kg/m^2. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Loughborough University Ethical subcommittee for human participants. The experimental procedures and possible risks were fully explained to the subjects before their written informed consent was given.

Table 4.1 Subject characteristics before and after 1-day of high-fat overfeeding

	Before overfeeding	**After overfeeding**
Age (years)	22.1 ± 0.5	-
Height (cm)	180.9 ± 2.1	-
Body mass (kg)	86.0 ± 3.2	86.8 ± 3.2 *
BMI (kg/m^2)	26.4 ± 1.1	26.6 ± 1.1*
Body fat (%)	16.2 ± 1.4	-

Data presented are means ± SEM. (n = 10) * denotes a significant change following the dietary intervention ($p < 0.05$).

4.3.2 Pre-testing

Prior to the start of the study, subjects attended the laboratory for an initial assessment of their baseline anthropometric characteristics (height, weight and BMI). This information was then used to estimate their resting energy expenditure (REE) according to the calculations described by Mifflin *et al.* (1990). A standard correction for physical activity level (1.7 times REE) was applied in order to estimate total daily energy requirements. This information was then used to determine individual energy intakes for the 1-day overfeeding period (diet details described later).

4.3.3 Experimental design

After the initial pre-testing visit, subjects attended the laboratory for an oral glucose tolerance test (OGTT; details of which can be seen in the experimental protocol below). Following this initial OGTT, subjects left the laboratory and continued their habitual food intake for 6 days. Subjects were then provided with all food to be consumed on the 7th day. The experimental diet was designed to be high in fat (73% total energy) and provide a severe energy excess (+ 80% kJ). All foods were purchased and prepared by the research team. Subjects were instructed to eat all of the food provided, and to maintain normal, habitual physical activity levels during the intervention period. No subjects reported any issues with diet compliance. Mean energy and macronutrient intake during the intervention period can be seen in Table 4.2. Saturated, monounsaturated and polyunsaturated fats made up 46 ± 0.9%, 37 ± 0.6%, and 9 ± 0.4% of the fat intake, respectively. An example diet plan for one of the subjects can be seen in Table 4.3. The next day (day 8) subjects returned to the laboratory for a second OGTT.

Table 4.2 Estimated daily energy intake and actual energy and macronutrient intake during the high-fat overfeeding period

	Estimated energy requirement (kJ)	**Experimental energy intake (kJ)**	**Experimental macronutrient intake (g)**	**Contribution to total energy intake (%)**
Total	14794 ± 299	26629 ± 483 *	-	-
Fat	-	19336 ± 316	523 ± 9	73
Carbohydrate	-	3307 ± 119	195 ± 7	12
Protein	-	3987 ± 71	235 ± 4	15

Data presented are means ± SEM. (n = 10). * denotes significantly different to estimated energy requirement ($p < 0.05$).

4.3.4 Experimental protocol

On the experimental days before (day 0) and after overfeeding (day 8), subjects reported to the laboratory between 07.00 and 09.00 h after an overnight fast of at least 10 h and having

refrained from physical activity for 48 h. After voiding and being weighed, a 20 gauge Teflon catheter (Venflon, Becton, Dickinson, Plymouth, UK) was inserted into an antecubital vein of one arm to allow for repeated blood sampling during the 2 h OGTT. A baseline (fasted) blood sample (10 mL) was obtained before subjects consumed a 25% glucose solution (75 grams of glucose dissolved in 300 mL of water). Additional 10 mL blood samples were obtained 15, 30, 45, 60, 90 and 120 minutes after glucose ingestion. Following the final blood sample, percentage body fat was assessed using bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat Ltd, Cronkbourne, Isle of Man). The timing of this measurement was selected in order to standardise fluid consumption and hydration status, which can influence the accuracy of BIA measurements (Kushner *et al.*, 1996). BIA measurements were made on the first trial only, as meaningful alterations in body composition would not be expected after a single day of high-fat overfeeding.

Table 4.3 Example 1-day diet plan for one subject

Breakfast	
Foods	Pork sausages (230 g), streaky bacon (120 g), fried eggs (180 g), fried white bread (36 g), whole milk (300 mL)
Protein (g)	86
Carbohydrate (g)	52
Fat (g)	127
Energy (kJ)	7045
% of the days intake	26
Lunch	
Foods	White bread (72 g), butter (15 g), cheddar cheese (70 g), mayonnaise (20 g), sausage roll (90 g)
Protein (g)	31
Carbohydrate (g)	65
Fat (g)	86
Energy (kJ)	4814
% of the days intake	17
Snack	
Foods	Pork pie (200 g)
Protein (g)	22
Carbohydrate (g)	47
Fat (g)	51
Energy (kJ)	3060
% of the days intake	11
Dinner	
Foods	Beef burgers (300 g), streaky bacon (120 g), cheddar cheese (90 g), coleslaw (150 g)
Protein (g)	95
Carbohydrate (g)	7
Fat (g)	173
Energy (kJ)	8135
% of the days intake	30

Dessert	
Foods	Chocolate chip muffin (70 g), double cream (150 mL)
Protein (g)	6
Carbohydrate (g)	37
Fat (g)	98
Energy (kJ)	4357
% of the days intake	16
Total intake	
Protein (g)	240
Carbohydrate (g)	209
Fat (g)	535
Energy (kJ)	27411

Water intake was allowed ad libitum throughout the day

4.3.5 Blood sampling

For analysis of glucose and non-esterified fatty acids (NEFA), whole blood samples were collected in 4.9 mL ethylenediaminetetraacetic acid (EDTA; 1.75 mg/mL) treated tubes (Sarstedt, Leicester, UK) and spun at 1,750 *g* in a refrigerated centrifuge (4°C) for 10 min. The resulting plasma was aliquoted into 1.5 mL Eppendorfs before being stored at -20°C until analysis. For analysis of insulin, whole-blood was collected in 4.5 mL tubes containing a clotting catalyst (Sarstedt, Leicester, UK). Samples were left at room temperature until complete clotting had occurred; after which they were centrifuged at 1,750 *g* in a refrigerated centrifuge (4°C) for 10 min. The resulting serum was aliquoted into 1.5 mL Eppendorfs before being stored at -20°C until analysis.

4.3.6 Analytical procedures

Plasma samples were analysed using commercially available spectrophotometric assays for glucose (Horiba Medical, Northampton, UK) and NEFA (Randox, County Antrim, UK) concentrations using a semi-automatic analyser (Pentra 400; Horiba Medical, Northampton, UK). Serum insulin concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA: EIA-2935, DRG instruments GmBH, Germany). To eliminate inter-assay variation, samples from each participant were analysed in the same run.

4.3.7 Calculations and statistics

Plasma glucose and serum insulin concentrations obtained before and during the OGTT were used to determine whole-body insulin sensitivity using the Matsuda insulin sensitivity index (ISI):

$$\text{ISI} = \frac{10000}{\sqrt{(FPG \times FSI) \times (mean\ OGTT\ insulin\ concentration) \times (mean\ OGTT\ glucose\ concentration)}}$$

Where FPG is the fasting plasma glucose concentration; FSI is the fasting serum insulin concentration; and 10000 represents a constant that allows numbers ranging between 1 and 12 to be obtained. The square root conversion is used to correct the nonlinear distribution of values (Matsuda, & DeFronzo, 1999).

Area under the curve (AUC) for glucose, insulin and NEFA was calculated using the trapezoidal rule with zero as the baseline. Data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS (V21.0) for windows (SPSS Inc, Chicago, IL). Fasting concentrations of glucose, insulin and NEFA before and after high-fat overfeeding were compared using a paired t-test, whereas the dynamic hormonal and metabolic responses to the OGTT were compared using a two-way (trial x time) repeated measures (0-120 min time points) analysis of variance (ANOVA) and Bonferroni *post hoc* analysis where appropriate. Statistical significance was set at $p < 0.05$.

4.4 Results

4.4.1 Weight gain and BMI

Subjects gained 0.85 ± 0.29 kg body mass following 1-day of high-fat overfeeding (p = 0.017, Table 4.1), and their BMI increased by 0.25 ± 0.08 kg/m^2 (p = 0.014, Table 4.1).

4.4.2 Fasting metabolic measures

Fasting substrate and hormone concentrations before and after overfeeding are presented in Table 4.4. Fasting plasma glucose and serum insulin were unaffected by 1-day of high-fat overfeeding (p = 0.48 and p = 0.39, respectively), while fasting plasma NEFA displayed a significant decrease (p = 0.011).

Table 4.4. Fasting metabolic measures before and after 1-day of high fat overfeeding

	Before overfeeding	**After overfeeding**
Plasma glucose (mmol/L)	5.6 ± 0.1	5.6 ± 0.1
Serum insulin (pmol/L)	70 ± 8	76 ± 10
Plasma NEFA (mmol/L)	0.48 ± 0.08	0.21 ± 0.03*

Data presented are means ± SEM. (n = 10). * denotes a significant change following the dietary intervention (p < 0.05).

4.4.3 Oral glucose tolerance test

Substrate and hormone responses to the 2 h OGTT are presented in figure 4.1. Plasma glucose and serum insulin concentrations increased in response to the OGTT, peaking 30-45 min after glucose ingestion (figure 4.1a and 4.1c, respectively). For plasma glucose, there was a significant trial x time interaction (p = 0.003, Figure 4.1a). Postprandial plasma glucose AUC was increased by 16.2% after overfeeding (from 798 ± 41 mmol/L per 120 min before overfeeding to 927 ± 45 mmol/L per 120 min after high-fat overfeeding; p = 0.008, Figure 4.1b). Serum insulin concentrations appeared to follow a similar trend, with AUC increasing by 14.1% after high-fat overfeeding (from 38221 ± 3147 pmol/L per 120 min before overfeeding to 43613 ± 3626 pmol/L per 120 min after overfeeding; Figure 4.1d) but this was not statistically significant (p = 0.085). A significant trial x time interaction was evident for plasma NEFA (p < 0.0001, Figure 4.1e). Nonetheless, average NEFA concentrations throughout the 2 h OGTT were not significantly different between trials (0.23 ± 0.04 mmol/L before overfeeding and. 0.20 ± 0.03 mmol/L after high-fat overfeeding, p = 0.407), and no

difference was evident in NEFA AUC (23 ± 3 mmol/L per 120 min before overfeeding and 22 ± 3 mmol/L per 120 min after overfeeding; $p = 0.974$, Figure 4.1f).

4.4.4 Insulin sensitivity

On average, whole-body insulin sensitivity decreased by 24% following 1-day of high-fat overfeeding (from 5.2 ± 0.5 Matsuda ISI before overfeeding to 4.0 ± 0.5 Matsuda ISI after overfeeding; $p = 0.021$, Figure 4.2).

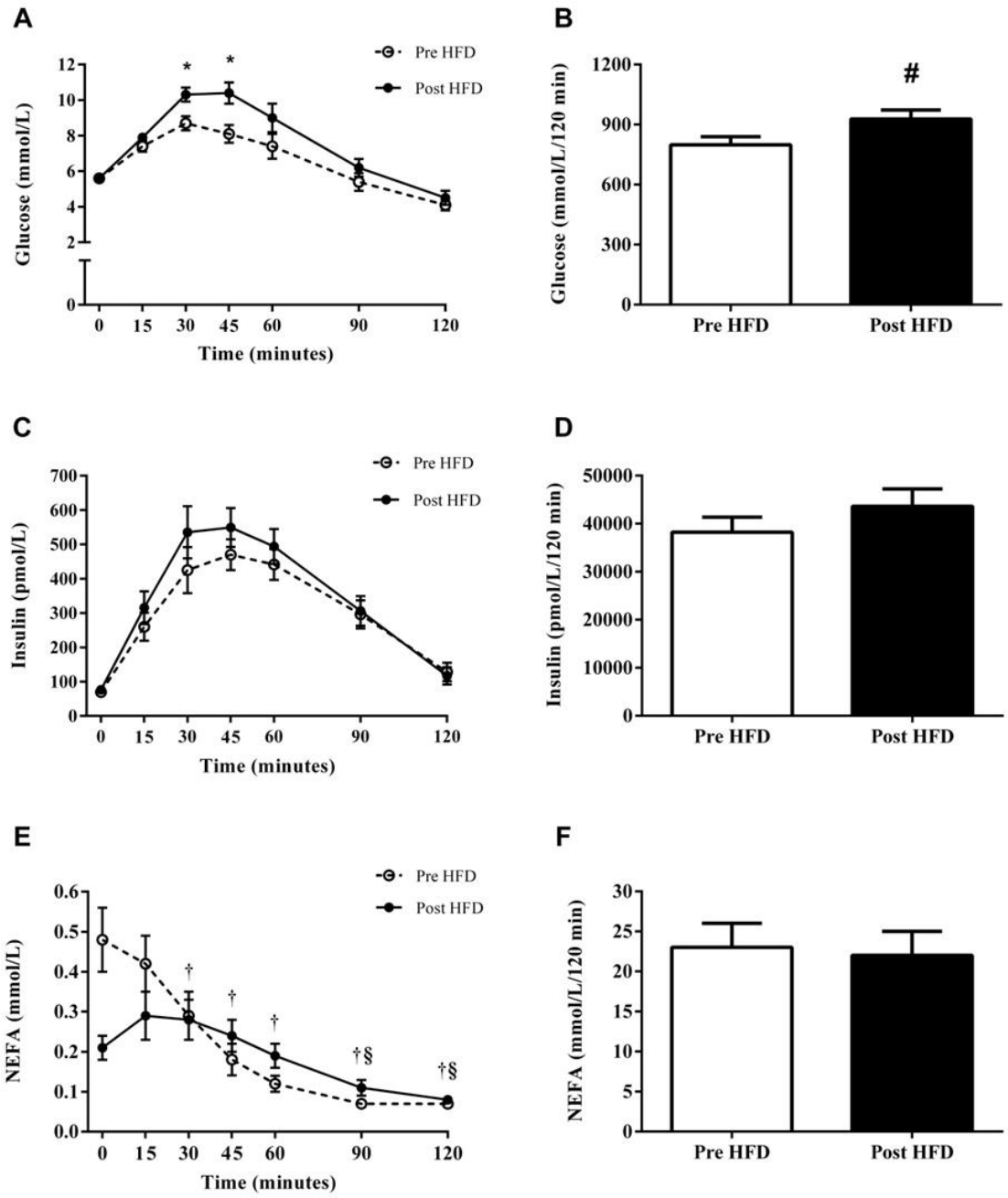

Figure 4.1 Fasting and postprandial plasma glucose (A), serum insulin (C) and plasma NEFA (E) concentrations; and postprandial plasma glucose (B), serum insulin (D) and plasma NEFA AUC (F), during a 2 hour oral glucose tolerance test (OGTT) conducted before (pre) and after (post) 1-day of high-fat overfeeding (HFD). Data presented are means ± SEM (n = 10). * denotes significant difference between trials at the annotated time point. #, denotes significant main effect of trial/high-fat overfeeding ($p < 0.05$). †, denotes significant difference between the annotated time point and 0 min within the pre-overfeeding trial ($p < 0.05$). §, denotes significant difference between the annotated time point and 0 min within the post-overfeeding trial ($p < 0.05$).

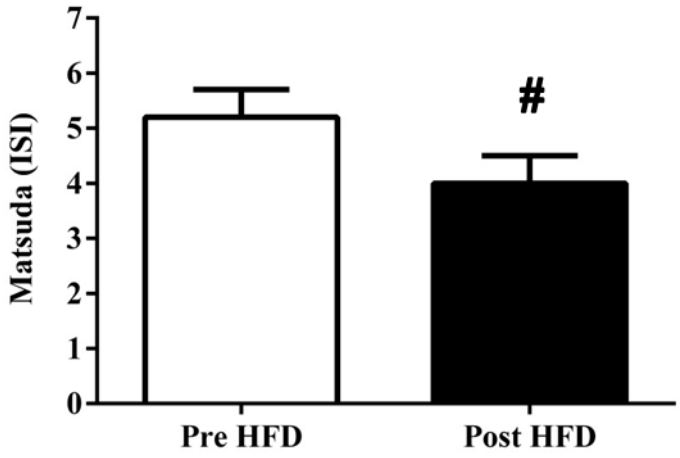

Figure 4.2 The Matsuda insulin sensitivity index (ISI) calculated during an oral glucose tolerance test, conducted before (pre) and after (post) 1-day of high-fat overfeeding (HFD). Data presented are means ± SEM (n = 10). #, denotes significant change following the dietary intervention ($p < 0.05$).

4.5 Discussion

The main finding of the present study was that 1-day of high-fat overfeeding led to a significant 24% reduction in whole-body insulin sensitivity in young, healthy, non-obese males. Intriguingly, this reduction in insulin sensitivity occurred despite a decrease in fasting NEFA concentrations and similar average concentrations throughout the 2 hour OGTT, possibly suggesting that experimental models which acutely elevate plasma NEFA levels may not accurately represent the development of metabolic diseases that are associated with excessive dietary fat intake. Our findings of increased postprandial glucose concentrations occurred alongside a non-significant increase in circulating insulin which indicates an insufficient adaptive response of pancreatic β-cells; a marker of reduced β-cell function.

Previous studies have demonstrated that short-term (3-14 days) high-fat overfeeding can impair glycaemic control (Cornford *et al.*, 2013; Parry *et al.*, 2017; Wulan *et al.*, 2014), and reduce whole-body (Hulston *et al.*, 2015), and hepatic (Brons *et al.*, 2009) insulin sensitivity in healthy individuals. There is, however, a paucity of information available delineating the time-course/initiation of these diet-induced impairments. Evidence from lipid-heparin infusion trials suggest that insulin resistance can be induced within a matter of hours in healthy, lean individuals (Boden *et al.*, 1994; Boden *et al.*, 1991). More recently, Nowotny *et al.* (2013) reported that ingestion of a single oral fat bolus reduced clamp-derived measures of whole-body insulin sensitivity to a comparable degree, and within a corresponding time-frame (6 h post ingestion/infusion), as that seen with iv lipid-heparin infusion, highlighting the rapidity with which dietary fat intake can alter glucose metabolism. A potential confounder of that study relates to the fact that participants were likely still in a postprandial state upon initiation of the hyperinsulinemic-euglycemic clamp, as evidenced by elevated plasma chylomicrons, GIP, and GLP-1 at this time point. It is consequently unclear if the observed reduction in insulin sensitivity in that study were simply a transient response related to the ongoing metabolism of dietary fat. For this reason, we chose to investigate the effects of a single day of high-fat food intake on whole-body insulin sensitivity when measured in the fasting/postabsorptive state. Our results demonstrate that only 1-day of high-fat overfeeding is sufficient to reduce whole-body insulin sensitivity in young, healthy, non-obese individuals.

It has been suggested that elevations in plasma NEFA concentrations are a primary mediator of insulin resistance (Eckel *et al.*, 2005). However, when comparing an oral fat challenge

(100 mL soyabean oil) with iv lipid-heparin infusion, Nowotny *et al.* (2013) observed a similar reduction in insulin sensitivity following both methods of lipid administration, despite divergent NEFA responses. In that study, iv lipid-heparin infusion doubled plasma NEFA concentrations from approximately 400 μmol/L to 800 μmol/L, whereas, fat ingestion had no discernible effect on plasma NEFA. Despite this, both experimental models activated protein kinase C theta (PKCθ) within skeletal muscle, which is suggested to impair insulin signalling by inhibiting the normal tyrosine kinase cascade via phosphorylation of the counter-regulatory serine residue of insulin receptor substrate-1 (IRS-1) (Zick, 2005). These findings suggest that different mechanistic pathways may be activated depending on the method of lipid administration. It also casts doubt on the physiological relevance/validity of using iv lipid-heparin infusion protocols when studying the mechanistic development of insulin resistance, and suggests that dietary models should be applied in order to enhance our understanding of human metabolic disease

In agreement with the findings of Nowotny *et al.* (2013), our data also suggest little, or no, relationship between plasma NEFA concentrations and insulin sensitivity, as we observed a reduction in whole-body insulin sensitivity after high-fat overfeeding despite reduced fasting NEFA concentrations, and similar average NEFA levels across the 2 h OGTT. The reduction in fasting plasma NEFA after high-fat overfeeding may be attributable to a sustained increase in insulin concentration throughout the dietary intervention period due to the large and frequent meals that are characteristic of our high-fat diet. This would lead to a suppression of adipose tissue lipolysis, and a reduction in circulating NEFA. It is also possible, however, that plasma NEFA levels may have been elevated on a meal-to-meal basis throughout the dietary intervention by way of the "spillover" phenomenon, which could explain the reduction in insulin sensitivity. Spillover refers to the proportion of fatty acids that escape into the circulation during the hydrolysis of dietary triglycerides by LPL. It has been shown that feeding repeated boluses of fat in combination with a continuous heparin infusion increases plasma NEFA concentrations (which is almost certainly attributable to spillover) and decreases whole-body insulin sensitivity (Beysen *et al.*, 2003; Beysen *et al.*, 2002). In the present study, the consumption of mixed composition meals during the dietary intervention would increase postprandial insulin concentrations, thereby activating LPL, which, due to the large quantity of dietary fat contained within these meals, may mimic the response seen in the studies of Beysen *et al*., (i.e. a progressive increase in plasma NEFA levels).

