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NUTRITIONAL STATUS, EXERCISE, AND INSULIN SENSITIVITY

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Public Health.

Massey University, Manawatu,
New Zealand.

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2011

Abstract

The insulin-glucose system in lean-healthy people adapts its normal function in the face of challenging metabolic conditions. To improve understanding of these adaptations, I exposed subjects to periods of starvation, high-protein-low-carbohydrate diet (HPLC) and overfeeding.

In six lean-healthy men, dietary carbohydrate was eliminated but gluconeogenic substrate supply was maintained by three-day HPLC diet, compared with three-day starvation and three-day mixed-carbohydrate diet. Insulin sensitivity, *vastus lateralis* intramyocellular lipid (IMCL) and fasting glucose were unaffected by HPLC diet, although they were significantly altered after starvation. These results indicate that dietary carbohydrate restriction does not trigger metabolic adaptations, although total metabolic carbohydrate supply remains likely to be important.

Six lean-healthy men underwent two three-day periods of starvation with either no exercise or daily endurance exercise (80 $min.day^{-1}$ at 50% VO_{2Max}) and a three-day mixed diet without exercise. Compared to mixed diet, starvation elevated fasting FFA and IMCL and decreased insulin sensitivity and fasting glucose. Exercise during starvation prevented the elevation of IMCL but did not prevent other metabolic disruption, in contrast with exercise during lipid infusion.

Maintaining high physical-activity may prevent the metabolic disruption associated with overfeeding, while insulin sensitivity may predict partitioning of fuel between tissues during overfeeding. Nine endurance-trained athletes maintained their normal physical-

activity while consuming a diet providing 90 kJ.(kg body mass)$^{-1}$.day^{-1} above their normal dietary intake for four weeks. Subjects' body-mass, fat-mass and body fat% increased while insulin sensitivity tended to decrease (14.5 ± 5.9 to 9.5 ± 4.1 min^{-1}.mU.l^{-1}, $p = 0.08$). Change in insulin sensitivity was correlated with change in body fat % ($r = -0.77$, $p < 0.023$). Initial insulin sensitivity was correlated with change in body fat% ($r = 0.90$, $p < 0.009$) and the proportion of mass gained as lean tissue ($r = 0.86$, $p < 0.024$). Maintenance of already high physical-activity cannot prevent metabolic disruption associated with overfeeding. These results also suggest that insulin sensitivity influences energy partitioning between tissues.

The results in this thesis suggest important interaction effects between exercise and diet. I propose that carbohydrate availability is a key modulator of the effects of exercise on metabolism in lean-healthy men.

Acknowledgements

The completion of this thesis would not have been possible without the generous assistance of many people. First and foremost I would like to thank my wife, Rose, for providing loving support and a sympathetic ear. I am also particularly indebted to my primary supervisor, Dr Stephen Stannard, who guided me away from many scientific pitfalls and made available whatever resources were needed. My co-supervisor Dr Chris Cunningham provided sage advice on the doctoral study process and co-supervisors Dr Nathan Johnson and Dr Peter Snell who provided physiological advice. I have enjoyed the collegial support of my fellow post-graduate students: Jon Hughes, Matt Barnes, Zac Schlader, Bob Stewart, Rachel Mason and Aaron Raman; and of Massey University staff Dr Jane Coad and Dr Toby Mundel. None of my research would have been possible without the technical assistance that was provided by Matt Barnes, Dr Pat Ruell, Dr Toos Sachinwalla, Chris Booth and Dr David Simcock. Perhaps most importantly, I am grateful to the subjects who endured unusually unpleasant interventions with stoic determination.

All the experiments presented in this thesis were approved by the local human ethics committee at the institution where they were performed, and complied with the Declaration of Helsinki.

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1 Background

The early 21st century sees the prevalence of type 2 diabetes is increasing worldwide with an estimated 23.5 million cases in the United States as of 2007 (United States of America Department of Health and Human Services - Center for Disease Control 2007). In most cases, the onset of the disease is slow and is preceded by a long period of insulin resistance (i.e. an inability of insulin to stimulate the disposal of glucose from the bloodstream) (United States of America Department of Health and Human Services - Center for Disease Control 2007). An understanding of the way in which insulin resistance develops is important in combating the current type 2 diabetes epidemic.