Chronic NEFA exposure results in a reduction in insulin synthesis and secretion and β-cell apoptosis (Giacca *et al.*, 2011; Natalicchio *et al.*, 2013; Natalicchio *et al.*, 2015; Poitout *et al.*, 2006; Sako, & Grill, 1990; Zhou, & Grill, 1994). This has been demonstrated in lipid infusion studies where not only do elevated NEFA concentrations induce peripheral insulin resistance, but they also impair the ability of the β-cell to mount a sufficient adaptive response resulting in hyperglycaemia (Carpentier *et al.*, 1999). An intriguing finding of the present study is the indication that β-cell function was reduced, evidenced by the insufficient compensatory increase in postprandial insulin secretion, despite a reduction in fasting NEFA, and similar postprandial NEFA levels. NEFA are an important mediator of β-cell function, potentiating insulin release in response to glucose and non-glucose stimulants (Dobbins *et al.*, 1998; Prentki *et al.*, 2002). It is therefore plausible that the reduction in fasting NEFA is associated with the failure of the β-cell to sufficiently augment insulin secretion and maintain glucose homeostasis. There are a number of potential mechanisms through which this might occur. Firstly, a reduction in the availability of NEFA to bind to the G-protein coupled receptor (GPR)40 on the β-cell membrane may impair intracellular signalling, resulting in a reduced intracellular calcium concentration and decreased exocytosis (Itoh *et al.*, 2003). Secondly, a reduction in NEFA uptake by the β-cell would reduce intracellular fatty acyl-CoA levels. As fatty acyl-CoA mediate insulin release both directly and indirectly (via activation of PKC isoforms) (Prentki *et al.*, 2002), reduced uptake may lead to a reduction in insulin secretion. Notably, our data are unable to determine the mechanisms responsible for the observed reduction in fasting NEFA. In animals models of diet-induced obesity an increased expression of cluster of differentiation (CD)36 in the liver is associated with increased NEFA uptake (Koonen *et al.*, 2007), and elevated expression levels of CD36 have been observed in human NAFLD patients compared to their healthy counterparts(Greco *et al.*, 2008; Mitsuyoshi *et al.*, 2009). It could therefore be hypothesised that our dietary intervention reduces circulating NEFA levels through increased hepatic uptake, this would have implications for hepatic fatty acid partitioning and may be a mechanism of hepatic steatosis (Yki-Jarvinen, 2015). This warrants further investigation.

The reduction in whole-body insulin sensitivity observed in this study is in accordance with previous findings from our laboratory (Hulston *et al.*, 2015; Parry *et al.*, 2017). Our previous work adopted a 7-day overfeeding period so that so that we could study the early metabolic responses to overnutrition without the confounding factor of excessive weight gain or an increase in adipose tissue mass that is seen in dietary interventions of longer duration.

Furthermore, this model consisted of realistic experimental meals, rather than isolated fat sources or loose association food components (e.g. supplementing habitual diets with oil and/or cream etc) which were selected to represent a typical Western diet (i.e. high in energy/total fat/saturated fatty acids). Whilst adherence to this diet-intervention is not typically a problem, participants often comment on the challenging nature of the 7-day overfeeding period. Thus, amending this model to just 1-day might reduce participant burden and further improve participation and retention, whilst also reducing experimental time and the costs associated with providing food. This seems like a feasible option given that the reductions in insulin sensitivity appear similar in magnitude regardless of whether 1 or 7 days of overfeeding was followed (24% in the current study and 27% in Hulston *et al*., 2015). One explanation for this might be the severe lipid overload that we provided in the present study (>500 g of fat intake). In our 7 day experiments we have overfed by ~50% and provided ~65% of the energy as fat, but the shorter duration of this study meant we were able to increase the magnitude of overfeeding to 80% additional energy with 73% of total energy coming from fat intake. Whilst few individuals are likely to consume this much fat in such a short timeframe, the results of this study demonstrate the speed in which insulin sensitivity is impaired through excessive fat intake. However, further characterisation of the metabolic responses associated with 1-day of high-fat overfeeding, and replication of our findings in a more heterogeneous cohort, is required before this model can be advocated for future use.

In conclusion, we have demonstrated that 1-day of high-fat overfeeding impairs whole-body insulin sensitivity in young, healthy, non-obese males. This finding is in accordance with previous studies from our laboratory following a similar dietary intervention, albeit for 7-days. Furthermore, this reduction in insulin sensitivity is seemingly unrelated to circulating plasma NEFA levels.

CHAPTER 5

Diet-induced impairments in glycaemic control do not influence the muscle protein synthetic response to carbohydrate and protein ingestion in young, healthy, lean individuals.

5.1 Abstract

Recent evidence indicates that lipid-induced insulin resistance is associated with insulin resistance and a blunted ability to stimulate muscle protein synthesis (MPS) following amino acid ingestion in young, healthy, lean individuals. Short-term (4-14 days) high-fat overfeeding has also been shown to reduce insulin sensitivity and impair glycaemic control in this population, however, it is currently unknown if the observed diet-induced changes in glucose metabolism impact upon MPS. Therefore, the aim of this study was to determine if 7-days of high-fat overfeeding influences fed-state MPS following carbohydrate and protein ingestion in young, healthy, lean individuals. Thirteen individuals (11 males and 2 females) underwent two experimental trials (before and after consumption of a 7-day high-fat [65%] high-energy [+50%] diet) during which glycaemic control, fed-state mixed muscle protein fractional synthesis rate (FSR) and associated signalling responses were assessed by stable isotope tracer and muscle biopsy techniques. Results demonstrate that 7-days of high-fat overfeeding was associated with an increase in postprandial glucose AUC of 10% ($p = 0.008$) and serum insulin AUC of 17% ($p = 0.086$). Analysis of fed-state mixed muscle protein FSR revealed similar values before and after overfeeding (0.073 ± 0.008 %/h before overfeeding and 0.066 ± 0.006 %/h after overfeeding; $p = 0.432$).The phosphorylation status of anabolic signalling proteins was similar before and after overfeeding, with the exception of $4EBP1^{Thr37/46}$ which was reduced by a mean difference of 8.8% after overfeeding ($p = 0.028$). These findings suggest that diet-induced impairments in glycaemic control do not affect the MPS response to nutrient stimulation in young, healthy, lean individuals. However, the reduction in 4EBP1 phosphorylation may suggest that MPS may be impaired if the diet was continued for a longer duration.

Acknowledgement: Stable isotope tracer analysis presented in this chapter was conducted by Professor Gerrit van Hall and his team at The Clinical Metabolomics Core Facility, Righospitalet.

5.2 Introduction

Skeletal muscle is a critical factor in whole body metabolic health, occupying a central role in the regulation of energy homeostasis (Tzankoff, & Norris, 1977), glycaemic control (Smith, 2002), and whole-body protein metabolism (Wolfe, 2006b). In addition, due to its central involvement in mobility and stability, skeletal muscle is also important in the performance of habitual daily living activities (Wolfe, 2006a). Consequently, the maintenance of muscle mass is vital to both metabolic health and quality of life in general. In humans the maintenance of skeletal muscle mass is dependent on the dynamic equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). On a day-to-day basis, MPS and MPB are largely dependent on food intake, with protein intake being particularly important (Atherton *et al.*, 2010; Wilkinson *et al.*, 2015). Following protein ingestion and absorption, plasma amino acid and insulin levels will increase, thereby stimulating MPS and suppressing MPB. This results in the rate of MPS exceeding MPB during the fed/postprandial state. Conversely, this balance is inverted in the fasting/postabsorptive state (i.e. MPB exceeds MPS) (Atherton, & Smith, 2012). Changes in skeletal muscle mass occur when one of these processes chronically exceed that of the other (i.e. skeletal muscle atrophy will occur if MPB is consistently greater than MPS).

In recent years, one of the emerging concepts in the field of health sciences has been that of "anabolic resistance"; defined as a reduced MPS response to external growth stimuli (Phillips *et al.*, 2012; Rennie, 2009b) Anabolic resistance has been implicated as a major contributor to the loss of skeletal muscle mass observed with aging (Cuthbertson *et al.*, 2005), immobility (Wall, & van Loon, 2013) and critical illness (Rennie, 2009a). Moreover, findings from recent studies suggest that anabolic resistance is also a comorbidity of obesity-induced insulin resistance. For instance, Guillet *et al.* (2009) report that obese, insulin-resistant men exhibit a reduced MPS response to insulin and amino acid stimulation when compared to their healthy-weight counterparts; a finding which is supported by some (Chevalier *et al.*, 2005; Murton *et al.*, 2015; Pereira *et al.*, 2008), but not all (Chevalier *et al.*, 2015) studies. Conversely, none of the above mentioned studies are able to determine the specific contribution of insulin resistance and excess adiposity *per se*, and can merely suggest that one, or both, of these factors may be associated with anabolic resistance. Intriguingly, when investigating differences in fat deposition, Liebau *et al.* (2014) report that upper-body obese women exhibit a greater degree of insulin resistance and a reduced postprandial anabolic response when compared to lower-body obese women who were matched for age, weight and BMI.

This suggests that it is insulin sensitivity, rather than obesity, that mediates anabolic resistance. However, this finding must be interpreted with caution as, in that study, anabolic response was assessed by investigating changes in whole-body protein balance, and direct measures of MPS were not obtained.

In an attempt to control for potentially confounding factors and investigate the impact of insulin resistance *per se* on MPS, Stephens *et al.* (2015) acutely impaired insulin sensitivity in young, healthy males by way of lipid-heparin infusion. In that study, MPS responses to insulin and amino acid stimulation were compared during either 10% Intralipid (100 mL/h), or normal saline infusion. Their results display that mixed-muscle fractional synthetic rate (FSR) increased 2.2-fold in response to insulin and amino acid ingestion during the saline trial. However, this increase in FSR was completely suppressed during the lipid infusion trial, indicating that lipid-induced insulin resistance reduces the anabolic sensitivity of skeletal muscle to nutrient stimulation. This impaired anabolic response appears to be underpinned by defects in translational efficiency, as evidenced by the failure of insulin and amino acids to increase phosphorylation of 4EBP1 above baseline/unstimulated values (Stephens *et al.*, 2015). While this work provides mechanistic insight into the association between lipid-induced insulin resistance and anabolic resistance, lipid infusion protocols represent an experimental situation where circulating NEFA is elevated to supraphysiological levels; a response which is not representative of obesity-induced insulin resistance in humans (Karpe *et al.*, 2011). Thus, it is currently not known as to whether similar responses are observed in experimental models which are more representative of the development of insulin resistance in humans (i.e. the high-fat diet model). Therefore, the purpose of this study was to determine whether 7-days of high-fat overfeeding, a model previously shown by our laboratory to reduce whole-body insulin sensitivity and impair glycaemic control (Hulston *et al.*, 2015; Parry *et al.*, 2017), influences the postprandial skeletal muscle protein FSR, and associated signalling responses, in young, healthy, lean individuals.

5.3 Methods

5.3.1 Subjects

Sixteen healthy individuals were initially recruited to participate in this study but two were excluded due to experimenter concerns regarding dietary compliance. Furthermore, a full data set is unavailable for one participant due to their aversion for the muscle biopsy procedure. Therefore, data is presented for 13 healthy individuals (11 males and 2 females; their physical characteristics can be seen in Table 6.1). Data from 12 of these subjects has already been presented in this thesis (Chapter 3). The inclusion criteria required subjects to be physically active (exercising at least 3 times per week for more than 30 minutes at a time), non-smokers, free from cardiovascular and metabolic disease, not taking any medication, weight stable for at least 6 months, and with a body mass index (BMI) below 30 kg/m^2. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Loughborough University Ethical subcommittee for human participants. The experimental procedures and possible risks were fully explained to the subjects before their written informed consent was given.

Table 5.1 Subject characteristics before and after 7-days of high-fat overfeeding.

	Before overfeeding	**After overfeeding**
Age (years)	23.2 ± 0.7	-
Height (cm)	175 ± 2.1	-
Body mass (kg)	76.4 ± 3.3	77.7 ± 3.4 *
BMI (kg/m^2)	24.7 ± 0.7	25.1 ± 0.7 *

Data presented are means ± SEM (n = 13). * denotes significant change following the dietary intervention ($p < 0.05$).

5.3.2 Pre-testing

Prior to the start of the study, subjects attended the laboratory for an initial assessment of their baseline anthropometric characteristics (height, weight and BMI). This information was then used to estimate their resting energy expenditure (REE) according to the calculations described by Mifflin *et al.* (1990). A standard correction for physical activity level (1.6 and 1.7 times REE for females and males, respectively) was applied in order to estimate total

daily energy requirements. This information was then used to determine individual energy intakes for the week-long overfeeding period (diet details described later).

5.3.3 Experimental design

Approximately 1-week after the initial pre-testing visit after the initial pre-testing visit, subjects reported to the laboratory for a battery of metabolic tests immediately before, and after, a 7-day high-energy, high-fat dietary intervention (described below). Briefly, during each visit, glycaemic control, and fed-state mixed muscle protein FSR and associated signalling responses were examined in response to carbohydrate and protein ingestion with the use of stable isotope tracer and muscle biopsy techniques (described in detail below). The dietary intervention was designed to be high in fat (65% total energy) and provide a severe energy excess (+ 50% kJ). Mean energy intake throughout the dietary intervention was 20018 ± 766 kJ, with 13012 ± 498 kJ provided as fat (more detailed information regarding the dietary intervention can be viewed in chapter 2). All foods were purchased and prepared by the research team. Subjects were instructed to eat all of the food provided, and maintain normal, habitual physical activity levels during the intervention period.

5.3.4 Experimental protocol

On the experimental days (before and after overfeeding) subjects reported to the laboratory between 07.00 and 09.00 h after an overnight fast of at least 10 h and having refrained from physical activity for 48 h. After voiding and being weighed, a 20 gauge Teflon catheter (Venflon, Becton, Dickinson, Plymouth, UK) was inserted into an antecubital vein on each arm to allow for repeated blood sampling and infusion of stable isotope tracers. A baseline, fasting venous blood sample (10 mL) was obtained to determine fasting metabolite concentrations and background isotopic enrichment before a primed constant infusion of [6,6-$^{2}H_{2}$]glucose (0.35 μmol/kg/min, prime 14 μmol/kg) and L-[*ring*-$^{13}C_{6}$]phenylalanine (0.05 μmol/kg/min, prime 2 μmol/kg) was initiated and continued for the duration of the experiment. Blood samples (10 mL) were obtained 90, 105 and 120 min into the infusion period to ascertain isotopic steady state, after which a resting (fasted) muscle biopsy was taken. Biopsies were obtained from the vastus lateralis under local anaesthesia (lidocaine 20 mg/mL) using a 5-mm Bergström needle, modified for use with manual suction. Two sections of muscle tissue, each weighing approximately 30-50 mg, were quickly blotted free of excess blood, snap-frozen in liquid nitrogen, and stored at -80ºC until analysis. A third section of muscle tissue, weighing approximately 10-30 mg, was mounted in Tissue-Tek

OCT (Sakura Finetek UK Ltd, Thatcham, UK) and frozen in isopentane for cryo-sectioning and histology analysis (data to be presented elsewhere). Within 10 min of the biopsy procedure, subjects ingested a mixed carbohydrate and protein beverage (CHO + PRO; described in detail below). Further venous blood samples (10 mL) were obtained at 15, 30, 45, 60, 90 and 120 min post-ingestion, and additional muscle biopsies were obtained at 30 and 120 min post-ingestion. Each biopsy was taken from a separate incision on the same leg, spaced ~3-4 cm apart. Biopsies were obtained from the opposite leg during the second experimental trial (after overfeeding). All stable isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA). A schematic of the experimental protocol can be viewed in Figure 6.1.

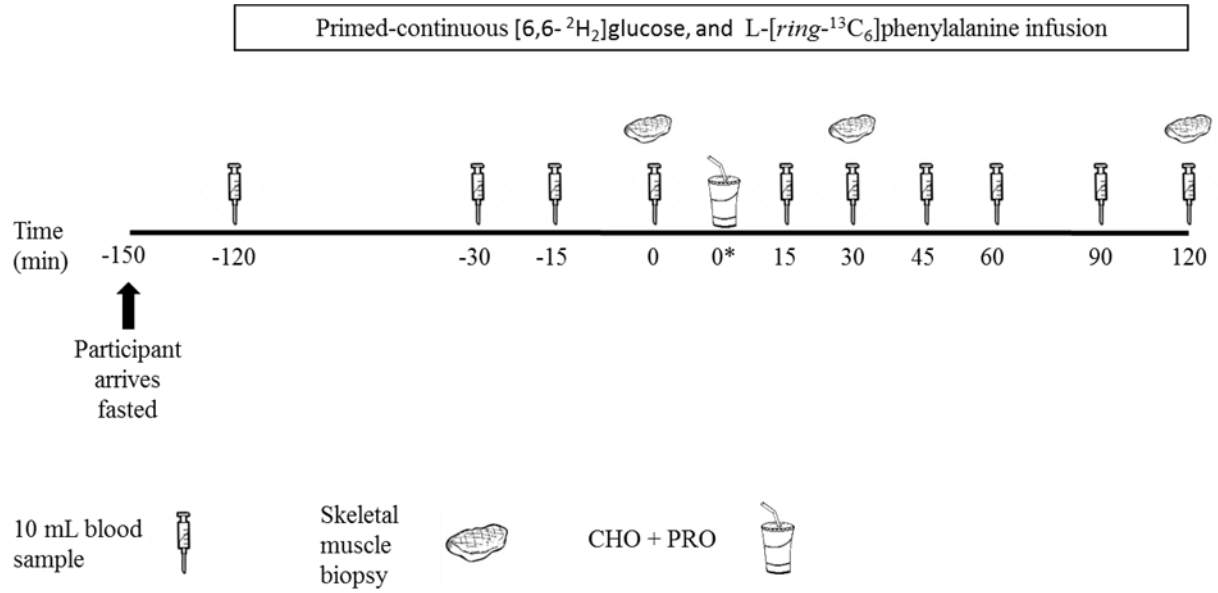

Figure 5.1 Schematic of experimental protocol. CHO + PRO = 50 g glucose (48.4 g unlabelled and 1.6 g [U-^{13}C]glucose) and 15 g whey protein with the addition of 27 mg L-[*ring*-$^{13}C_6$]phenylalanine. * denotes an approximate 10 minute delay between sampling (skeletal muscle and blood) and ingesting the CHO + PRO beverage.

5.3.5 Beverages

The CHO + PRO beverage was a 12.5% glucose solution (50 g glucose [48.4 g unlabelled glucose and 1.60 g [U-^{13}C]glucose] dissolved in 400 mL of water) with the addition of 15 g whey protein in the form of a commercially available protein supplement (Volac; UltraWhey 90, Hertfordshire, UK). The amino acid content of the protein was (in percent content wt:wt): Alanine, 5; Arginine, 2.1; Aspartic acid, 11; Cystine, 2.2; Glutamic acid, 18.1; Glycine, 1.4;

Histidine, 1.7; Isoleucine, 6.4; Leucine, 10.6; Lysine, 9.6; Methionine, 2.2; Phenylalanine, 3; Proline, 5.5; Serine, 4.6; Threonine, 6.7; Tryptophan, 1.4; Tyrosine, 2.6, and Valine, 5.9. An additional 27 mg of L-[*ring*-$^{13}C_6$]phenylalanine was added to the beverage in order to minimise shifts in isotopic steady state as a result of consuming unlabelled phenylalanine in the protein supplement. The amount of labelled phenylalanine added to the test solution was determined assuming that the tracer infusion rate would elicit a plasma TTR of approximately 0.06.

5.3.6 Blood sampling

For analysis of glucose and NEFA concentrations and phenylalanine concentration and enrichment, whole blood samples were collected in 4.9 mL Ethylenediaminetetraacetic acid (EDTA; 1.75 mg/mL) treated tubes (Sarstedt, Leicester, UK) and spun for 10 min at 1,750 *g* in a refrigerated centrifuge (4°C). The resulting plasma was aliquoted into 1.5 mL Eppendorfs and stored at -20°C until analysis. For analysis of insulin, whole blood was collected in 4.5 mL tubes containing a clotting catalyst (Sarstedt, Leicester, UK). Samples were left at room temperature until complete clotting had occurred; after which they were also spun for 10 min at 1,750 *g* in a refrigerated centrifuge (4°C). The resulting serum was aliquoted into 1.5 mL Eppendorfs and stored at -20°C until analysis.

5.3.7 Analytical procedures

Plasma NEFA concentrations were analysed using commercially available spectrophotometric assays (Randox, County Antrim, UK). Plasma glucose concentrations were analysed by the addition of a tracer (M+13) internal standard and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Chapter 3). Serum insulin concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA: EIA-2935, DRG instruments GmBH, Germany).

5.3.8 Plasma phenylalanine concentration and enrichment analysis

A 200 µl aliquot of plasma was mixed with 50 µL of internal standard (105 µmol/L, L-[*ring*-$^{13}C_9$]phenylalanine) and 500 µL of 50% acetic acid before being passed through a strong cation exchange column (Dowex AG 50W-X8; BioRad, Hemel Hempstead, UK). The purified amino acids were eluted with 3 mL of 2 M ammonium hydroxide (NH_4OH), dried under a stream of nitrogen, and converted to their t-butyldimethylsilyl derivatives by adding 200 µL of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide and acetonitrile (1:3) and

heating for 1 h at 70ºC. The enrichment of the sample and the internal standard was determined using gas chromatography-tandem mass spectrometry (GC–MS/MS) with electron impact ionisation and selective ion monitoring for 336, 342 and 345 m/z, for phenylalanine, L-[*ring*-$^{13}C_6$]phenylalanine, and L-[*ring*-$^{13}C_9$]phenylalanine, respectively.

5.3.9 Muscle phenylalanine concentration and enrichment analysis

At least 30 mg of wet weight tissue was freeze-dried and dissected free from visible blood, fat and connective tissue. From the cleaned fibre mass, 3-5 mg was used for analysis. The precise mass of each sample was recorded before adding 100 µL of L-[*ring*-$^{13}C_9$]phenylalanine. The sample was then extracted three times with 500 µL of 2% perchloric acid. Supernatants were then pooled and processed in the same manner as plasma (described above) for determination of intracellular phenylalanine concentration and enrichment. The remaining protein pellet was then washed twice with 1 mL of 70% EtOH, once with 1 mL of 100% EtOH, dried under a stream of N_2 and hydrolysed in 1 mL of 6 M HCl that was heated to 120ºC for 15-18 h. Once hydrolysed, the protein fraction was dried at 120ºC and dissolved in 500 µL of 50% acetic acid. Samples were then treated in the same manner as plasma samples except that column purified amino acids were then converted to their N-acetyl N-propyl esters for determination of protein bound phenylalanine enrichment using gas chromatography-combustion-isotope ratio mass spectrometry (GC–C–IRMS, Hewlett Packard 5890-Finnigan GC combustion III-Finnigan Deltaplus; Finnigan MAT, Bremen, Germany). As fed-state FSR was calculated using the 0 and 120 minute samples phenylalanine concentration and enrichment analysis was only performed on these samples.

5.3.10 Muscle protein fractional synthesis rate

Mixed muscle protein FSR was determined using the standard precursor-product method:

$$\text{FSR (\%/h)} = \frac{\Delta E_{p\ phe}}{(E_{ic\ phe} t)} \text{ X } 100$$

Where, $\Delta E_{\text{p phe}}$ is the change in protein bound enrichment between the muscle biopsies at time point 1 and 2, $E_{\text{ic phe}}$ is the mean intracellular phenylalanine enrichment between muscle biopsies at time point 1 and 2, and *t* is the period of tracer incorporation in time (hours). A factor of 100 is used to express FSR in percent per hour (%/h).

5.3.11 Western blotting

The optimisation procedures for Western blotting analysis performed in this thesis are presented in Appendix A. Approximately 30-50 mg of frozen muscle tissue was homogenised in 10 µL/mg ice-cooled buffer (1 x PBS containing 1 % Triton X-100, 1% protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL), and 1% 0.5 M EDTA (Thermo Scientific, Rockford, IL) using a TissueLyser II (Qiagen, Hannover, Germany). All samples were disrupted by the TissueLyser II for 2 x 2 min at 20 Hz. Homogenates were then centrifuged for 10 min at 13,300 *g*. The resulting supernatant was removed and aliquoted into 1.5 mL Eppendorfs prior to being stored at -80˚C until analysis. Protein concentration was determined in aliquots of supernatant diluted 1:5, 1:10, and where necessary 1:20 in 1 x PBS using the Pierce 660 nm protein assay (Thermo Scientific, Rockford, IL, USA). After protein determination, homogenates were mixed in sample buffer containing 25% NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA), 5% β-mercaptoethanol and distilled water. The ratio of lysate to distilled water was amended for each sample to produce two distinct gel samples with protein concentrations of 1.5 µg/µL and 2.5 µg/µL. Samples were then vortexed, and heated at 95˚C for 5 minutes in order to denature proteins.

For analysis of p-Akt^{Ser473}, p-eEF2^{Thr56}, and p-4EBP1$^{Thr37/46}$, 15 µg (10 µL of the1.5 µg/µL gel samples) of protein was loaded onto NuPAGE 10% Bis-Tris Gels (Invitrogen, UK). Due to being lowly expressed, 40 µg (16 µL of the 2.5 µg/µL gel samples) of protein was loaded for analysis of p-P70S6K1^{Thr389}. Gels were then run for 2 h at 125 V in 1 x NuPAGE MOPS SDS running buffer (Invitrogen, UK) to allow for ample separation of proteins. For p-mTOR^{Ser2448}, 15 µg of protein was loaded onto NuPAGE 3-8% Tris-Acetate Gels (Invitrogen, UK), before being ran for 35 min at 150 V in 1 x NuPAGE Tris-Acetate SDS running buffer (Invitrogen, UK). Following separation, all proteins were transferred onto PVDF membrane (Invitrogen, UK) for 1 h (p-Akt^{Ser473}, p-eEF2^{Thr56} and p-mTOR^{Ser2448}) or 2 h (p-P70S6K1^{Thr389} and p-4EBP1$^{Thr37/46}$) at 30 V in 1 x NuPAGE Transfer Buffer (Invitrogen, UK) containing 10% methanol, with the exception of p-mTOR^{Ser2448} for which no methanol was added. After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS: 50 mmol/L Tris base, 150 mmol/L NaCl, pH 7.6) containing 0.5 % Tween-20 (TBST), and either 5% BSA (p-eEF2^{Thr56} and p-mTOR^{Ser2448}) or 5% NFDM (p-Akt^{Ser473}, p-4EBP1$^{Thr37/46}$ and p-P70S6K1^{Thr389}). Membranes were then incubated overnight at 4˚C in 1 x TBST and 2-5% BSA or NFDM with commercially available primary antibodies (Table 6.2). The next morning, membranes were serially washed in TBST (3 x 5 min and 1 x 15 min) before being

incubated for 1 h at room temperature in 1 x TBST and 2-5% BSA or NFDM with a commercially available secondary antibody (Table 6.2). Following secondary antibody incubation, membranes were serially washed (3 x 5 min and 1 x 15 min) in TBST before being incubated in enhanced chemiluminescent substrate at room temperature in the dark for 5 minutes. Finally, membranes were blotted free of excess chemiluminescent and antibodies bound to target proteins were visualised on a Bio-Rad Chemi-doc imaging system. Membranes were exposed long enough to ensure clear, non-saturated bands for all samples. Bands apparent at expected molecular weights were manually defined with sample boxes fitted as tightly as possible and the volume of band densities were determined using Quantity One image-analysis software. All samples were normalised to Coomassie staining of membranes to control for loading. The phosphorylation of target proteins was used as an indirect measure of their activity.

5.3.12 Calculations and statistics

Area under the curve (AUC) for glucose, insulin and NEFA was calculated using the trapezoidal rule with zero as the baseline. All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS (V21.0) for windows (SPSS Inc, Chicago, IL). Paired t-tests were used to compare differences in body mass, BMI, fasting glucose, NEFA and insulin concentrations, and fed-state skeletal muscle FSR before and after overfeeding. All remaining data were compared using a two-way (trial x time) repeated measures analysis of variance (ANOVA) and Bonferroni *post hoc* analysis where appropriate. Statistical significance was set at $p < 0.05$.

Table 5.2 Primary and secondary antibody information.

Antibody	**Product No.**	**Isotype**	**Molecular weight (kDa)**	**Antibody dilution (w/v with diluent)**	**Diluent in TBST**
p-Akt^{ser473}	4060	Rabbit	60	1:5000	5% NFDM
p-mTOR^{Ser2488}	5536	Rabbit	289	1:2000	2.5% BSA
p-eEF2^{Thr56}	2331	Rabbit	95	1:1000	5% BSA
p-P70S6K1^{Thr389}	9234	Rabbit	70, 85	1:1000	2% NFDM
p-4EBP1$^{Thr37/46}$	2855	Rabbit	15-20	1:1000	2% NFDM
Secondary Antibody					
Anti-rabbit IgG, HRP-linked	7074	Goat	--	1:2000	Equivalent to the primary antibody of interest.

All antibodies were purchased from Cell Signaling Technology (Danvers, USA)

5.4 Results

5.4.1 Weight gain and BMI

All 13 subjects gained body mass following the overfeeding intervention (increasing by 1.4 ± 0.3 kg; $p < 0.0001$, Table 6.1), leading to an increase in BMI of 0.44 ± 0.09 kg/m^2 ($p < 0.0001$, Table 6.1).

5.4.2 Fasting metabolic responses

Fasting substrate and hormone concentrations are presented in table 6.3. Fasting plasma glucose displayed a tendency to increase after overfeeding, but this did not reach significance ($p = 0.08$). Serum insulin increased after overfeeding ($p = 0.026$), while plasma NEFA decreased ($p = 0.004$).

Table 5.3 Fasting substrate and hormone concentrations before and after 7-days high-fat overfeeding.

	Before overfeeding	**After overfeeding**
Glucose (mmol/L)	4.93 ± 0.09	5.09 ± 0.08
Insulin (pmol/L)	67 ± 7	82 ± 8 *
NEFA (mmol/L)	0.58 ± 0.08	0.35 ± 0.05 *

Data presented are means ± SEM (n = 13). Fasting values represent mean values across the -30 - 0 min period before CHO + PRO ingestion. * denotes significant change following the dietary intervention ($p < 0.05$).

5.4.3 Substrate and hormone responses to carbohydrate and protein ingestion

Substrate and hormone responses to CHO + PRO ingestion before and after overfeeding are presented in Figure 6.2. Plasma glucose concentrations increased after CHO + PRO ingestion, peaking at the 30 minute time point in both trials. Seven days of high-fat overfeeding increased plasma glucose AUC by 10% (from 610 ± 23 mmol/L per 120 min before overfeeding to 672 ± 19 mmol/L per 120 min after overfeeding; $p = 0.008$, figure 6.2b). A significant trial x time interaction was evident for plasma glucose ($p = 0.043$, figure 6.2a). Serum insulin AUC tended to increase by 17% after overfeeding (from 34554 ± 4200 pmol/L per 120 min before overfeeding to 40518 ± 3382 pmol/L per 120 min after overfeeding), but this did not reach statistical significance ($p = 0.085$, figure 6.2d). Consistent with plasma glucose, a significant trial x time interaction was also evident for serum insulin

(p = 0.006, figure 6.2b). Plasma NEFA concentrations decreased in response to CHO + PRO ingestion, reaching a nadir at 90 minutes post-ingestion in both trials (figure 6.2e). Plasma NEFA also displayed a significant time x trial interaction (p < 0.0001, figure 6.2e). Serum NEFA AUC was reduced by 19.5% after overfeeding (from 26.6 ± 2.5 mmol/L per 120 min before overfeeding to 21.4 ± 1.8 mmol/L per 120 min after overfeeding; p = 0.039, figure 6.2f).

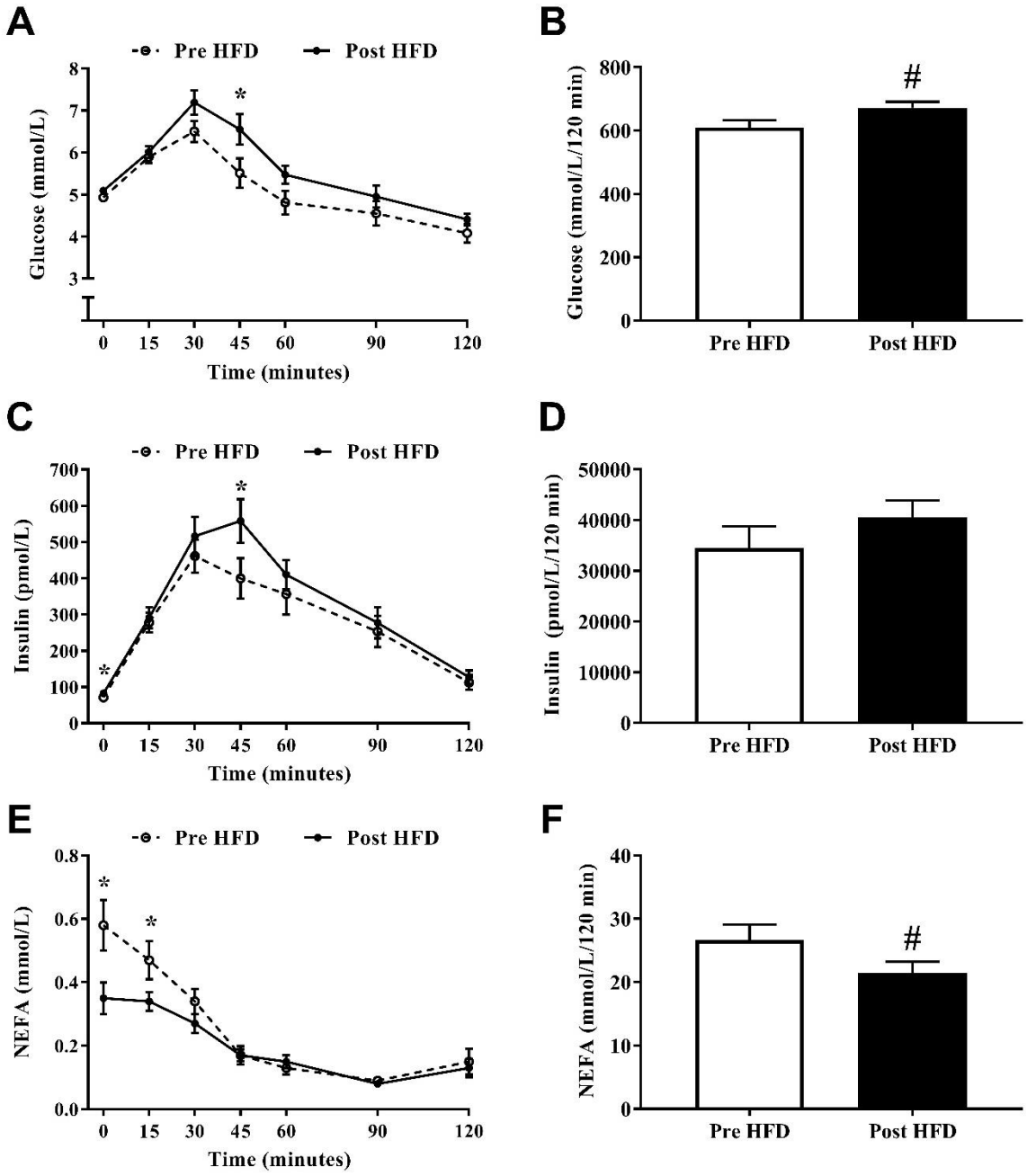

Figure 5.2 Fasting and postprandial plasma glucose (A), serum insulin (C), and plasma NEFA (E) concentrations, and plasma glucose (B), serum insulin (D), and plasma NEFA

AUC (F), before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Time point 0 represents mean (-30-0 minutes) fasting values. Data presented are means ± SEM (n = 13). * denotes significant difference between trials at the annotated time point ($p < 0.05$). # denotes significant main effect of trial/high-fat overfeeding ($p < 0.05$).

5.4.4 Plasma and intracellular phenylalanine concentration

Ingestion of CHO + PRO led to an increase in plasma phenylalanine concentrations, reaching a peak at the 30-45 minute time point (figure 6.3). No difference in plasma phenylalanine concentrations were evident between trials ($p = 0.244$). Intracellular phenylalanine concentrations also exhibited a main effect of time, ($p < 0.0001$), with the biopsy taken 120 minutes post CHO + PRO ingestion exhibiting a lower concentration than the baseline/fasted biopsy (Table 6.4). Again, no difference in intracellular phenylalanine concentrations were evident between trials ($p = 0.696$).

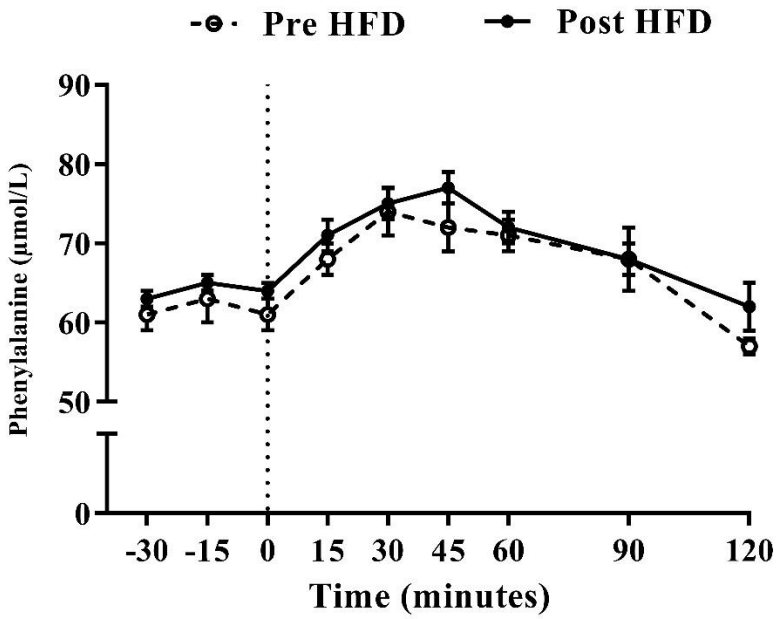

Figure 5.3 Fasting and postprandial plasma phenylalanine concentrations before (pre) and after (post) 7-days of high-fat overfeeding (HFD). Time points -30 – 0 min represent the final 30 min of the 2-h pre-infusion period. All subsequent time points are following the ingestion of CHO + PRO (indicated by dotted line). Data presented are means ± SEM (n = 13).

5.4.5 Plasma and intracellular [$^{13}C_6$]phenylalanine enrichment

Plasma [$^{13}C_6$]phenylalanine enrichment displayed a progressive increase over time, peaking at 120 minutes in both trials (figure 6.4a). This response was not influenced by high-fat overfeeding ($p = 0.244$). Intracellular [$^{13}C_6$]phenylalanine enrichment also displayed an increase over time (figure 6.4b). Again, this response was not influenced by high-fat overfeeding ($p = 0.659$). The gradual increase in isotopic enrichment within both plasma and intracellular amino acid pools is most likely attributable to an insulin-induced suppression of whole-body protein breakdown following ingestion of the test beverage. This would reduce isotopic dilution due to a decreased rate of release of unlabelled amino acids from the protein pool.