Chronic insulin resistance, as present in many sedentary and overweight individuals, is a pathological condition. However, non-pathological acute insulin resistance also develops in lean-healthy people undergoing short periods of starvation (Peters 1945; Reaven 1998) as were commonly experienced by our hunter-gatherer ancestors (Speth and Spielmann 1983). The great majority of insulin resistance research has focussed on obese sedentary individuals in whom insulin resistance is a major health risk factor. However, to fully understand the pathological condition it is important to also understand how healthy insulin resistance develops under the same environmental pressures that caused it to evolve.

It is not practical to study insulin resistance in the few remnant hunter-gatherer societies that still exist today, but study is practical in a readily available model of this lifestyle: the modern endurance athlete. Like hunter-gatherer peoples, endurance athletes are characterised by lean phenotype, high daily energy expenditure, and superb metabolic

flexibility (Eaton, Eaton III et al. 1997; van Loon, Jeukendrup et al. 1999). This thesis focuses on the flexibility of insulin sensitivity (the ability of insulin to stimulate the disposal of glucose from the bloodstream) in lean, physically active people. In doing so it provides a healthy baseline for comparison with the pathological insulin resistance that is so prevalent in modern society.

2 Issues associated with human research

Research on human subjects provides physiological information that cannot be confirmed by using other methods. While animal models or human cell culture experiments provide direction for investigation in human subjects, the only way to positively confirm a physiological phenomenon in humans is to observe it *in vivo*. However, working with human subjects also presents some important issues not encountered in other research.

All scientific research must be carried out in an ethical manner, whether or not humans are being investigated. In addition to these general scientific ethics, the Massey University Human Ethics Committee code of conduct (Massey University Human Ethics Committee 2010) lists eight principles that should be adhered to when planning or conducting human research:

a) Respect for persons.

b) Minimisation of harm to subjects, researchers, institutions and groups.

c) Informed and voluntary consent.

d) Respect for privacy and confidentiality.

e) The avoidance of unnecessary deception.

f) Avoidance of conflict of interest.

g) Social and cultural sensitivity to the age, gender, culture, religion, social class of the subjects.

h) Justice.

When performing invasive human physiological research, the principle of minimisation of harm is of particular concern. In accordance with this principle, risk to subjects may

be minimised by careful choice of experimental techniques, the use of best practice in applying those techniques, and designing experiments to minimise the number of measurements. In the research presented in this thesis, intramuscular measurements were made non-invasively using NMR spectroscopy, thus avoiding the risks associated with a muscle biopsy procedure. Insulin sensitivity was assessed using an intravenous glucose tolerance test which, unlike the hyperinsulinaemic-euglycaemic clamp or the insulin suppression test, does not require an insulin infusion in the highly insulin sensitive subjects used. All testing was performed in accordance with best practice procedures and was overseen by a registered medical practitioner. To minimise subject exposure, *a priori* power calculations were used to estimate the minimum number of subjects required to give an 80% chance of detecting the expected change in insulin sensitivity.

Physiological research on human subjects also poses practical difficulties. Although the risk to the subjects' health may be small, participating in research often involves considerable inconvenience and disruption to normal living. Participating in the research presented in this thesis required athletes to either fast for three days or overeat for a month. Not only is such disruption unpleasant and inconvenient for research subjects, it also makes it difficult for researchers to recruit subjects and ensure compliance with experimental protocols. For the experiments presented in this thesis, subject recruitment was slow and markedly increased the time required to complete each study. However, as interventions were designed to produce large changes in the primary outcome variable, only small subject numbers were required and the intended number of subjects was able to be recruited. To aid subject compliance, protocols were designed to minimise disruption to normal living while still achieving experimental aims. Ensuring compliance with experimental protocol was also helped by limiting the number of subjects simultaneously participating in each intervention. During the starvation experiments only

one subject was fasting at any one time, while during the overfeeding experiment, no more than four subjects were performing the intervention simultaneously. By limiting the number of subjects simultaneously performing difficult interventions I was able to ensure that I had adequate time to perform weighing sessions; design, purchase and deliver the required extra meals; and monitor subjects' dietary intake and physical activity throughout the interventions.

Although working with small subject numbers minimises exposure to the risks of experimental procedures, it also raises questions regarding the generalisability of the results, particularly if post-hoc analyses are used. The results of the research presented in this thesis were used only to test prospective hypotheses (although one post-hoc multiple regression was used to determine the amount of variation explained by variables already identified as important in *a priori* correlation analyses). All data were examined both visually and using statistical tests to confirm that there was no evidence of violation of the assumptions required by the tests used. By ensuring that rigorous statistical procedures were followed, the potential generalisability of the results was maintained.

Research on human subjects results in extra difficulty and expense compared to research not involving humans. However, by careful management of the challenges of working with people, human research can be both rigorous and productive.

3 Introduction: Energy substrates, exercise and insulin sensitivity

The human body's insulin-glucose system is a finely tuned feedback loop that regulates the concentration of glucose in the bloodstream. This regulation is particularly important for the function of the central nervous system, which will only operate within a narrow range of blood glucose concentrations (Pramming, Thorsteinsson et al. 1986). The insulin-glucose system adapts its normal regulation to maintain homeostasis in abnormal metabolic conditions. Understanding the nature of these adaptations is important to improve understanding of both general human physiology and the physiological dysregulation that occurs during the development of modern chronic diseases such as metabolic syndrome and type-2 diabetes.

One situation that poses a challenge to glucose homeostasis is acute starvation. During starvation, the lack of dietary carbohydrate intake means the body must rely on stored carbohydrate and endogenous carbohydrate production. Stored carbohydrate is quickly depleted and endogenous production is expensive in terms of both energy cost and degradation of protein rich functional tissue (Owen, Morgan et al. 1967). As a result, the insulin-glucose system must adapt to conditions of low glucose input so that mental and physical function are preserved. Such adaptation is partially achieved by reducing the circulating insulin concentration to reduce glucose uptake (Unger, Eisentraut et al. 1963). However, if insulin concentrations fall too low then rampant proteolysis will ensue (Mortimore and Poso 1987; Fryburg, Barrett et al. 1990; Klein, Sakurai et al. 1993), quickly degrading functional tissue such as skeletal muscle and decreasing physical

ability to obtain food. Instead, the main insulin-stimulated glucose consuming tissues become less sensitive to the insulin signal to take up glucose (Mansell and Macdonald 1990; Webber, Taylor et al. 1994; Johnson, Stannard et al. 2006). The reduction in insulin sensitivity allows blood glucose disposal to be minimised while sustaining a maintenance concentration of insulin to regulate proteolysis.

This model of insulin-glucose homeostasis predicts that a lack of glucose (i.e. dietary carbohydrate intake) is able to trigger 'insulin resistance'. That is, like starvation, other forms of dietary carbohydrate restriction should also be able to trigger a similar reduction in insulin sensitivity. This prediction is supported by reduced insulin sensitivity in response to a very-low-carbohydrate, high-fat diet (Johnson, Stannard et al. 2006), but has not been investigated using other forms of carbohydrate restriction such as a very-low-carbohydrate, high-protein diet.

Another situation that challenges glucose homeostasis is exercise. Sustained endurance exercise greatly increases skeletal muscle carbohydrate oxidation and can deplete muscle carbohydrate stores within a few hours (Gollnick, Armstrong et al. 1973). When carbohydrate intake is high and blood glucose is plentiful, exercise causes skeletal muscle to increase its insulin sensitivity, allowing the muscle to sustain the physical activity and quickly replenish muscle carbohydrate stores after exercise (Richter, Mikines et al. 1989). However, it is not clear what will be the effect of exercise superimposed upon carbohydrate deficit. If insulin sensitivity were to be increased by exercise while blood insulin remains at a tissue maintenance concentration then blood glucose levels would quickly fall resulting in unconsciousness or death. Tales of human endurance throughout history demonstrate that exercise during starvation is possible without these dire consequences, but the mechanisms responsible remain to be described.

Counter-intuitively given the response to starvation, insulin sensitivity is also reduced in the face of sustained positive energy balance (Faeh, Minehira et al. 2005; Erdmann, Kallabis et al. 2008). Human cells have only a finite capacity for carbohydrate storage, so if glucose uptake unremittingly exceeds glucose oxidation then cells must reduce their insulin sensitivity so as to preserve cell integrity. Exercise increases the oxidation of glucose and stimulates an increase in carbohydrate storage capacity of muscle cells so is able to alleviate this reduction in insulin sensitivity. However, if cellular glucose uptake remains greater than glucose oxidation despite exercise, insulin sensitivity would be expected to eventually fall.