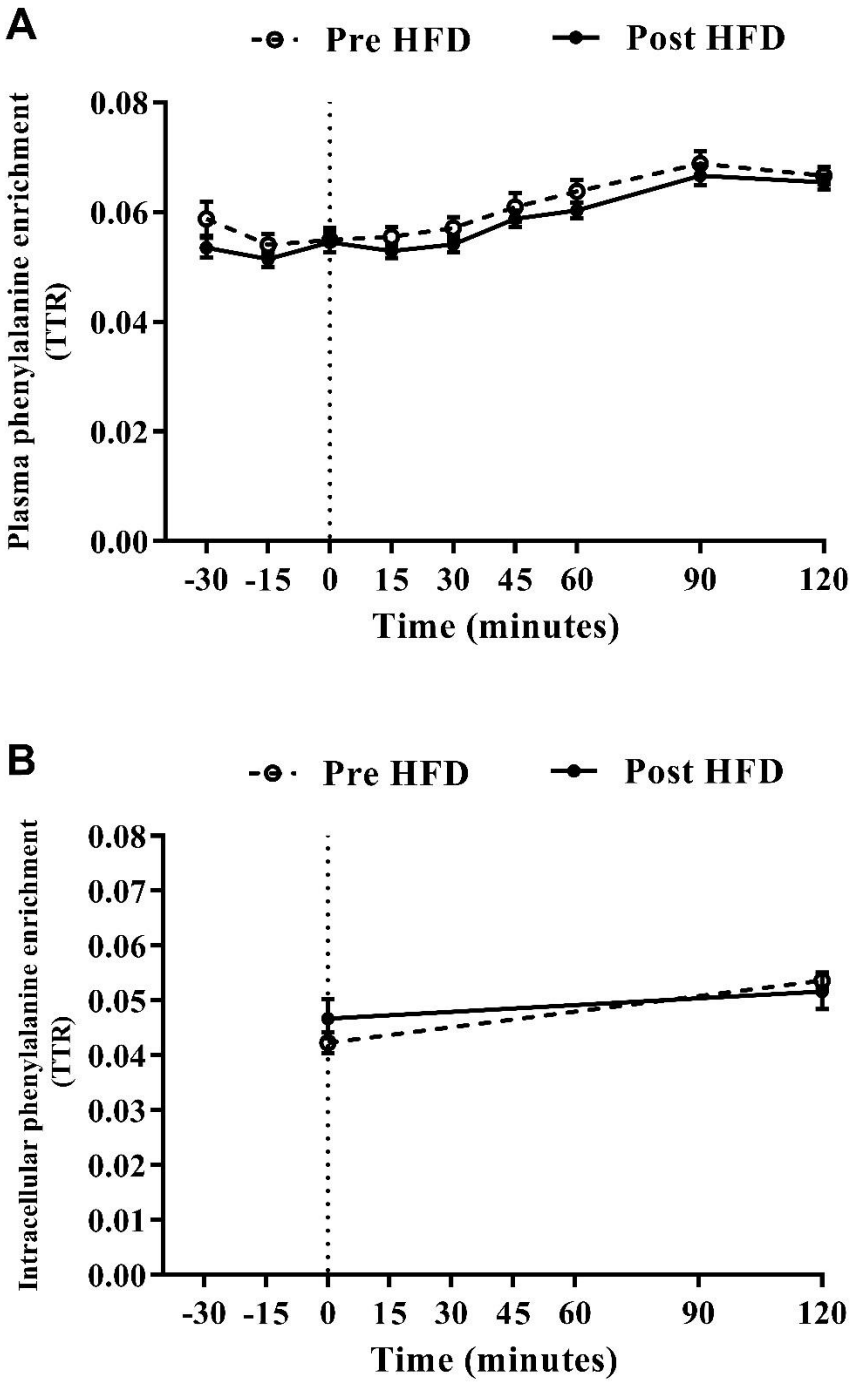

Figure 5.4 Plasma (A) and intracellular (B) [$^{13}C_6$]phenylalanine enrichment before (pre) and after (post) 7-days high-fat overfeeding (HFD). Time points -30 – 0 min represent the final 30 min of the 2-h pre-infusion period. All subsequent time points are following the ingestion of CHO + PRO (indicated by dotted line). Data presented are means ± SEM (n = 13).

Table 5.4 Intracellular phenylalanine concentration, [$^{13}C_6$]phenylalanine enrichment and change in muscle bound protein enrichment before (0), and 120 minutes after CHO + PRO ingestion, before and after 7-days of high-fat overfeeding.

	Before overfeeding		**After overfeeding**	
	0	**120**	**0**	**120**
Intracellular concentration (μmol/L)	54 ± 2	42 ± 2 *	51 ± 2	43 ± 2 *
Intracellular enrichment (TTR)	0.042 ± 0.002	0.054 ± 0.002	0.047 ± 0.004	0.052 ± 0.003
Δ muscle protein enrichment (TTR)	—	0.000067 ± 0.000007	—	0.000070 ± 0.000006

Data presented are means ± SEM (n = 13). * denotes significant difference between the fasting (0) and 120 minute biopsies ($p < 0.05$).

5.4.6 Mixed muscle protein synthesis

Fed-state mixed muscle protein FSR were similar before and after high-fat overfeeding (0.073 ± 0.008 %/h before overfeeding and 0.066 ± 0.006 %/h after overfeeding; $p = 0.432$, figure 6.5). This suggests that MPS is not affected by high-fat, overfeeding-induced impairments in glycaemic control.

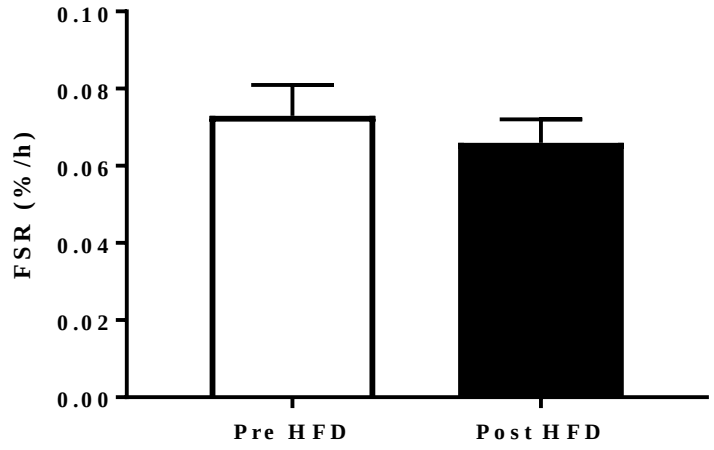

Figure 5.5 Mixed muscle protein FSR following CHO + PRO ingestion before (pre) and after (post) 7-days high-fat of high-fat overfeeding (HFD). Data presented are means ± SEM (n = 13).

5.4.7 Anabolic signalling

The phosphorylation status of skeletal muscle Akt^{ser473}, $mTOR^{Ser2488}$, $eEF2^{Thr56}$, $P70S6K1^{Thr389}$, and $4EBP1^{Thr37/46}$ are presented in figure 6.6a-e respectively. Phosphorylation of Akt^{ser473} increased approximately 2-fold 30 minutes after CHO + PRO ingestion, before decreasing again at 120 minutes. Phosphorylation of $mTOR^{Ser2488}$ and $P70S6K1^{Thr389}$ also increased 30 minutes post ingestion by approximately 20%, and 2-fold, respectively. The phosphorylation status of these signalling intermediates remained elevated above baseline at the 120 minute time point. Phosphorylation of $eEF2^{Thr56}$ was slightly (7.4%) decreased at the 120 minute time point when compared to baseline. Seven days of high-fat, overfeeding did not alter the phosphorylation status of Akt^{ser473}, $mTOR^{Ser2488}$, $eEF2^{Thr56}$, and $P70S6K^{Thr389}$. However, there was an overall main effect of trial/high-fat overfeeding for $4EBP1^{Thr37/46}$ phosphorylation, which was reduced by a mean difference of 8.8% after overfeeding (p = 0.028). No difference in $4EBP1^{Thr37/46}$ phosphorylation was evident in response to CHO + PRO ingestion. Representative western blot images are presented in Figure 6.7.

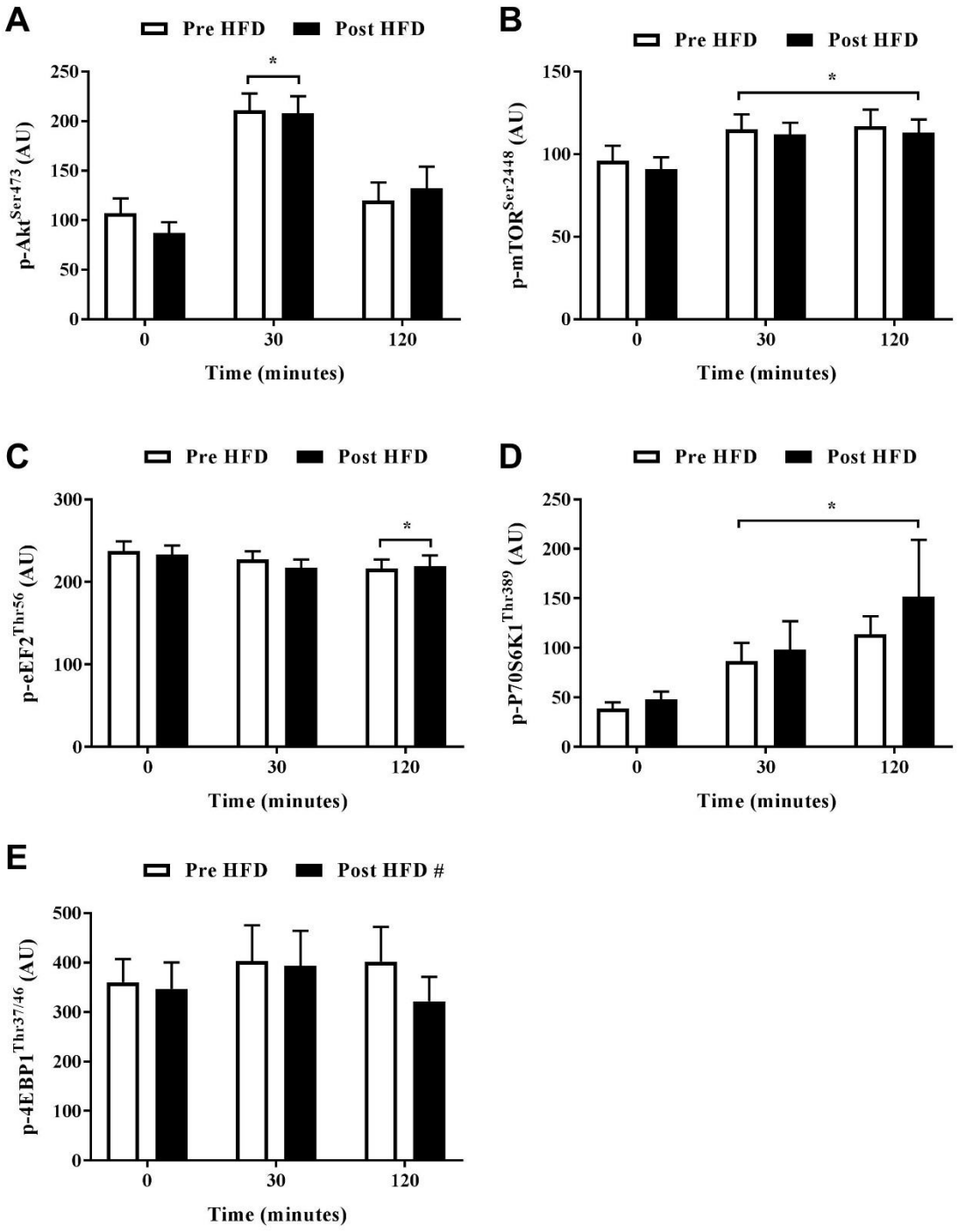

Figure 5.6 Phosphorylation of skeletal muscle Akt^{ser473} (A), mTOR^{Ser2488} (B), eEF2^{Thr56} (C), P70S6K1^{Thr389} (D) and 4EBP1$^{Thr37/46}$ (E) at baseline (0) and 30 and 120 min after CHO + PRO ingestion before (pre) and after (post) high-fat overfeeding (HFD). Data presented are means ± SEM (n = 13). AU, arbitrary units. # denotes significant main effect of trial/high-fat overfeeding ($p < 0.05$). *, denotes significant difference from baseline measurement (time 0) ($p < 0.05$).

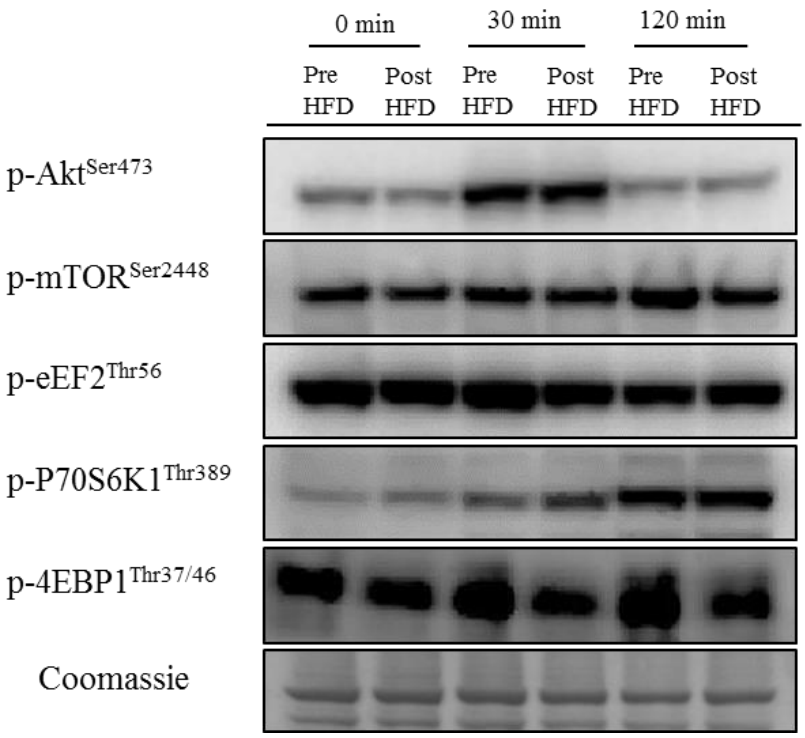

Figure 5.7 Representative blots of phosphorylated (p) Akt^{Ser473}, p-mTOR^{Ser2488}, p-eEF2^{Thr56}, p-P70S6K1^{Thr389} and p-4EBP1$^{Thr37/46}$, and Coomassie staining at baseline (0), 30, and 120 min after CHO + PRO ingestion, before (pre) and after (post) high-fat overfeeding (HFD).

5.5 Discussion

The main finding of the present study was that 7-days of high-fat overfeeding impaired glycaemic control in young, healthy, lean individuals as evidenced by a significant 10% increase in postprandial glucose AUC and similar (arguably elevated) serum insulin AUC (17% non-significant increase). In addition, overfeeding was also associated with an overall reduction in the phosphorylation status of 4EBP1$^{Thr37/46}$. However, while 4EBP1 is considered one of the principal regulators of translation initiation, this reduction in phosphorylation did not influence fed-state skeletal muscle protein FSR, with similar values observed before and after the overfeeding intervention. These findings suggest that diet-induced impairments in glycaemic control do not affect the MPS response to nutrient stimulation in young, healthy, non-obese individuals.

There is currently contention in the literature as to whether insulin resistance impairs the anabolic sensitivity of skeletal muscle, with some (Chevalier *et al.*, 2005; Guillet *et al.*, 2009; Murton *et al.*, 2015; Pereira *et al.*, 2008), but not all (Chevalier *et al.*, 2015), studies reporting that obese, insulin resistant individuals exhibit a reduced MPS response to nutrient stimulation. In humans the maintenance of skeletal muscle mass is dependent on the balance between MPS and MPB (Atherton, & Smith, 2012). Thus, reductions in MPS may lead to an imbalance between these processes resulting in skeletal muscle atrophy. This is important as relative muscle mass is inversely related to whole-body insulin resistance (Srikanthan, & Karlamangla, 2011) and reductions in mass may therefore further exacerbate the metabolic derangements seen in obesity. Furthermore, T2DM is associated with impaired skeletal muscle function and an accelerated loss of lean mass with aging, this would have implications for mobility and stability and has been reported to be a key regulator of morbidity and mortality in the elderly (Kim *et al.*, 2010; McLeod *et al.*, 2016; Park *et al.*, 2009; Park *et al.*, 2006; Park *et al.*, 2007). However, studying obese insulin resistant individuals makes it difficult to disentangle the effects of insulin resistance from the other metabolic alterations seen in obesity. In an attempt to address this question, Stephens *et al.* (2015) investigated the MPS response in healthy, lean individuals during a hyperinsulinemic-euglycaemic clamp with supplementary amino acid ingestion, and concomitant intravenous (iv) infusion of either 10% Intralipid (100 mL/h) or normal saline. The findings of that study demonstrate that lipid infusion was associated with a 20% reduction in peripheral glucose disposal, and a complete absence of the 2.2-fold increase in FSR observed during saline infusion. From this finding, the authors conclude that lipid-induced insulin resistance blunts

the anabolic response to insulin and amino acid stimulation. However, an alternative model of investigating the progression of insulin resistance is through the consumption of a high-fat diet for several days/weeks, and it is unclear as to whether diet-induced alterations in glucose metabolism influence MPS. Thus, in the present study we have investigated the effect of 7-days of high-fat overfeeding on skeletal muscle FSR in young, healthy, non-obese individuals. In contrast to the findings reported by Stephens *et al.* (2015), we observed impairments in postprandial glycaemic control, whereas fed-state skeletal muscle FSR was seemingly unaffected.

The differences between the results reported in the present study and those of Stephens *et al.* (2015) are likely attributable to the different models of insulin resistance utilised (i.e. iv lipid heparin infusion and high-fat overfeeding). It has previously been shown that iv lipid heparin infusion can rapidly (within 3-4 h) induce insulin resistance in healthy individuals, which is associated with a supraphysiological rise in circulating NEFA concentrations (Boden, & Jadali, 1991; Roden *et al.*, 1996; Szendroedi *et al.*, 2014). This method was developed based on early observations that enlarged adipose tissue mass results in elevated plasma NEFA levels (Opie, & Walfish, 1963), and has thus provided a hypothetical mechanistic link between obesity and insulin resistance (Eckel *et al.*, 2005). However, a recent systematic review by Karpe *et al.* (2011) demonstrates that plasma NEFA concentrations are not simply determined by increased adiposity, and even severe obesity-induced insulin resistance can occur without elevations in circulating NEFA levels. Moreover, our data demonstrate impaired glycaemic control despite a reduction in fasting and postprandial NEFA concentrations, a finding which is in accordance with previous observations from our laboratory (Parry *et al.*, 2017), and other short-term overfeeding studies (Brons *et al.*, 2009; Cornford *et al.*, 2013). Combined, these findings cast doubt on the NEFA hypothesis of insulin resistance and the physiological relevance of lipid infusion protocols. Furthermore, intravenous infusion of fatty acids has previously been demonstrated to reduce MPB (Gormsen *et al.*, 2008; Keller *et al.*, 2002). Increased circulating insulin concentrations are also known to reduce MPB (Fryburg *et al.*, 1990; Gelfand, & Barrett, 1987; Greenhaff *et al.*, 2008). Thus, it is possible that the reduction in fed-state MPS observed by Stephens *et al.* (2015) is attributable to a decrease in intracellular amino acid availability resulting from the combined suppressive effects of elevated concentrations of NEFA (from the iv lipid heparin infusion) and insulin (from the hyperinsulinemic-euglycaemic clamp) on MPB. Indeed, the reduction in postprandial plasma phenylalanine concentrations observed in that study during

the lipid infusion trial are indicative of reduced MPB. However, this hypothesis cannot be confirmed as the authors did not present intracellular amino acid concentrations, although intracellular tracer enrichment (which would be influenced by changes in amino acid concentration) were similar between the lipid and saline infusion trials.