While energy balance clearly affects insulin sensitivity, epidemiological evidence suggests that insulin sensitivity also affects energy balance, at least in a free living situation with plentiful food availability. Long term follow-up studies demonstrate that high insulin sensitivity is associated with future weight gain (Swinburn, Nyomba et al. 1991; Pannacciulli, Ortega et al. 2007; Boule, Chaput et al. 2008; Chaput, Tremblay et al. 2008). As individual insulin sensitive tissues have unique insulin response curves (Kraegen, James et al. 1985), it is possible that differences in insulin sensitivity could alter energy partitioning between tissues. While this phenomenon may explain the association between insulin sensitivity and weight gain, it has not been tested in an experimental setting.

This thesis focuses on the competing challenges of exercise and dietary imbalance. As such, I undertake to answer the following questions:

1. Will insulin sensitivity be reduced in the face of a very-low-carbohydrate high-protein diet, as it is in the face of starvation or very-low-carbohydrate high-fat diet?
2. How is blood glucose maintained in the face of simultaneous challenge by exercise and starvation? Is low insulin sensitivity maintained or is insulin concentration reduced?
3. Is continued exercise able to prevent the decrease in insulin sensitivity that usually occurs in the face of a sustained oversupply of energy substrate?
4. Is insulin sensitivity able to predict changes in body composition in response to a continuous oversupply of energy substrate?

4 Literature Review

4.1 Introduction

This literature review is divided into four sections. The first section provides background on the operation of the insulin-glucose system and the final three sections roughly correspond to the three experimental studies included in this thesis. There is considerable overlap between sections and while each section introduces a new area, it also draws heavily on literature already covered in previous sections. The final three sections are each concerned with the effect of some stimulus on insulin sensitivity. The first examines changes in insulin sensitivity induced by acute dietary interventions. The second examines how exercise affects insulin sensitivity, particularly in the face of carbohydrate deprivation or very high lipid diet. The last section presents research concerning insulin sensitivity in the face of excess energy intake.

This review focuses on the metabolism of healthy people. As such, research involving lean, healthy subjects is given priority. Where data from lean, healthy people is unavailable or incomplete, information from other human populations or animal models is used and a description of the experimental subjects is given.

4.2 The insulin-glucose system

4.2.1 Requirement to maintain blood glucose concentration

The human central nervous system requires a continuous supply of glucose for normal operation (Owen, Morgan et al. 1967). If the glucose supply is insufficient then the brain's ability to control other systems, and the organism's ability to obtain further glucose, is diminished (Pramming, Thorsteinsson et al. 1986). Although the brain is a highly oxidative tissue it has a strong preference for oxidation of glucose (Cryer 2007) and the other most abundant metabolic substrates, free fatty acids (FFA), are unable to cross the blood brain barrier so cannot be utilised by the brain (Dhopeshwarkar 1973). On the other hand, red blood cells are entirely anaerobic entities without mitochondria. As such they exclusively rely on glycolysis for their energy production and must be supplied with glucose to remain healthy (Rastogi 2005). Thus, the metabolic requirement to supply fuel for red blood cells and the brain equates to a need to maintain blood glucose concentration above a critical minimum level at all times.

Hyperglycaemia, on the other hand, increases free radical production, non-enzymatic protein glycosylation, and the osmolarity and viscosity of the blood (Sheetz and King 2002). These changes cause damage to various tissues including the vascular endothelium, retina and kidney (Klein 1995; Sheetz and King 2002) and increase the risk of infection, necrosis, cardiac arrest and all cause mortality (Klein 1995; Umpierrez, Isaacs et al. 2002). As such, it is important that the body maintains blood glucose concentration within a narrow range at all times.

4.2.2 Hormonal regulation of blood glucose

Blood glucose concentration is primarily regulated by the circulating concentrations of the hormones insulin and glucagon. Both hormones are produced in the pancreas, with pancreatic α-cells releasing glucagon and β-cells releasing insulin. When blood glucose concentration becomes elevated, pancreatic insulin release is stimulated, glucagon release is inhibited, and these hormonal changes stimulate other tissues to act to decrease blood glucose concentration. When blood glucose concentration falls, pancreatic insulin release is inhibited, glucagon release is stimulated, and blood glucose concentrations rise (Tortora and Grabowski 2003).

Insulin stimulates the uptake of glucose by skeletal muscle, adipose tissue and liver (Barbour 1926; Tortora and Grabowski 2003). Insulin also decreases hepatic glucose output directly by inhibition at the liver (Steele 1959) and indirectly by inhibition of glucagon release by the pancreatic α-cells (Maruyama, Hisatomi et al. 1984) and inhibition of peripheral proteolysis, which would otherwise supply gluconeogenic substrates (Mortimore and Poso 1987; Fryburg, Barrett et al. 1990). While all the actions of insulin contribute to reducing blood glucose concentration, the quantitatively most important action is increased glucose disposal by skeletal muscle; in healthy humans skeletal muscle accounts for around 75 % of whole body insulin stimulated glucose disposal (DeFronzo, Gunnarsson et al. 1985). The quantitative importance of skeletal muscle glucose disposal compared to that of the liver is unsurprising given that an average human has approximately 20 times more skeletal muscle mass than liver mass (Tortora and Grabowski 2003).

To accommodate the increased glucose uptake by insulin sensitive tissues, insulin encourages glucose oxidation and storage as glycogen while it simultaneously inhibits glycogenolysis in liver and skeletal muscle (Banting, Best et al. 1922; Cohen 1992; Petersen, Laurent et al. 1998). Likewise, insulin inhibits functional tissue proteolysis and hepatic gluconeogenesis (Mortimore and Poso 1987; Fryburg, Barrett et al. 1990). As glucose is the primary fuel of peripheral tissues when insulin concentrations are high, there is less need for lipid fuel so skeletal muscle and adipose tissue lipolysis is decreased, preserving lipid stores and decreasing circulating FFA concentration (Anthonsen, Rannstrand et al. 1998). The corollary of the anabolic actions of insulin is that when insulin concentration is low, fuel stores and functional tissue are catabolised (Garlick, Fern et al. 1983).

The actions of glucagon are much less extensive than those of insulin, acting only on the liver and pancreas. Glucagon acts on the liver to stimulate hepatic glycogenolysis, gluconeogenesis and glucose output (Tortora and Grabowski 2003), and on the pancreatic β-cells to stimulate insulin release (Kawai, Yokota et al. 1995). Thus, an increase in blood glucagon concentration increases the total blood glucose flux by directly promoting hepatic glucose output and by indirectly promoting blood glucose disposal via increased insulin release (Rizza, Cryer et al. 1979). The ability of glucagon to increase total glucose flux is particularly important in response to a high protein meal as gluconeogenesis must be increased to dispose of excess amino acids and glucose disposal must be increased to match the corresponding increase in hepatic glucose output (Krezowski, Nuttall et al. 1986).

4.2.3 Insulin signalling of glucose uptake in skeletal muscle

Insulin stimulates skeletal muscle to increase glucose uptake via the GLUT4 transport protein. While an in-depth discussion of the biochemical signalling involved is beyond the scope of this literature review, a brief summary of the current knowledge is presented in this section and in Figure 4-1. A more complete discussion of the biochemistry of insulin signalling can be found in Saltiel *et al.* (2001) or Taniguchi *et al.* (2006).

The GLUT4 protein is the primary insulin-stimulated glucose transporter present in skeletal muscle (James, Brown et al. 1988), and is therefore responsible for the majority of whole body glucose disposal following a carbohydrate meal or glucose bolus. It allows the polar glucose molecule to cross the plasma membrane by facilitated transport (Huang and Czech 2007). Once inside the muscle cell, glucose is rapidly and irreversibly phosphorylated to glucose-6-phosphate (G6P) by hexokinase. As G6P cannot cross the cell membrane, this rapid phosphorylation traps the glucose within the cell and maintains the concentration gradient of glucose across the cell membrane, ensuring that the rate of transport across the membrane is the rate limiting step in myocellular glucose uptake (Dresner, Laurent et al. 1999). Under low-insulin conditions, the majority of GLUT4 is stored in an intracellular compartment so muscle glucose uptake remains at basal levels. However, upon stimulation by insulin, GLUT4 is translocated to the plasma membrane allowing rapid myocellular glucose uptake (James, Brown et al. 1988; Huang and Czech 2007).