Notably, our choice of experimental cohort may have influenced findings. In the present study we chose to investigate the impact of 7-days of high-fat overfeeding in young, healthy, lean participants who were recreationally active (performing moderate to vigorous intensity exercise at least 3 times per week for more than 30 minutes at a time). It has previously been demonstrated that a potent inhibitor of MPS is immobility (Ferrando *et al.*, 1996; Ferrando *et al.*, 2010; Ferrando *et al.*, 1997; Gibson *et al.*, 1987; Kortebein *et al.*, 2007; Symons *et al.*, 2009), and increased physical activity levels would appear to be protective of sarcopenia in the elderly (Mijnarends *et al.*, 2016; Park *et al.*, 2010; Ryu *et al.*, 2013). Taken together these findings indicate that habitual physical activity levels *per se* are a key regulator of MPS. Therefore the physical activity status of our participants may have had a protective effect. Similar findings have previously been reported regarding whole-body insulin sensitivity (i.e. physical activity blunts the deleterious effects of overfeeding) (Krogh-Madsen *et al.*, 2014; Walhin *et al.*, 2013), and human muscle satellite cells donated from active individuals appear to be more protected from palmitate-induced insulin resistance than those from sedentary donors (Green *et al.*, 2013). It would be of interest to repeat this experiment in combination with a model of reduced physical activity, or in cohorts who are characterised by sedentary lifestyles.

Alongside elevating circulating NEFA levels, iv lipid heparin infusions also increase intramyocellular lipid (IMCL) concentrations (Bachmann *et al.*, 2001). Indeed, while IMCL content was not measured in Stephens *et al.* (2015), the increase in intramuscular long-chain acyl-CoA and acetylcarnitine concentrations observed in that study suggests augmented lipid content in skeletal muscle. Thus, the findings of Stephens *et al.* (2015) may not be attributable to reductions in insulin sensitivity *per se*, but rather the direct effect of specific lipid subspecies (i.e. ceramide and diacylglycerol) on the mechanisms responsible for MPS. The findings regarding alterations in IMCL content after short-term overfeeding interventions are inconclusive, with both increases (Adochio *et al.*, 2009), and no change (Cornford *et al.*, 2013) previously reported; and the impact of our model of high-fat overfeeding on IMCL content is still unknown. Interestingly, the accumulation of IMCL is a characteristic

metabolic feature of obesity (Goodpaster *et al.*, 1999), insulin resistance (Perseghin *et al.*, 1999), and aging (Rivas *et al.*, 2012); all conditions which have previously been postulated to reduce anabolic sensitivity. Furthermore, *in vitro* evidence suggests treating cultured skeletal muscle cells with a cell-permeable ceramide attenuates mTOR and P70S6K1 phosphorylation and impairs the cellular protein synthesis response to nutrient stimulation (Hyde *et al.*, 2005). The hypothesis that IMCL accumulation impairs MPS may explain the findings seen in obese, insulin-resistant individuals (Chevalier *et al.*, 2005; Guillet *et al.*, 2009; Murton *et al.*, 2015; Pereira *et al.*, 2008), who are likely to demonstrate increased IMCL compared to their healthy-weight counterparts. However, this is speculative as none of the above mentioned studies compared IMCL content between their study cohorts. Therefore, while it is possible that increased IMCL accumulation impairs MPS, further investigations and direct measurements of IMCL content are required.

The stimulation of MPS following nutrient ingestion is reliant upon increased ribosomal activity (also referred to as "translational efficiency"). Central to this increase in ribosomal activity is the activation of the mTOR signalling pathway, which increases translation initiation and elongation through the phosphorylation of downstream signalling intermediates (namely 4EBP1 and P70S6K1) (Drummond *et al.*, 2009). The suppression of postprandial MPS in response to lipid infusion (Stephens *et al.*, 2015), skeletal muscle disuse (Wall *et al.*, 2016), and aging (Cuthbertson *et al.*, 2005), have previously been characterised by a reduction in 4EBP1 phosphorylation, suggesting that impairments in intracellular signalling underpin reductions in anabolic sensitivity in a variety of circumstances. In the present study, 7-days of high-fat overfeeding was associated with reductions in 4EBP1 phosphorylation. However, this reduction in phosphorylation status was relatively minor (~9%), and did not seem to impact upon postprandial MPS, with similar skeletal muscle protein FSR observed before and after overfeeding. It is unclear why the observed reductions in 4EBP1 did not translate into differences in MPS in this study, although a potential explanation comes from Masgrau *et al.* (2012) who, when investigating the chronological changes in MPS induced by overnutrition in rodents, demonstrate a time-course response whereby MPS was only impaired after prolonged overfeeding/obesity. To elaborate, Masgrau *et al.* (2012) compared MPS rates in rats fed a 16 week control diet, to those fed a high-fat, high-sucrose diet for either 16 or 24 weeks. The results of that study demonstrate that despite significant increases in body weight and fat mass after 16 weeks of dietary intervention, mixed muscle protein FSR were well maintained. However, when feeding was continued until 24 weeks, a 26%

reduction in mixed muscle protein FSR was observed. Intriguingly, in line with the discussion point above this reduction in FSR coincided with a significant increase in IMCL content. Thus, it is possible that the reduction in 4EBP1 phosphorylation observed in the present study represents an early-phase response to short-term high-fat overfeeding; a response which could be augmented if the dietary intervention was continued for a longer duration, and may eventually lead to impairments in MPS.

We must acknowledge certain limitations within our study, the first of which is the decision to limit our comparisons to fed-state skeletal muscle protein FSR. For practical reasons, we did not include an assessment of basal/fasting protein FSR, meaning we may have missed potential diet-induced differences at this time. Conversely, available evidence would suggest that the impairments in MPS seen in obesity and/or insulin resistance are limited to postprandial responses, whereas basal/fasting rates of MPS are unaffected (Murton *et al.*, 2015; Pereira *et al.*, 2008; Stephens *et al.*, 2015). Based on this information we are therefore confident that 7-days of high-fat overfeeding did not influence basal/fasting muscle protein FSR in this cohort. In addition, the lack of basal/fasting muscle protein FSR measurements in this study mean we are unable to categorically determine the ability of our carbohydrate and protein beverage to upregulate postprandial MPS. However, with reference to the literature (Kumar *et al.*, 2009; Stephens *et al.*, 2015; Volpi *et al.*, 2001), we can reasonably assume that the fed-state FSR observed in this study are increased above fasting/postabsorptive levels. Further support for this assumption comes from the observation that phosphorylation of Akt^{ser473}, $mTOR^{Ser2488}$, and $P70S6K1^{Thr389}$ was increased in the postprandial period after carbohydrate and protein ingestion, and phosphorylation of $eEF2^{Thr56}$ was decreased; responses which are consistent with the nutritional stimulation of human MPS (Drummond *et al.*, 2009).

In conclusion, 7-days of high-fat overfeeding impaired postprandial glycaemic control in young, healthy, non-obese individuals. Furthermore, high-fat overfeeding also led to a reduction in the phosphorylation status of $4EBP1^{Thr37/46}$. However, this attenuation in 4EBP1 phosphorylation did not influence postprandial MPS responses, with similar fed-state skeletal muscle protein FSR observed before and after the overfeeding intervention. These findings suggest that diet-induced impairments in glycaemic control do not affect the MPS response to nutrient stimulation in young, healthy, non-obese individuals. However, the reduction in

4EBP1 phosphorylation noted in the current study possibly suggests that reductions in fed-state FSR may be induced had the dietary intervention been continued for a longer duration.

CHAPTER 6

General discussion.

6.1 Reiteration of aims and objectives

Short-term (3-28 days) high-fat overfeeding is associated with negative alterations in glucose metabolism in healthy, lean subjects, including reductions in whole-body insulin sensitivity and impairments in glycaemic control (Hulston *et al.*, 2015; Samocha-Bonet *et al.*, 2010; Tam *et al.*, 2010; Wulan *et al.*, 2014), increased EGP (Brons *et al.*, 2009) and defects in skeletal muscle insulin signalling (Adochio *et al.*, 2009). However, the mechanisms underpinning these impairments and the time course of development are still unknown. There is particular contention as to which tissues are the first to demonstrate diet-induced impairments, with some reports implicating changes in hepatic insulin sensitivity and increased endogenous glucose production (EGP) (Brons *et al.*, 2009; Cornier *et al.*, 2006), whereas others suggest that skeletal muscle is the primary site of metabolic dysfunction (Adochio *et al.*, 2009; Knudsen *et al.*, 2012). Additionally, evidence suggests that obese insulin resistant individuals also demonstrate "anabolic resistance" (i.e. a reduced muscle protein synthetic response to anabolic stimuli) (Chevalier *et al.*, 2005); Guillet *et al.* (2009); (Murton *et al.*, 2015; Pereira *et al.*, 2008). A response which has been attributed to the onset of insulin resistance (Stephens *et al.*, 2015). However, the impact of diet-induced impairments in whole-body insulin sensitivity on skeletal muscle protein metabolism has not been addressed. With this in mind, the overall aim of this thesis was to address current gaps in the literature regarding the impact of short-term high-fat overfeeding on whole-body and tissue-specific metabolic control. This aim was to be achieved by successful completion of the following objectives:

1. To corroborate and further characterise a model of short-term, high-fat overfeeding, previously shown by our laboratory to reduce whole-body insulin sensitivity in young, healthy, lean individuals.

2. To investigate tissue-specific insulin sensitivity in response to diet-induced impairments in glycaemic control.

3. To delineate the developmental time-course of diet-induced impairments in whole-body insulin sensitivity.

4. To determine whether diet-induced impairments in glycaemic control impair the anabolic response to nutrient stimulation in young, healthy, lean individuals.

6.2 Summary of findings

This section will summarise the key findings from the studies described in Chapters 2,3,4, and 5 of this thesis, and how they relate to the objectives outlined above.

6.2.1 Chapter 2. Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal weight individuals.

This chapter demonstrates that 7-days of high-fat (65% total energy), overfeeding (+50% energy) impairs glycaemic control in healthy, lean individuals as evidenced by a significant increase in postprandial glucose and insulin area under the curve (AUC). This confirms a previous finding from our laboratory (Hulston *et al.*, 2015), thus completing objective 1, and validating the use of this model in future mechanistic investigations. In addition, this chapter also demonstrates that the postprandial plasma ghrelin, and glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) responses were unaffected by the diet intervention. Indicating that these orexigenic, and anorexigenic/insulintropic hormones are not major regulators of the early adaptive responses to overnutrition.

6.2.2 Chapter 3. Short-term, high-fat overfeeding impairs glycaemic control in young, healthy, lean individuals by altering the coordinated processes regulating plasma glucose flux.

In an attempt to investigate the mechanisms underpinning the early, diet-induced impairments in glycaemic control, dual-glucose tracer methodology was combined with the 7-day model of high-fat overfeeding utilised in chapter 2. This chapter again demonstrates that our model of high-fat overfeeding impairs glycaemic control in young, healthy, lean individuals. Furthermore, the use of the dual-glucose tracer in this study enabled us to investigate the individual processes which contribute to whole-body glycaemic control (i.e. intestinal absorption of glucose following carbohydrate ingestion, endogenous glucose production (EGP), and peripheral uptake of glucose), and thus meet objectives 1 and 2. Our data demonstrate no significant differences in any of these individual processes, but did reveal a postprandial imbalance between the rate of glucose appearance and disappearance which would favour the accretion of plasma glucose. This suggests that the observed impairments in glycaemic control are attributable to subtle alterations in the coordinated processes regulating

plasma glucose flux, rather than overt tissue-specific alterations in glucose metabolism which have previously been reported (Brons *et al.*, 2009; Cornier *et al.*, 2006; Knudsen *et al.*, 2012).

6.2.3 Chapter 4. A single day of excessive dietary fat intake impairs whole-body insulin sensitivity in healthy, non-obese, young men.

Chapters 2 and 3 demonstrate that 7-days of high-fat overfeeding impair glycaemic control in young, healthy, non-obese individuals. This finding is in accordance with a number of other short-term (3-14 day) overfeeding studies (Cornford *et al.*, 2013; Hulston *et al.*, 2015; Lagerpusch *et al.*, 2012; Olefsky *et al.*, 1975; Tam *et al.*, 2010). However, the developmental time-course of these impairments is currently unknown. Therefore, in an attempt to complete objective 3, we herein investigated glucose tolerance in healthy, non-obese males before, and after a 1-day, high-fat (73%), high-energy (+80%) diet. Our data demonstrate that 1-day of high-fat overfeeding significantly reduced whole-body insulin sensitivity (assessed by the Matsuda insulin sensitivity index) in this cohort by 24%. A further important finding from this study is that, in line with the observations from our 7-day model, the reduction in whole-body insulin sensitivity occurred alongside an insufficient compensatory increase in insulin secretion, indicating some degree of β-cell dysfunction. These alterations occurred alongside reduced fasting, and unchanged postprandial non-esterified fatty acid (NEFA) concentrations. This finding highlights potential mechanistic differences between intravenous (iv) lipid infusion protocols and dietary models of insulin resistance, which along with the recent change in consensus that obesity and elevated NEFA concentrations may in fact not be directly related (Karpe *et al.*, 2011), advocates the future use of dietary models in investigations aimed at enhancing our understanding of human metabolic disease.

6.2.4 Chapter 5. Diet-induced impairments in glycaemic control do not influence the muscle protein synthetic response to carbohydrate and protein ingestion in young, healthy, lean individuals.

It has recently been demonstrated that iv lipid heparin infusions can induce insulin resistance and impair the muscle protein synthesis (MPS) response to anabolic stimuli in healthy individuals (Stephens *et al.*, 2015). However, as demonstrated in chapters 2, 3, and 4, short-term high-fat, overfeeding impairs glycaemic control and reduces insulin sensitivity independently of changes in circulating NEFA concentrations, potentially highlighting distinct mechanistic differences between dietary models of insulin resistance and iv lipid infusion protocols. It is currently not known if diet-induced alterations in glucose metabolism

influence MPS responses. In this chapter we investigated whether 7-days of high-fat (65% total energy), overfeeding (+50% energy), which can be considered a more physiological/applied model of insulin resistance, impaired the MPS response to a mixed carbohydrate and protein beverage in young, healthy, lean individuals. Our findings demonstrate that the fed-state MPS response is maintained in response to diet-induced impairments in glycaemic control, thus completing objective 4.

6.3 Limitations

There are a number of limitations of the work contained in this thesis, some of which have been briefly discussed in the relevant experimental chapters. However, there are also a number of limitations which are common across studies, the first of which being the lack of a diet control. It is challenging to determine what the most appropriate experimental design for a control diet/group would be, as a change in any dietary component ultimately results in changing the composition of the diet in its entirety, and altering the energy content makes it difficult to disentangle the influence of macronutrients *per se*. It is plausible that any dietary change may elicit unknown alterations in metabolism. Based on this we chose to compare our experimental diet against subjects' habitual diet. We are therefore unable to determine whether the findings outlined in this thesis are attributable to the high-energy content of the diet, the high-fat content, or the high-saturated fat content. It would be of interest to investigate the influence of each specific dietary component on glycaemic control and whole-body insulin sensitivity and this has been outlined in the future directions section below.

A further limitation is that the physical activity levels of our participants was not measured before or during the dietary intervention phase. It has previously been demonstrated that physical activity level is a prime determinant of the deleterious effects of overfeeding, and those with increased physical activity levels are able to somewhat maintain whole-body insulin sensitivity in response to an overfeeding intervention compared to those who are sedentary (Krogh-Madsen *et al.*, 2014; Walhin *et al.*, 2013). This protective effect of physical activity is associated with changes in body-fat distribution and adipose tissue lipogenic gene expression. Furthermore, it would appear that skeletal muscle satellite cells maintain this metabolic phenotype *ex vivo* (Green *et al.*, 2013). Green *et al*., (2013) demonstrated that even after multiple passages, myotubes from physically active donors are protected from palmitate-induced insulin resistance compared to those from sedentary donors. Furthermore, physical activity seems to be a key mediator of skeletal muscle protein metabolism

(Mijnarends *et al.*, 2016; Park *et al.*, 2010; Ryu *et al.*, 2013). It is therefore possible that the physical activity status of our participants (who were classed as recreationally active and performed moderate to vigorous intensity exercise at least 3 times per week for more than 30 minutes at a time) may have influenced the findings of all experimental chapters in this thesis, but in particular those of chapters 3 and 6 where we have demonstrated that short-term high-fat overfeeding does not influence whole-body glucose uptake (which is predominantly governed by skeletal muscle glucose uptake) or mixed muscle fractional protein synthesis rate. It remains unclear as to whether our findings would hold true for those who are less active. Unfortunately, as no quantifiable measures of physical activity were obtained we are unable to investigate this notion in any detail. It would be of interest to investigate whether differences in physical activity within our experimental cohort influence findings (i.e. do those who are more active display a different response to those who are less active), or whether our dietary intervention independently influences physical activity levels, which could be a contributing factor to our findings.

A number of methodological tools have also been used in this thesis to investigate glycaemic control/whole-body insulin sensitivity (e.g. meal tolerance test, oral glucose tolerance test, dual-glucose tracer method). While these methodological tools were chosen to answer specific questions regarding the influence of short-term high-fat overfeeding, they make comparisons between experimental chapters difficult, as altering the amount of ingested glucose has previously been shown to cause a dose-dependent increase in postprandial insulin response (de Nobel, & van't Laar, 1978; Mosora *et al.*, 1981; Pan *et al.*, 1982), and ingesting carbohydrates with other macronutrients, particularly protein, typically potentiates the insulin response in humans (Bock *et al.*, 2007). Furthermore, the difference in solid and liquid feeding of test meals would have implications for gastric emptying (Achour *et al.*, 2001) and the resulting postprandial substrate and hormonal milieu with which changes in gastric emptying are associated. Nevertheless, it is notable that a similar pattern of response was observed in all chapters, i.e. an insufficient compensatory insulin response resulting in increased postprandial glucose levels. This finding would suggest some form of impaired β-cell function. This response was observed irrespective of the testing method utilised. No direct measures of β-cell function were performed in this thesis.

6.4 Future directions

Alongside the findings of the experimental chapters above, samples obtained in this thesis have been provided to collaborators at Loughborough University, Liverpool John Moores University, and The University of Sheffield in order to determine hepatokine secretion, lipid droplet formation, microvascular function and immune cell-derived microvesicle production, responses to a severe lipid overload. However, there are still a number of questions which remain to be answered;

6.4.1 Does 7 days of high-fat overfeeding influence ectopic lipid accumulation?

In the experimental chapters of this thesis we have demonstrated that short-term, high-fat, overfeeding impairs glycaemic control. However, what is not known is the fate of the ingested dietary fat. Previous research has hypothesised that dietary interventions of this nature increase the deposition of lipids in ectopic sites such as the liver and skeletal muscle; a response which may be critical in the development of metabolic disorders. Conversely, data outlining the influence of short-term overfeeding protocols on liver, and/or skeletal muscle lipid accumulation is relatively sparse. Whilst our collaborators from Liverpool John Moores University may be able to provide some insight into this area, future research should focus on establishing whether there is a relationship between the site of lipid deposition and whole-body glucose metabolism.

6.4.2 Blood lipids and lipoproteins: Dietary adaptations and clinical implications?

Where dietary interventions have been performed in this thesis, we have seen consistent alterations in lipid and lipoprotein metabolism. However, the nature of these alterations are somewhat surprising, with high-fat, overfeeding associated with reductions in fasting plasma NEFA, and triglyceride (TG), and increased high-density lipoprotein (HDL) concentrations, and reduced, or unchanged postprandial NEFA concentrations (Chapters 2,3, and 4); all responses which would typically be considered clinically favourable. However, the fact these responses have been observed in the presence of impaired glycaemic control questions this favourable assumption. Elucidating both the mechanisms underpinning these responses, and their clinical relevance, was beyond the scope of this thesis and would require in depth investigations that are currently beyond the capabilities of our laboratory. Nevertheless, these are important questions that should be addressed in order to further our knowledge regarding the development of metabolic disease.