The insulin receptor is a tetrameric protein consisting of two α-subunits and two β-subunits. Insulin binds to the α-subunit causing a conformation change which allows the β-subunit to activate by autophosphorylation of a β-subunit tyrosine residue (Luo, Beniac

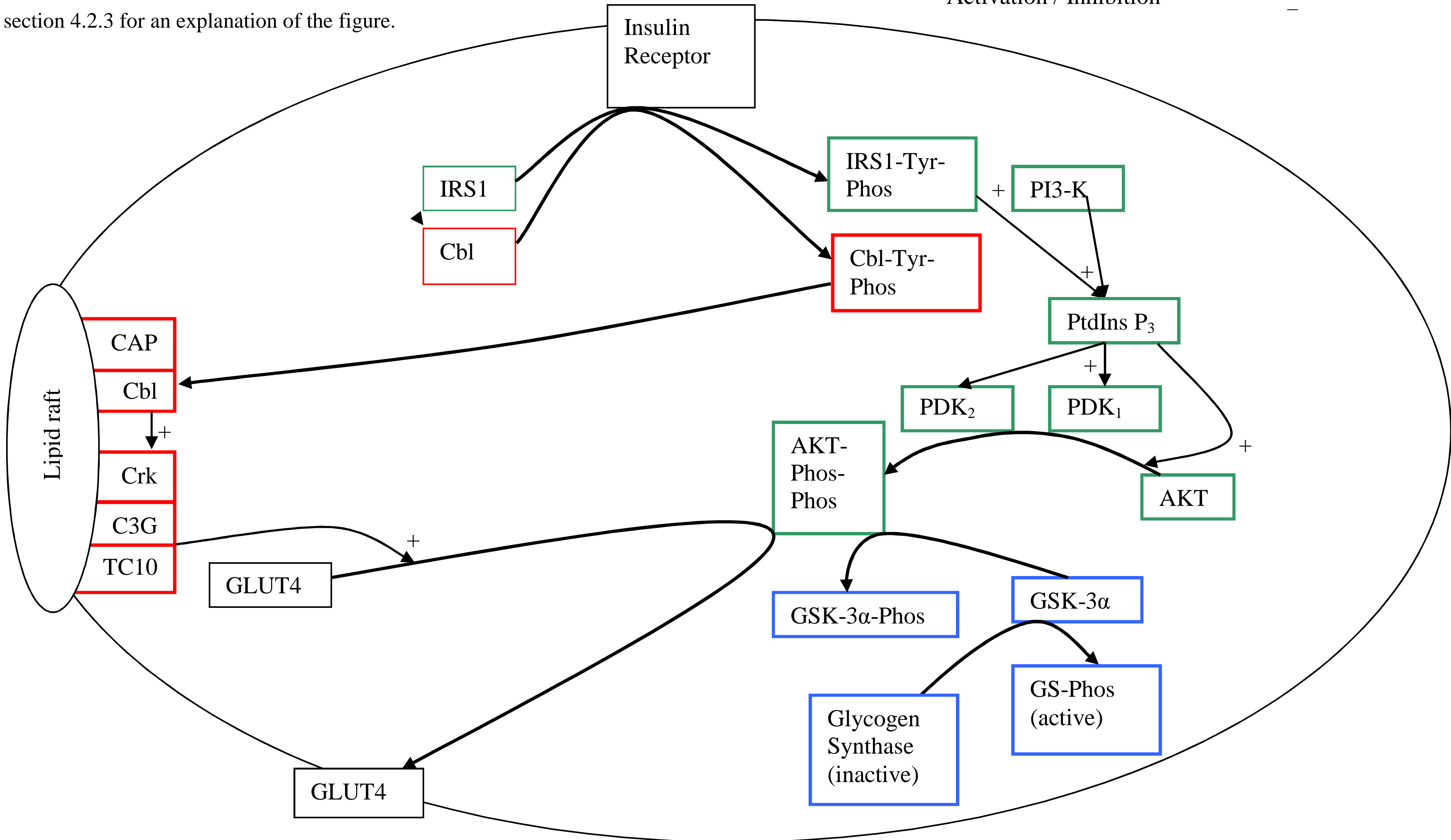
et al. 1999; Huang and Czech 2007). The activated β-subunit can then phosphorylate tyrosine residues on many different substrates, with insulin receptor substrate 1 (IRS1) being the primary substrate involved in signalling of glucose uptake in skeletal muscle (White 1998; Huang and Czech 2007).