6.4.3 Is it high-energy intake *per se* that impairs glycaemic control, or excessive fat intake?

All the experimental chapters of this thesis involved the provision of experimental diets aimed at impairing glycaemic control/reducing insulin sensitivity. These diets were characterised by being both high in total energy and fat content and achieved their intended aim. In future work, it would be particularly interesting to determine if it is excess energy intake, or excess dietary fat intake, which negatively impacts glucose metabolism; and if similar effects can be elicited when overfeeding other macronutrients (e.g. carbohydrates and added sugars in particular). Furthermore, our model of high-fat overfeeding consisted of realistic experimental foods that were representative of typical Western dietary habits (i.e. high in saturated fat), and it is unclear if the observed responses were driven by high intakes of total fat, or saturated fat *per se*. Alongside investigating the effects of specific dietary macronutrients, attention should be given to the composition of the dietary fat and the specific contributions of saturated, polyunsaturated, and monounsaturated fat to impairments in glycaemic control.

6.4.4 Metabolic responses to short-term high-fat overfeeding: Population specific differences?

In this thesis, all experimental chapters involved the recruitment of young, healthy, lean individuals who engaged in regular physical activity. These individuals were chosen to allow for investigation into the early metabolic responses to short-term high-fat overfeeding which may provide information regarding the onset of insulin resistance. It is therefore unclear as to how short-term high-fat overfeeding would impact populations who a characterised by existing metabolic derangements, such as those who are obese and/or insulin resistant, individuals with type 2 diabetes mellitus (T2DM), or those who exhibit sedentary lifestyles. It is plausible that these individuals may demonstrate a more exaggerated deleterious response to high-fat overfeeding than the individuals examined in this thesis. Delineating the response to high-fat overfeeding in these individuals may reveal important information about the progression of metabolic disease.

6.5 General conclusions

Based on the results of this thesis, it is clear that even extremely brief periods (1-7 days) of high-fat overfeeding negatively impact glucose metabolism, as demonstrated by impairments in glycaemic control and reductions in whole-body insulin sensitivity. Our data suggest that

these impairments are attributable to subtle changes in the coordinated processes regulating plasma glucose flux. Intriguingly a common finding of the experimental chapters seen in this thesis was an insufficient compensatory insulin response resulting in increased postprandial glucose levels after high-fat overfeeding. This would suggest that short-term high-fat overfeeding reduces β-cell function which would have severe implications for the development of T2DM. Conversely, our data also demonstrate that whilst short-term high-fat overfeeding impairs glycaemic control, it does not influence the MPS response to nutrient stimulation. Therefore, while it would appear that short-term high-fat overfeeding negatively impacts whole-body glucose and lipid metabolism, skeletal muscle protein synthetic responses appear to be relatively unaffected in young, lean, healthy humans.

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APPENDIX A

Western blotting: an overview of the basic principles and the optimisation procedures specific to this thesis.

A.1 Abstract

The use of western blotting (WB) techniques is now commonplace in life science and biochemistry laboratories. WB allows for the separation and analysis of specific proteins, and is therefore considered an extremely useful tool in physiological research, providing mechanistic insight into a diverse range of regulatory processes. However, while WB can be separated into a number of simple theoretical steps, each of these steps can be performed in a variety of ways. Furthermore, variation in each of these steps may dramatically impact upon the standard of the analysis. Thus, each step of the WB procedure must be carefully considered and tailored for the individual proteins of interest. In this thesis WB has been utilised to investigate the phosphorylation status of proteins postulated to be involved in the anabolic signalling cascade (i.e. the Akt/mTOR signalling pathway). This chapter outlines any optimisation work that was undertaken to improve the quality and repeatability of blots presented in Chapter 5.

A.2 Introduction

Although it was not named until 1981 (Burnette, 1981), Western blotting (WB) evolved from DNA (Southern) blotting and RNA (Northern) blotting and was first described by Towbin *et al.* (1979). This multistep technique allows for the transfer of proteins, separated by their molecular mass through electrophoresis, from polyacrylamide gels to a solid support membrane. This creates a replica of the original gel pattern, from which a wide variety of analytical procedures can then be applied to the immobilised proteins. This includes the investigation of protein abundance, kinase activity, cellular localisation, and protein-protein interactions (Bass *et al.*, 2017), alongside post-translational modifications such as phosphorylation (Nairn *et al.*, 1982), ubiquitinylation (Paul *et al.*, 2012), glycosylation (Pere-Brissaud *et al.*, 2015), methylation (Voelkel *et al.*, 2013), and sumoylation (Park-Sarge, & Sarge, 2009). This diverse range of applications has led to the widespread use of WB in life science and biochemistry laboratories. In the context of this thesis, the WB technique has been employed in order to investigate the phosphorylation status of proteins postulated to be involved in the anabolic signalling response to both nutrient and exercise stimulation (i.e. the Akt/mTOR signalling pathway). Of particular interest is the question of whether these anabolic signalling responses differ following acute or chronic (obesity) periods of overnutrition.

The basic principles of WB can be separated into a number of general steps: (i) sample preparation, including the extraction and quantification of cellular proteins from tissues and cells etc.; (ii) separation of proteins by gel electrophoresis; (iii) transfer of proteins to a solid support (membrane); (iv) "blocking" of the membrane to reduce non-specific binding; (v) initial detection of target(s) of interest by incubation with a specific (primary) antibody; (vi) detection of primary antibodies by incubation with a secondary antibody conjugated to a label (e.g. horseradish peroxidase [HRP]); (vii) production and detection of a signal, which is proportional to the degree of antibody binding, through catalysing an interaction with the conjugated label (e.g. chemiluminescent); and (viii) imaging and quantification of the resulting signal (bands). However, while almost all WB procedures adhere to these basic steps, each individual protein/target of interest has its own inherent considerations that must be addressed in order to provide reproducible and reliable results. Thus, each aspect of the WB process must be carefully considered and tailored for the individual targets of interest. This chapter describes the principles and objectives of each step in the WB procedure and outlines any optimisation work undertaken to improve the quality and repeatability of blots.

This chapter is not an exhaustive list of all potential considerations and avenues for optimisation, rather a specific list of the key steps undertaken by our laboratory with regards to our targets of interest. The reader is directed to a number of relevant papers for a more comprehensive overview of possible technical considerations (Bass *et al.*, 2017; Kurien, & Scofield, 2006; Mahmood, & Yang, 2012).

A.3 Sample preparation

In this thesis, sample preparation concerns the extraction and quantification of cellular proteins from skeletal muscle tissue samples. This occurs via the lysis and disruption of cell membranes using homogenisation techniques, for which possible options are sonication, manual grinding with a pestle and mortar and mechanical grinding. While manual grinding with a pestle and mortar is considered the most flexible and cost effective method (Size *et al.*, 2011), it is also time consuming, and therefore, mechanical homogenisation with a TissueLyser II (Qiagen, Hannover, Germany) was explored whilst undertaking this PhD. In this method, homogenisation occurs as a result of stainless steel beads being "thrown" against the tissue by mechanical shaking of an Eppendorf containing the beads, skeletal muscle sample and lysis buffer. Samples are separated as a result of the impact force of the accelerated beads grinding the tissue (Goldberg, 2008). As can be seen in figure A.1, performing a WB with tissue homogenised by manual grinding *vs*. the TissueLyser II results in blots which are remarkably similar. Therefore, due to the ability of the TissueLyser II to homogenise up to 36 samples at once, this method was chosen for subsequent analysis based on its time effectiveness.

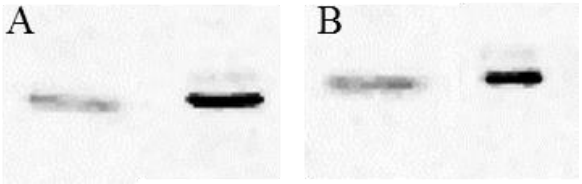

Figure A.1 Representative image of p-P70S6K1^{Thr389} blots produced from manual tissue grinding using a pestle and mortar with the tissue submerged in liquid nitrogen (A) or using stainless steel ball bearings and the TissueLyser II (Qiagen, Hannover, Germany) (B). Lanes 1 and 3 are from a resting/fasted (unstimulated) muscle biopsy, whereas lanes 2 and 4 are following 3 hours of cycling exercise with co-ingestion of carbohydrate and protein (stimulated). Each muscle biopsy was sectioned into two pieces for homogenisation via the

two different techniques. Tissue samples were obtained from a previous study (Hulston *et al.*, 2011).

Protein lysates are complicated in their composition, containing contaminants such as cellular or tissue debris, fats, hydrophobic protein aggregates, nucleic acids, and proteases that can all affect the data obtained from WB (Taylor, & Posch, 2014). Thus, it is vitally important that an appropriate lysis buffer, which ensures efficient extraction of proteins but maintains the conformation of the target(s) of interest, is used in order to eliminate these effects (MacPhee, 2010). Lysis buffer components used within this thesis can be seen in table A.1. While this buffer may differ from other variants described in the literature (e.g. radioimmunoprecipitation assay [RIPA] and Nonidet P-40 [NP-40] buffers), it does adhere to the recommended composition of variables laid out in Harlow, and Lane (1999) (e.g. salt concentrations 0-1 M, non-ionic detergents 0.1-2%, EDTA concentrations 0-5 mM and pH 6-9). In addition, this buffer contains a protease and phosphatase inhibitor (table A.2) which is recommended for use in all lysis buffers (MacPhee, 2010). Based on advice and guidance from colleagues at the John Walls renal unit (University Hospitals of Leicester) and in an attempt to standardise protein concentrations between lysates, all samples were weighed and homogenised in 10 µL of lysis buffer for every mg of wet weight tissue.

Table A.1 Lysis buffer components used for tissue homogenisation in this thesis.

Component	Purpose	Concentration
Phosphate buffered saline (PBS [0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4])	Helps to disengage proteins whilst maintaining their conformation and prevents protein aggregation.	97%
Triton x-100	Increase the solubility of non-polar proteins.	1%
0.5 M EDTA	Inhibit metalloproteases and prevent changes in protein phosphorylation.	1%

Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL)	Inhibit specific proteases and phosphatases (Table 5.2).	1%

Table A.2 Protease and phosphatase inhibitors included in the protease and phosphatase inhibitor cocktail used within this thesis, and their targeted enzyme class. Data obtained from the manufacturer (Thermo Scientific, Rockford, IL).

Inhibitor	Target
Sodium fluoride	Serine/Threonine and Acidic Phosphatases
Sodium orthovanadate	Tyrosine and Alkaline Phosphatases
β-glycerophosphate	Serine/Threonine Phosphatases
Sodium pyrophosphate	Serine/Threonine Phosphatases
Aprotinin	Serine Proteases
Bestatin	Amino-peptidases
E64	Cysteine Proteases
Leupeptin	Serine/Cysteine Proteases

Following the extraction of proteins from skeletal muscle, it is vitally important to determine the concentration of the protein yield in order to standardise the amount of protein loaded per gel well, and thus the amount of total protein being compared between samples. Quantification of protein content in this thesis was determined by the Pierce 660 nm protein assay (Thermo Scientific, Rockford, IL, USA). This assay utilises a colorimetric change caused by deprotonation of a dye-metal complex (polyhydroxybenzenesulfonephthalein-type dye and a transition metal) in acidic conditions. As this interaction is facilitated by positively charged amino acids groups in proteins, the dye mainly interacts with amino acids such as Histidine, Arginine and Lysine and to a lesser extent Tyrosine, Tryptophan and Phenylalanine. The colorimetric change produced by the assay is measured at 660 nm, and increases relative to increases in protein concentration. This assay was chosen due to its compatibility with a wide range of detergents (e.g. Triton x-100), and its reported linear detection range of 25-2000 µg/mL (Antharavally *et al.*, 2009). However, experimental observations revealed that the assay absorbance becomes saturated at a protein concentration of ~1000 µg/mL, leading to a plateau in values irrespective of increasing protein

concentrations (Figure A.2). Therefore, standards for this assay were prepared by serially diluting a 1 mg/mL BSA stock 5 times in PBS to produce standards ranging from 31.25-1000 µg/mL (31.25, 62.5, 125, 250, 500 and 1000 µg/mL), and a standard curve was generated by measuring standards in duplicate and plotting the average blank-corrected 660 nm measurement for each standard against its concentration in µg (Figure A.2). However, following homogenisation, skeletal muscle tissue samples typically display protein concentrations 5-20 fold greater than the working range of the standard curve. Thus, homogenised samples were diluted 1:5, 1:10 and where necessary 1:20, in 1 x PBS in order to allow for detection (Figure A.3). Typical protein yields per skeletal muscle sample observed in this thesis were 5-9 µg/µL.

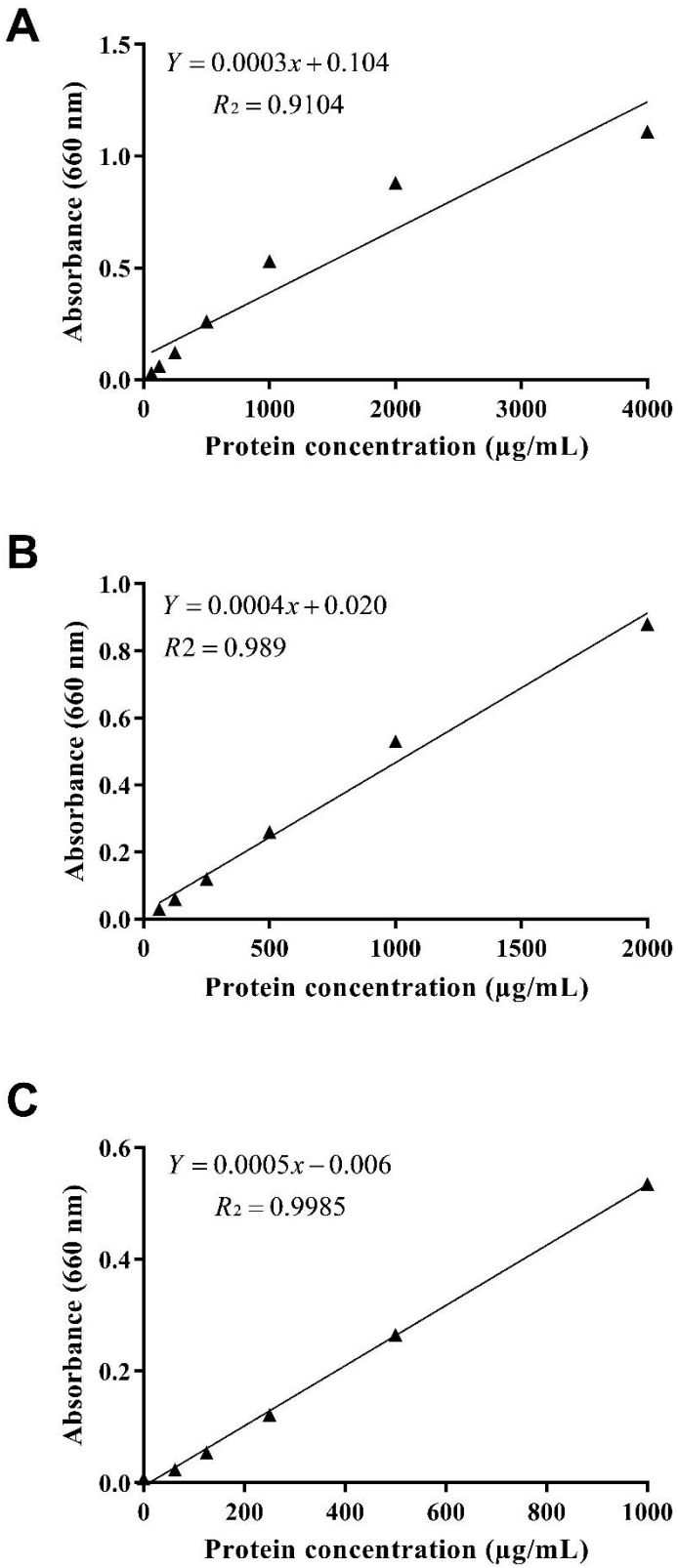

Figure A.2 Comparison of standard curve linearity using the Pierce 660 nm assay when using a top standard of 4000 µg/mL (A), 2000 µg/mL (B), and 1000 µg/mL (C).

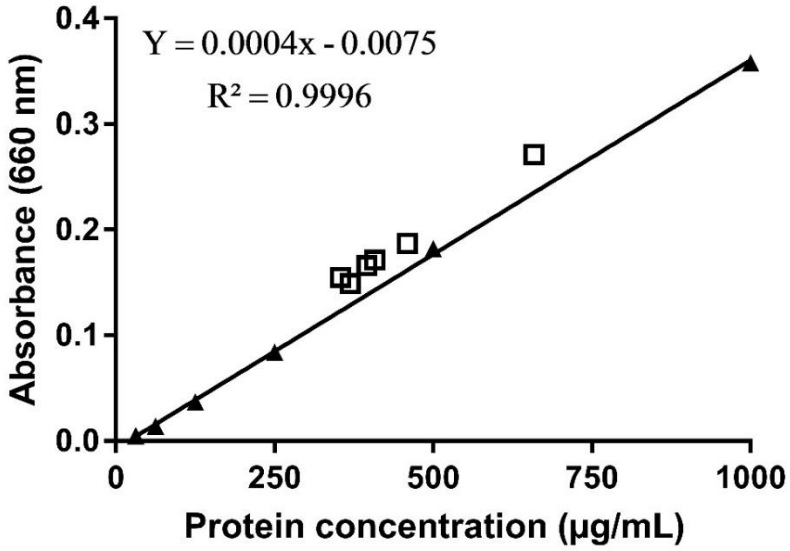

Figure A.3 Example of skeletal muscle tissue samples and their interaction with a standard curve. In this instance, samples were diluted to 1:20 in 1 x PBS. Final concentrations of lysates will be corrected according to their dilution. Filled triangles denote protein standards, while empty squares denote protein lysates from 6 separate muscle biopsies. All samples fall within the range of the standard curve.

After protein determination, samples were diluted to the desired concentration in sample buffer, β-mercaptoethanol and distilled water (Table A.3), before being vortexed and heated at 95˚C for 5 minutes in order to denature the proteins. The ratio of lysate to distilled water was amended for each sample to ensure a final protein concentration of 1.5 µg/µL. It is commonplace in the literature to see laboratories loading between 10-80 µg of protein per sample (Taylor *et al.*, 2013). There is no scientific basis for choosing an appropriate loading volume other than ensuring it is sufficient to allow detection of the target of interest and does not lead to oversaturation of images. Therefore, each target of interest may require different loading volumes dependent upon their expression and/or performance of the primary antibody. In our laboratory, a final protein concentration of 1.5 µg/µL allows for a sufficient amount of protein (15 µg or 10 µL of gel sample) to be loaded onto gels for analysis of phosphorylated (p) Akt^{Ser473}, p-eEF2^{Thr56}, p-4EBP1$^{Thr37/46}$, and p-mTOR^{Ser2448}. However, as demonstrated in figure A.4, due to the lower expression of p-P70S6K1^{Thr389}, it has been requisite to increase the amount of protein loaded onto gels to 40 µg to ensure adequate detection of all samples. Thus, due to the maximum well capacity of the gel system utilised (20 µL), more concentrate gel samples of 2.5 µg/µL were needed for analysis of p-

P70S6K1^{Thr389}. It is vitally important that the concentration of gel samples is consistent to ensure the total amount of protein loaded into each well is standardised; this factor will be covered in more detail in the analysis section below.

Table A.3 Buffer components used in gel samples.

Component	Purpose	Concentration
LDS sample buffer (Containing; 141 mM Tris base 106 mM Tris HCl 2% LDS 10% Glycerol 0.51 mM EDTA 0.22 mM SERVA Blue G 0.175 mM Phenol Red pH 8.5)	To denature proteins making them negatively charged, to increase the density of the sample relative to the surrounding buffer for ease of loading into gel wells (glycerol), and to allow for visualisation of the run progression (SERVA Blue G and Phenol Red)	25%
β-mercaptoethanol	Cleavage of disulphide bonds and protein denaturation	5%
Distilled water	Dilute sample to desired concentration.	Dependent on protein concentration of skeletal muscle sample and desired sample concentration.

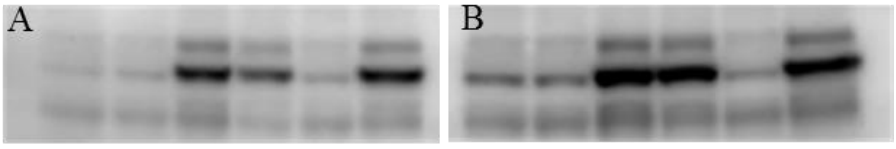

Figure A.4 Representative image of the same sample blotted for p-P70S6K1^{Thr389}, when loading 22.5 µg of protein (A) and 40 µg of protein (B) onto gels. As can be seen with the lower loading condition it becomes difficult to quantify unstimulated samples (lanes 1 and 2).

A.4 Polyacrylamide gel electrophoresis (PAGE)

Following preparation, samples are loaded onto polyacrylamide gels and separated by electrophoresis. The SDS-polyacrylamide gel electrophoresis (PAGE) procedure has been documented in detail elsewhere (Burnette, 1981). Briefly, following the denaturing process protein samples become anionic and thus migrate toward a positive electrode as an electrical current is applied. Polyacrylamide gels contain a matrix through which pores are formed between polymer chains (Ornstein, 1964). The ability of proteins to migrate through these pores is dependent on protein size (molecular weight), meaning smaller proteins migrate more rapidly than larger proteins leading to separation. Altering the bis-acrylamide concentration of the gel regulates pore size. Therefore, increasing the acrylamide concentration of the gel reduces the pore size and increases the frictional resistance applied on proteins slowing their migration. The choice of gel type (composition) and concentration was determined by the molecular weight of the target(s) of interest. Commercial pre-cast gels have been reported to be more consistent than hand-cast gels (Bass *et al.*, 2017), and were therefore utilised in this thesis for ease of use, and in an attempt to remove some of the variability in gel quality seen by other members of our laboratory when casting gels in-house.

Electrophoretic separation of protein samples requires a discontinuous buffer system (Ornstein, 1964). The discontinuous buffer system is designed to improve the focus and resolution of proteins, and works by utilising gels separated into two regions; a "stacking" gel and a "resolving" gel. Initially, protein samples are loaded onto the large pore "stacking" gel, where differences in buffer ions and pH between the gel and the electrode reservoirs results in an ion gradient. Ions present within the gel (e.g. chloride or acetate) display a high affinity to the anode relative to the denatured proteins and therefore migrate faster, forming a lead ion boundary. Ions present within the running buffer (e.g. 3-[*N*-morpholino]propanesulfonic acid [MOPS] or Tricine) serve as the trailing ion as they display reduced affinity/mobility. Thus, migrating protein samples are compressed between the two ion boundaries, concentrating the proteins into sharp bands. Due to the large pore size of the stacking gel, all proteins are migrating at the same speed at this stage. However, the "resolving" gel consists of smaller pores which lead to the sieving effect (regulated by protein size) previously described. The higher pH of the resolving gel also leads to ionization of the trailing ions, increasing their mobility to the point where they will migrate past the protein samples, liberating the proteins from their focused bands allowing their unimpeded separation (Bass *et al.*, 2017). Thus, in addition to the acrylamide concentration of the gels, the efficiency of protein migration and

resolution is also governed by the pH and ionic strength of the buffers in the electrode reservoir (running buffers), and those used to cast gels (Sambrook, & Russell, 2001).

Taking the above points and manufacturer recommendations into consideration, and in an attempt to run multiple proteins on the same gel minimising time and cost, NuPAGE 10% Bis-Tris Gels (Invitrogen, UK) with MOPS running buffer were used for analysis of p-Akt^{Ser473}, p-eEF2^{Thr56}, p-4EBP1$^{Thr37/46}$, and p-P70S6K1^{Thr389}. Whereas NuPAGE 3-8% Tris-Acetate Gels (Invitrogen, UK) with Tris-Acetate running buffer were utilised for analysis of p-mTOR^{Ser2448} (Figure A.5).

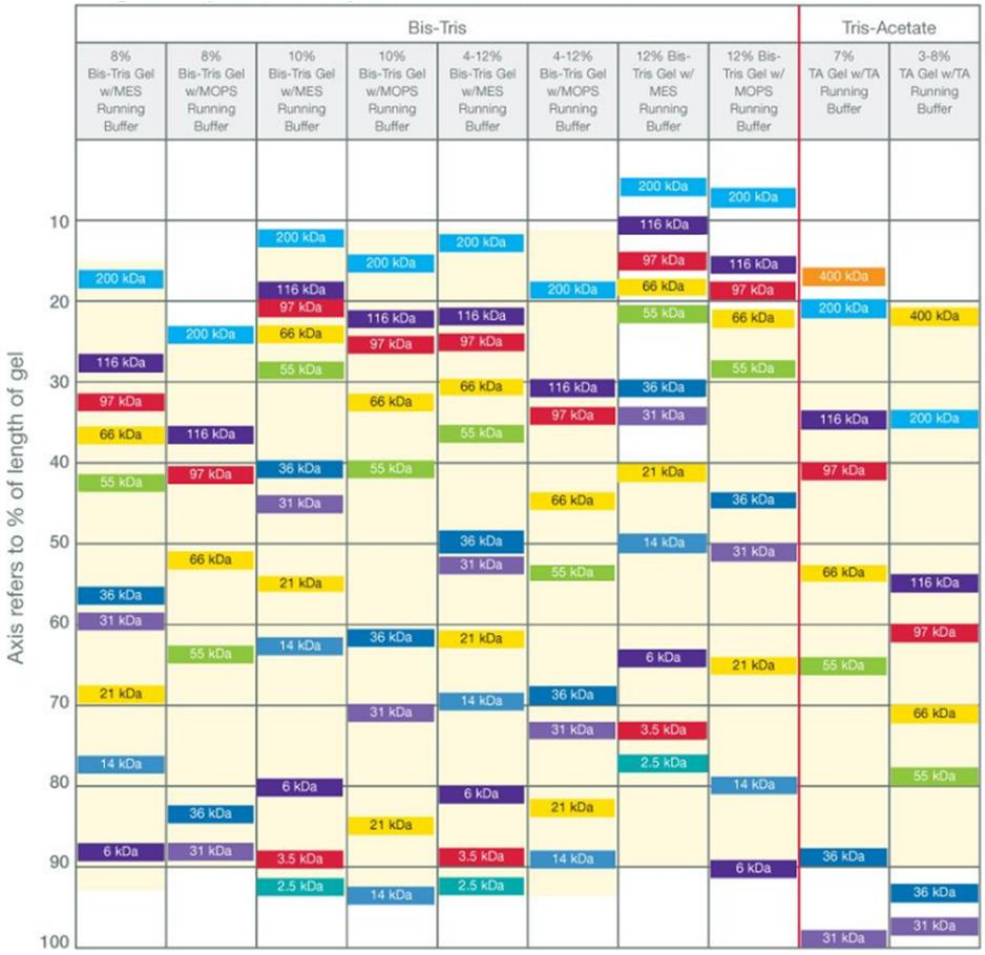

Figure A.5 Protein migration chart outlining the separation of different molecular weight proteins by specific gel and buffer systems. Table taken from gel manufacturer (Invitrogen, UK).

Selection of appropriate running conditions are also an important aspect of electrophoresis, as running at too high a voltage can distort bands, or running for too short or long a duration

may lead to inadequate protein separation or the running of proteins through the bottom of the gel, respectively. Protein migration displays a linear relationship with voltage. Thus, electrophoresis should occur at a constant voltage, as current is dependent on voltage and resistance. Therefore, as changes in resistance are possible during the process of electrophoresis (e.g. due to warming of buffers), the use of a constant current will cause the voltage to fluctuate leading to variability in protein migration (Bass *et al.*, 2017). In this thesis electrophoresis was performed for 120 minutes with a constant voltage of 125 V for 10% Bis-Tris gels with MOPS running buffer. This is based on the recommendations of other individuals within the department and was sufficient to give suitable separation of all targets of interest. However, due to the different gel and buffer system used to run p-mTOR^{Ser2448} (3-8% Tris-Acetate gels with Tris-Acetate running buffer), electrophoresis was performed for 35 minutes with a constant voltage of 150 V. The manufacturer recommends a run time of 1 h at 150 V for the optimal use of this gel-buffer system. However, we have observed that running in accordance with these recommendations results in distorted bands. Therefore, the run time was reduced to 35 minutes, which was sufficient to allow for suitable separation in the region of interest (~ 250 kDa). This significantly improved the linearity and sharpness of bands (Figure A.6).

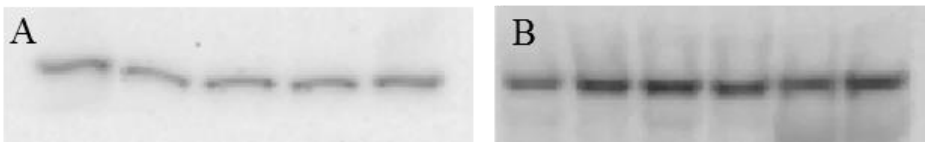

Figure A.6 Example of typical p-mTOR^{Ser2448} blots following electrophoretic separation at a constant voltage of 150 V for 60 minutes (A) and 35 minutes (B).

A.5 Membrane transfer

Following separation of proteins, a replica of the gel can be made on a solid support structure (membrane). This occurs through construction of a "gel sandwich" (Figure A.7) and utilises the same methodological principles as gel electrophoresis; that is the negatively charged proteins in the gel are transferred onto the membrane when a lateral electrical current is applied while immersed in a buffered solution (wet transfer). Transfer efficiency can vary between proteins due to their characteristically differing ability to migrate from the gel and their propensity to bind to the membrane. In addition, factors such as gel composition, the gel-membrane contact, transfer duration, protein size, and the presence of detergents (e.g. Triton x-100) can all further effect protein transfer efficiency (Alegria-Schaffer *et al.*, 2009).

While nitrocellulose membranes are commonly used, they are potentially disadvantageous since the proteins are not covalently bound and therefore only a small fraction of total protein actually binds. Furthermore, these membranes can be brittle and prone to damage when dry. Polyvinylidene fluoride (PVDF) membranes, on the other hand, exhibit a high binding capacity and are physically strong (Kurien, & Scofield, 2006). Additionally, PVDF membranes are amenable to coomassie staining while nitrocellulose membranes are not, a point which will become more pertinent in the analysis section (below). Therefore, in this thesis all proteins were transferred onto PVDF membranes. PVDF membranes bind proteins through hydrophobic interactions. This hydrophobic nature of PVDF membranes means an initial pre-soaking in methanol is required to allow the infiltration of the buffer and the binding of proteins (Mansfield, 1995).

Optimal transfer of most proteins occurs in the presence of low-ionic strength buffers, under a low electrical current (Alegria-Schaffer *et al.*, 2009). All proteins within this thesis were transferred using 1 x NuPAGE Transfer Buffer (Invitrogen, UK) at 30 V. The addition of methanol to the transfer buffer is known to improve the absorption of protein to the membrane, and prevent gel swelling and protein distortion during electro-transfer (Towbin, & Gordon, 1984). However, methanol also has the potential to shrink gels, reducing their pore size and inhibiting the transfer of higher molecular weight proteins. The inclusion of methanol to the transfer buffer is therefore not recommended for proteins >100 kDa (MacPhee, 2010). All proteins examined within this thesis were transferred with 1 x NuPAGE Transfer Buffer (Invitrogen, UK) containing 10% methanol, with the exception of p-mTOR^{Ser2448}, for which no methanol was added as this protein possesses a molecular weight of approximately 289 kDa. Efficient transfer of p-Akt^{Ser473}, p-eEF2^{Thr56}, and p-mTOR^{Ser2448} was achieved after 1 h. However, in an attempt to increase the signal intensity of p-4EBP1$^{Thr37/46}$, and p-P70S6K1^{Thr389}, by increasing the quantity of protein transferred from gels, transfer time was extended to 2 h for these proteins.

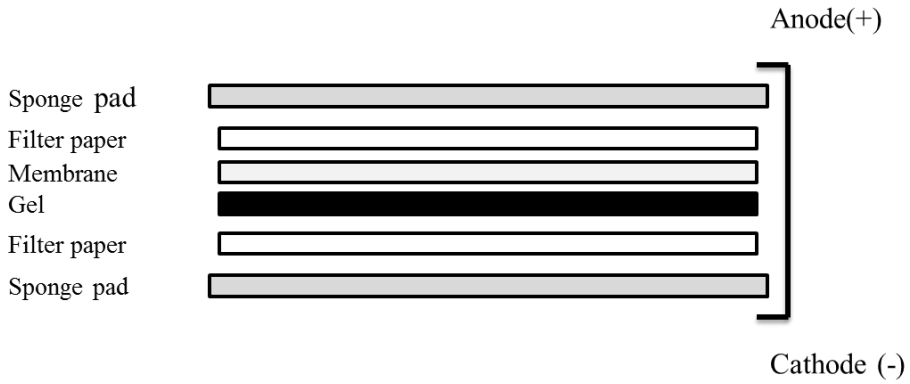

Figure A.7. Diagram of a gel "sandwich" used to transfer proteins from polyacrylamide gels to a solid support membrane. The entire gel sandwich is subsequently soaked in transfer buffer.

A.6 Blocking of membranes

Following transfer of proteins, membranes were incubated in blocking solution at room temperature for 1 h. Blocking refers to the incubation of membranes in an abundance of protein in an attempt to fill vacant membrane binding sites (i.e. sites which are not occupied by proteins transferred from the gel). Different reagents can be used to block membranes, and choosing an appropriate blocking buffer is essential in improving the clarity and sensitivity of the WB. Appropriate guidance on blocking conditions is provided by the commercial companies alongside the antibody specific information, and both non-fat dried milk (NFDM) and bovine serum albumin (BSA) diluted in tris-buffered saline with 1% Tween (TBST) are typically recommended as membrane-blocking reagents. However, not all blocking solutions are suitable for all targets of interest, and certain membranes may perform better with one blocking solution compared to another (i.e. the use of BSA has previously been reported to lead to inadequate blocking of PVDF membranes [Macphee *et al.*, 2010]). Therefore, validation and testing of blocking reagents for any blots which exhibit high background noise is required. Figure A.8 demonstrates the impact a change in blocking reagent can have on image clarity. In this instance, blocking membranes in 5% BSA led to a large degree of non-specific binding when analysing p-Akt^{Ser473}. Simply changing the blocking solution to 5% NFDM significantly improved this issue and vastly improved the clarity of the blots. Table A.4 outlines the blocking reagents used for final analysis of all proteins of interest contained in this thesis.

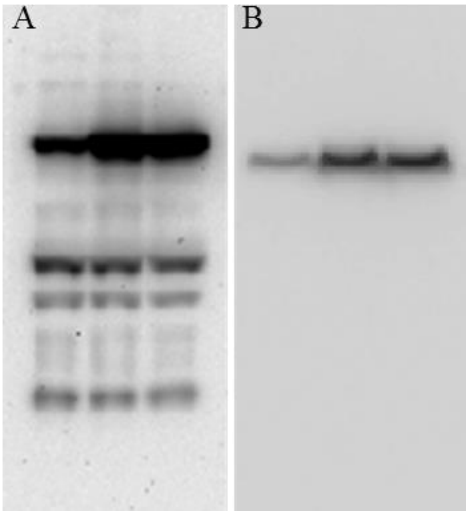

Figure A.8 Representative image of a single sample blotted for p-Akt^{Ser473} with 5% BSA (A) and 5% NFDM (B) used as blocking solutions.

Table A.4 Proteins analysed within this thesis and their respective blocking buffers.

Protein of interest	Blocking solution (w/v in TBST)
p-Akt^{Ser473}	5% NFDM
p-mTOR^{Ser2448}	5% BSA
p-eEF2^{Thr56}	5% BSA
p-P70S6K1^{Thr389}	5% NFDM
p-4EBP1$^{Thr37/46}$	5% NFDM

A.7 Primary antibody incubation

Following incubation in blocking solutions, membranes were incubated in primary antibodies overnight at 4˚C. Primary antibodies are a critical part of the WB procedure, as the affinity of the antibody to bind to the target of interest is a prime determinant of the assay (Burnette, 1981). Therefore, validating the specificity and sensitivity of a primary antibody is vital to the success of WB. Both monoclonal and polyclonal antibodies can be used for WB analysis, with each variety having their own inherent advantages and disadvantages. Polyclonal antibodies (pAb) are produced from different β-cell lineages, and therefore recognise multiple epitopes on any one antigen. This generally results in a stronger signal due to the greater number of antibody molecules available for binding to the target of interest, although a consequence of this may be an increase in non-specific binding (MacPhee, 2010). Monoclonal antibodies (mAb), on the other hand, are manufactured from a single cell line against an individual epitope and are therefore more specific and sensitive to their target of

interest. However, the binding affinity of mAb can be reduced if the antigenic site has been significantly altered through the denaturing process or electrophoretic separation (Lipman *et al.*, 2005; MacPhee, 2010). Based on advice and guidance from others within the department and assessment of previous literature, both pAb and mAb were chosen for use in this thesis, (Table A.5), and early pilot work revealed these were appropriate in the majority of cases. However, initial investigations of p-P70S6K1^{Thr389} using a pAb (product #9205, Cell Signalling Tech, Danvers, MA, USA) revealed poor detection of samples in the region of interest, irrespective of the primary antibody concentration (Figure A.9). Therefore, a comparison was made with a mAb, which demonstrated an improved ability to detect both a positive control lysate, and our samples of interest (Figure A.10). This antibody was therefore used for future investigations (Table A.5).

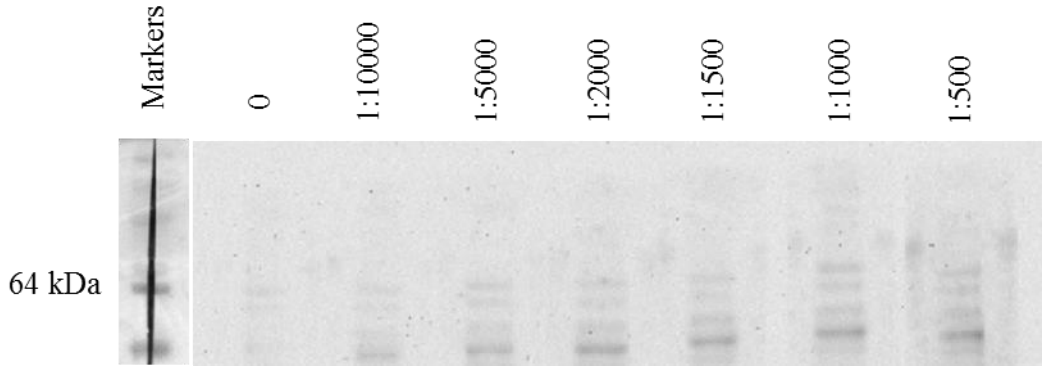

Figure A.9 Representative image of sequential primary antibody dilutions for p-P70S6K1^{Thr389} with a polyclonal antibody (product #9205, Cell Signalling Tech, Danvers, MA, USA). 'Markers' refers to the molecular weight protein ladder run on the gel.

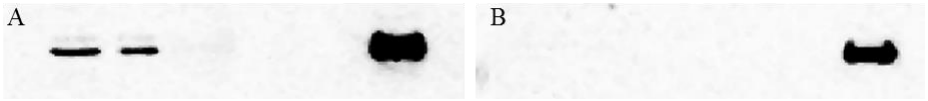

Figure A.10 Representative image of detection of p-P70S6K1^{Thr389} with both a monoclonal [A] (product #9234, Cell Signalling Tech, Danvers, MA, USA) and polyclonal [B] (product #9205, Cell Signalling Tech, Danvers, MA, USA) primary antibody. Lanes 1 and 2 (A) and 7 and 8 (B) are muscle samples obtained after 3 hours cycling exercise with co-ingestion of carbohydrate and protein. Lanes 3 and 4 (A) and 9 and 10 (B) are muscle samples from the Derby laboratory of Dr Philip Atherton, but these did not provide a detectable signal, possibly due to the age of the samples. Lanes 5 (A) and 11 (B) are a negative control and lanes 6 (A) and 12 (B) are a positive control. The polyclonal antibody was only able to detect the positive

control sample, whereas the monoclonal antibody provided a clear signal for our muscle samples and the positive control.

Alongside validating the type of primary antibody appropriate for analysis, a further key optimisation step involves determining the optimal concentration of primary antibodies in order to avoid issues such as weak signal, oversaturation, and high background presence (MacPhee, 2010). Guidelines for primary antibody concentrations are provided by the manufacturer. However, antibodies may differ in their performance dependent on the tissue being analysed or as a result of differences in sample preparation (Murphy, & Lamb, 2013). It is therefore essential to ascertain an appropriate primary antibody concentration for the samples of interest. Figure A.11 outlines the sequential analysis of primary antibody dilutions for p-Akt^{Ser473}, p-mTOR^{Ser2448}, p-eEF2^{Thr56}, and p-4EBP1$^{Thr37/46}$, and final antibody concentrations used during the analysis stage can be seen in table A.5.

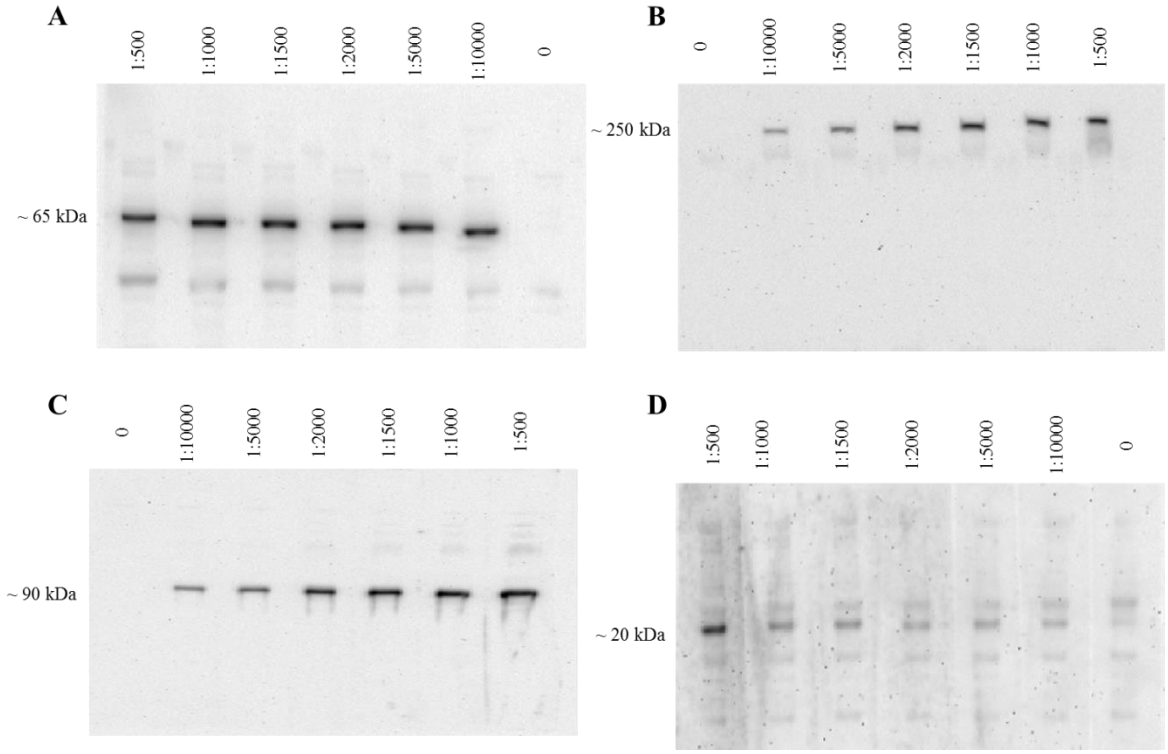

Figure A.11 Representative image of sequential primary antibody dilutions for p-Akt^{Ser473} (A), p-mTOR^{Ser2448} (B), p-eEF2^{Thr56} (C), and p-4EBP1$^{Thr37/46}$ (D) using the antibodies outlined in table A.5.

A further factor that can affect the performance of a primary antibody is the concentration of the primary antibody dilution buffer (diluent). It is commonplace in the literature to dilute primary (and secondary) antibodies in the same buffer used to block membranes; typically 5% NFDM or BSA in TBST. However, while incubation in the blocking buffer may prevent non-specific binding of the antibody, we have observed that too high a concentrate of block reagent in the buffer solution can prevent efficient binding of the antibody, leading to a weak signal (faint bands). Therefore, during the optimisation phase, where targets of interest exhibited a weak signal the concentration of the blocking reagent in the antibody dilution buffer was reduced, leading to improved signal intensities (Figure A.12). Final antibody dilution buffer concentrations can be seen in table A.5.

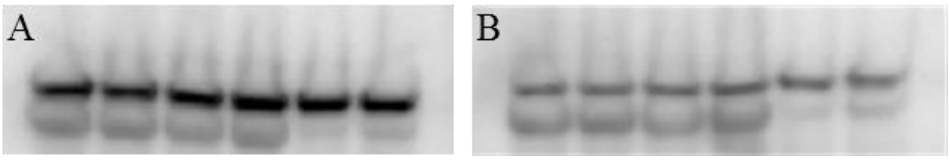

Figure A.12 Representative image of a single sample blotted for p-mTOR^{Ser2448} by incubation in a 1:2000 primary antibody dilution with a 2.5% BSA diluent (A) and a 5% BSA diluent (B).

Table A.5 Antibody specific information for all targets of interest investigated within this thesis. All antibodies were purchased from Cell Signalling Tech (Danvers, MA, USA).

Target of interest	**Antibody information**	**Product number**	**Antibody dilution (v/v)**	**Antibody diluent (w/v)***
p-Akt^{Ser473}	Rabbit mAb	4060	1:5000	5% NFDM
p-mTOR^{Ser2448}	Rabbit mAb	5536	1:2000	2.5% BSA
p-eEF2^{Thr56}	Rabbit pAb	2331	1:1000	5% BSA
p-P70S6K1^{Thr389}	Rabbit mAb	9234	1:1000	2% NFDM
p-4EBP1$^{Thr37/46}$	Rabbit mAB	2855	1:1000	2% NFDM

*Antibody diluents were used for both primary and secondary antibody incubations.

A.8 Secondary antibody incubation

Following primary antibody incubation, membranes were incubated in secondary antibody for 1 h at room temperature. As primary antibodies are generally not engineered with a

reporter function to allow detection, secondary antibodies are used as a means of detecting the target of interest. This antibody recognises and binds to the primary antibody and is conjugated to a specific label (e.g. horseradish peroxidase [HRP]) which allows for detection. The choice of secondary antibody is dependent upon the primary antibody isotype and the animal within which it was raised. Antibodies derived from mammals contain a variety of antibody (immuno-globulin [Ig]) molecules that differ in their target specificity and *in vivo* function as a result of variations in their heavy chain formations (Manning *et al.*, 2012). These antibodies will constitute one of five classes, IgM, IgD, IgE, IgA, or the most predominant of the classes, IgG. Furthermore, certain mammals such as rats, mice and humans produce different subclasses of IgG, which would require secondary antibodies be selected for the specific isotype (Bass *et al.*, 2017). However, as all primary antibodies used in this thesis were raised in rabbit, who produce only a single isotype of IgG {Manning, 2012 #184, a single anti-rabbit IgG secondary antibody was suitable for all targets of interest (product #7074, Cell Signalling Tech, Danvers, MA, USA).

Appropriate secondary antibody concentrations will depend upon the affinity for the primary antibody and expression levels of the targets of interest {Bass, 2017 #175}. In this thesis it was observed that a secondary antibody dilution of 1:2000 was sufficient to induce a detectable signal for all targets of interest following the optimisation procedures outlined above. All secondary antibodies were diluted in the same block buffer as the primary antibodies for which they were targeted towards (Table A.5).

A.9 Chemiluminescent detection

The principles of detection for WB are similar to those seen in traditional assays such as ELISA; as in the secondary antibody is conjugated with a labelled compound or enzyme that allows for detection. The principle of chemiluminescent detection is that HRP catalyses the oxidation of luminol in the presence of hydrogen peroxide. The luminol then forms an excited state product (3-aminophthalate) immediately following this reaction and emits light (at 425 nm) as it decays (Kurien, & Scofield, 2006). The chemiluminescent substrate is thus a limiting reagent and light production ends as the substrate is oxidised. Although a chemiluminescent signal can last for 6-24 h depending on the substrate used, all blots were imaged after 5 min of incubation with the substrate in line with manufacturer recommendations. The volume of substrate used was dependent on the size of the cut membrane being analysed, with sufficient volume to ensure that the blot is completely

covered with substrate. Blots were detected in this thesis through use of a Bio-Rad Chemi-doc imaging system (Thermo Scientific, Rockford, IL). The use of an imaging system has a number of benefits over traditional film-based methods of detection, including a larger dynamic range, higher degree of exposure control, and the ability to use the accompanying software to perform densitometry analysis (Alegria-Schaffer *et al.*, 2009) discussed in more detail below. Loading too much protein and/or chemiluminescent substrate can result in oversaturation of the image (Figure A.13). Furthermore, attention must be given to optimising detection parameters, as exposing for too short or long duration may lead to under- or overexposure, respectively. It is therefore advisable to take multiple images over an extended duration in order to obtain an optimal image for analysis.

Figure A.13 Representative image of a sample blotted for GAPDH when loading 15 µg of protein. Red marking indicates the area is outside the range of detection (oversaturation). Furthermore, this image contains what are typically referred to as "ghost bands", while not completely understood, these bands occur when the chemiluminescent substrate is depleted as a result of over-expression of the target of interest (Vattem, & Mathrubutham, 2005).

A.10 Analysis

Following chemiluminescent detection and image acquisition, quantification of WB data can be performed through computer-aided determination of the optical density of selected bands, allowing for the measurement of phosphorylation status of specific protein(s) between samples (e.g. comparison between specific time points in an experiment). However, there are a variety of densitometric methods available depending on the software package being utilised, such as peak height, the integral, average band density and band volume (Gassmann *et al.*, 2009). Unfortunately, there exists no scientific means of choosing a method of densitometry (Gassmann *et al.*, 2009). Therefore band volume (intensity x mm^2) was chosen for use in this thesis as early investigations suggested this method of quantification was the most representative of our visualised blots.

Appropriate selection of a normalisation method must also be considered to assure that any responses observed between samples are not a result of experimental errors in sample

preparation or pipetting (loading) into the gel lanes (Taylor, & Posch, 2014). It is common to use antibodies targeted towards so called "housekeeping" proteins that are (supposedly) consistently expressed as internal loading controls; common examples of which are GAPDH, tubulin, β-actin and pan-actin. However, experimentation suggests the expression of these commonly used proteins is altered by certain experimental conditions, potentially masking results and eliminating their usefulness as loading controls. For example, figure A.14 contains a representative image of skeletal muscle samples taken at rest, immediately post exercise and 3 h post cycling exercise with coingestion of protein and carbohydrate blotted for both p-mTOR^{Ser2448} and total pan-actin. As can be seen, the increased expression of p-mTOR^{Ser2448} over time is matched by increased total pan-actin expression. In this instance, pan-actin is completely unsuitable as a loading control as its use would mask the increase in mTOR^{Ser2448} phosphorylation. This is a consistent observation from other laboratories (Taylor, & Posch, 2014). In addition we have seen that GAPDH is easily oversaturated when loading "typical" volumes of protein (e.g. 10-15 µg) (Figure A.13), and loading less protein may reduce the ability to detect the actual proteins of interest. In fact, it has previously been reported that loading more than 0.5 µg of protein leads to saturation of GAPDH, suggesting this protein is likely only suitable as a loading control for highly abundant proteins which require little sample loading (Taylor *et al.*, 2013). Similar responses have been observed for both β-actin and tubulin which are also highly expressed in muscle samples, limiting their usefulness as loading controls (Aldridge *et al.*, 2008; Dittmer, & Dittmer, 2006).

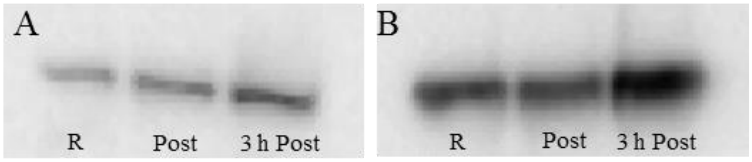

Figure A.14 Representative image of p-mTOR^{Ser2448} (A) and pan-actin (B) at rest (R), immediately (post) and 3 hours post cycling exercise with co-ingestion of carbohydrate and protein. Tissue samples were obtained from a previous study (Hulston *et al.*, 2011)

Coomassie staining is a common, simple approach that has been demonstrated to be an unbiased method of total protein assessment with a high linear range of detection (Welinder, & Ekblad, 2011). Staining both gels and membranes with coomassie allows for evaluation of the overall WB process and transfer efficiency (Taylor *et al.*, 2013). If this approach is taken, the quantification of a single random band that is consistent across each lane may be used for

normalisation (Bass *et al.*, 2017). Unequal loading will be evident as noticeable differences between protein band(s)/lane(s) intensities. This variability may be due to errors in the loading of samples into gels (incorrect volumes), or errors in determination of protein quantification. For example, when comparing the p-mTOR^{Ser2448} blots presented in figure A.15 independently, they would appear to suggest that consuming a high-fat diet leads to reduced phosphorylation of mTOR^{Ser2448} at 120 minutes post carbohydrate and protein ingestion. However, as can be seen from the corresponding coomassie stain, it would appear as though there was a reduction in the amount of protein loaded for this specific sample; likely due to this sample being a particularly bloody biopsy, resulting in an overestimation of protein concentration due to the presence of haemoglobin interacting with the Pierce 660 nm assay (Table A.6). This example highlights the importance of utilising an appropriate method of correction. A further added benefit of using coomassie as a loading control relates to the expense of not having to purchase additional housekeeping antibodies. As already noted, this method is only applicable with PVDF membranes as nitrocellulose membranes react with the coomassie stain producing a dense background (Welinder, & Ekblad, 2011).

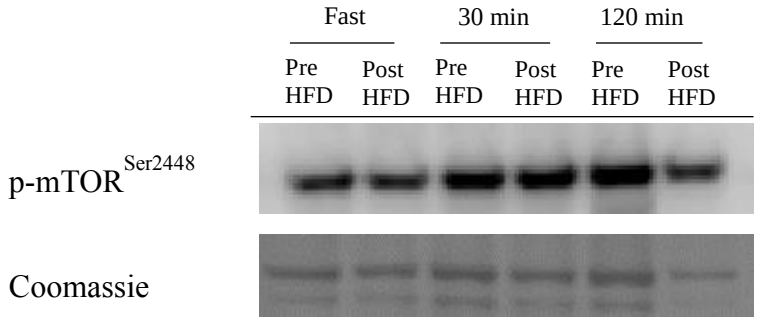

Figure A.15 A representative image of p-mTOR^{Ser2448} and the corresponding coomassie stain in the fasted state (fast) and 30 and 120 min post carbohydrate and protein ingestion before (pre) and after (post) 7-days of high-fat overfeeding (HFD).

Table A.6 Protein concentration determined via the Pierce 660 nm protein assay and the standard curve presented in figure A.3.

Sample	Protein lysate concentration (µg/mL)	Dilution correction *	Protein concentration (µg/µL)
Pre-0	396	7924	7.92
Pre-30	354	7090	7.09
Pre-120	369	7373	7.37
Post-0	449	8986	8.99
Post-30	408	8158	8.16
Post-120	659	13184	13.18

* samples were diluted 1:20 in order to determine protein concentration.

During quantification, even blots which have been fully optimised may still display a visible background (Bass *et al.*, 2017). Therefore, a decision regarding background correction must be made. However, methods of removing background may differ between software programs, and traditional methods, such as choosing a "representative background" may induce error as the selected background may differ from that associated with individual bands. Therefore, in this thesis sample boxes were manually fitted as tightly as possible and it was accepted that boxed bands will always contain both the band density and a small quantity of the associated background.

A.11 Conclusions and final protocols

In conclusion, while WB may be a useful tool in molecular physiology, important considerations must be made, and validation steps performed, for each individual target of interest in order to produce reliable and reproducible data. In theory, every aspect of the WB procedure has the potential to be amended, and the aim of this chapter was not to discuss all potential avenues for optimisation, but rather the specific alterations made during our optimisation phase which have improved the quality of blots. An outline of the entire WB process used for analysis of all targets of interest investigated within this thesis can be seen below.

Approximately 30-50 mg of frozen muscle tissue was homogenised in 10 µL/mg ice-cooled buffer (1 x PBS containing 1 % Triton X-100, 1% protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL), and 1% 0.5 M EDTA (Thermo Scientific, Rockford, IL) using a TissueLyser II (Qiagen, Hannover, Germany). All samples were disrupted by the TissueLyser II for 2 x 2 min at 20 Hz. Homogenates were then centrifuged for 10 min at 13,300 *g*. The resulting supernatant was removed and aliquoted into 1.5 mL Eppendorfs prior to being stored at -80˚C until analysis. Protein concentration was determined in aliquots of supernatant diluted 1:5, 1:10, and where necessary 1:20 in 1 x PBS using the Pierce 660 nm protein assay (Thermo Scientific, Rockford, IL, USA). After protein determination, homogenates were mixed in sample buffer containing 25% NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA), 5% β-mercaptoethanol and distilled water. The ratio of lysate to distilled water was amended for each sample to produce two distinct gel samples with protein concentrations of 1.5 µg/µL and 2.5 µg/µL. Samples were then vortexed, and heated at 95˚C for 5 minutes in order to denature proteins.

For analysis of p-Akt^{Ser473}, p-eEF2^{Thr56}, and p-4EBP1$^{Thr37/46}$, 15 µg (10 µL of the1.5 µg/µL gel samples) of protein was loaded onto NuPAGE 10% Bis-Tris Gels (Invitrogen, UK). Due to being lowly expressed, 40 µg (16 µL of the 2.5 µg/µL gel samples) of protein was loaded for analysis of p-P70S6K1^{Thr389}. Gels were then run for 2 h at 125 V in 1 x NuPAGE MOPS SDS running buffer (Invitrogen, UK) to allow for ample separation of proteins. For p-mTOR^{Ser2448}, 15 µg of protein was loaded onto NuPAGE 3-8% Tris-Acetate Gels (Invitrogen, UK), before being ran for 35 min at 150 V in 1 x NuPAGE Tris-Acetate SDS running buffer (Invitrogen, UK). Following separation, all proteins were transferred onto PVDF membrane (Invitrogen, UK) for 1 h (p-Akt^{Ser473}, p-eEF2^{Thr56} and p-mTOR^{Ser2448}) or 2 h (p-P70S6K1^{Thr389} and p-4EBP1$^{Thr37/46}$) at 30 V in 1 x NuPAGE Transfer Buffer (Invitrogen, UK) containing 10% methanol, with the exception of p-mTOR^{Ser2448} for which no methanol was added. After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS: 50 mmol/L Tris base, 150 mmol/L NaCl, pH 7.6) containing 0.5 % Tween-20 (TBST), and either 5% BSA (p-eEF2^{Thr56} and p-mTOR^{Ser2448}) or 5% NFDM (p-Akt^{Ser473}, p-4EBP1$^{Thr37/46}$ and p-P70S6K1^{Thr389}). Membranes were then incubated overnight at 4˚C in 1 x TBST and 2-5% BSA or NFDM with commercially available primary antibodies (Table A.5). The next morning, membranes were serially washed in TBST (3 x 5 min and 1 x 15 min) before being incubated for 1 h at room temperature in 1 x TBST and 2-5% BSA or NFDM with a commercially available secondary antibody (anti-rabbit IgG product # 7074, Cell Signalling

Tech, Danvers, MA, USA). Following secondary antibody incubation, membranes were serially washed (3 x 5 min and 1 x 15 min) in TBST before being incubated in enhanced chemiluminescent substrate at room temperature in the dark for 5 minutes. Finally, membranes were blotted free of excess chemiluminescent and antibodies bound to target proteins were visualised on a Bio-Rad Chemi-doc imaging system. Membranes were exposed long enough to ensure clear, non-saturated bands for all samples. Bands apparent at expected molecular weights were manually defined with sample boxes fitted as tightly as possible and the volume of band densities were determined using Quantity One image-analysis software. All samples were normalised to coomassie staining of membranes to control for loading. The phosphorylation of target proteins was used as an indirect measure of their activity.