Once phosphorylated, IRS1 interacts with phosphatidylinositol-3-kinase (PI3K) to phosphorylate phosphatidylinositol (PtdIns), producing PtdIns-P_3 (Myers and White 1996). PtdIns-P_3 in turn interacts with phosphoinositide-dependent kinase 1 and 2 (PDK1 and PDK2) to enable phosphorylation and activation of RAC-beta serine/threonine-protein kinase (AKT2 or PKB, Alessi and Cohen 1998). The PtdIns-P_3 protein also interacts directly with AKT2 to increase its activity and target it to the cell membrane (Alessi and Cohen 1998). The activated and localised AKT2 then enables increased GLUT4 translocation from the intracellular compartment to the cell membrane (Cong, Chen et al. 1997; Wang, Somwar et al. 1999).

A second signalling pathway that is also required for GLUT4 translocation begins with tyrosine phosphorylation of the Crk associated substrate NS-1 B-lineage proto-oncogene (Cbl) by the insulin receptor (Ribon and Saltiel 1997). Phosphorylated Cbl, with its associated adaptor protein (c-Cbl-associated protein, CAP) translocates to lipid rafts on the plasma membrane (Baumann, Ribon et al. 2000) and binds with a CrkII protein and guanyl nucleotide exchange protein (C3G) complex (Chiang, Baumann et al. 2001). C3G then draws the GTPase protein TC10 into the lipid rafts, which is permissive of GLUT4 translocation to the plasma membrane (Baumann, Ribon et al. 2000; Chiang, Baumann et al. 2001). Translocation of GLUT4 and subsequent glucose uptake is also stimulated by skeletal muscle contraction, independent of insulin (see section 4.4.1).

Figure 4-1. Insulin signalling of GLUT4 translocation and glycogen synthesis.

See section 4.2.3 for an explanation of the figure.



4.3 Acute dietary interventions and insulin sensitivity

4.3.1 Starvation

Starvation presents a formidable challenge to homeostasis in the human body. On the one hand, tissue must be broken down to provide a continuing supply of glucose for the central nervous system. On the other, functional tissue must be preserved to maintain physical and mental capacity (Cahill 1998). The ability of humans to survive prolonged periods of starvation demonstrates that there are effective regulatory mechanisms to balance the need for catabolism of energy stores with the need for preservation of function.

Small total glucose storage capacity

The total glycogen stored in an average 70 kg human male is usually less than 300 g (Owen, Morgan et al. 1967), although this can be increased by carbohydrate loading (Tarnopolsky, Atkinson et al. 1995) and exercise training (Bergstrom and Hultman 1966). Similarly, the total protein mass is approximately 11 kg (Wang, Shen et al. 2003), about half of which can be catabolised before death occurs (Owen 2005), although mobility will be significantly impaired after much smaller protein loss. Assuming that it takes about two grams of protein to produce one gram of glucose (Owen, Morgan et al. 1967), the total available glucose for an average human undergoing starvation is about 3000 g.

In normal post-absorptive conditions an average human brain consumes about 130 g of glucose per 24 hours (Reinmuth, Scheinberg et al. 1965). This means that in normal resting conditions, the brain alone could consume the body's entire store of carbohydrate within a little over two days, or enough protein to cause death within 23 days.

Additionally, carbohydrate is also consumed by other tissues, especially skeletal muscle. During heavy physical activity, glucose oxidation by skeletal muscle can functionally exhaust the body's glycogen stores in less than two hours (Gollnick, Armstrong et al. 1973). However, humans are able to survive over 60 days without eating (Owen 2005), so glucose oxidation must be reduced during fasting.

Reduced glucose oxidation

In the brain a reduction in glucose oxidation is achieved via substitution by ketone bodies. During prolonged periods of starvation, the liver converts FFA derived acetyl-CoA into ketone bodies (acetoacetate and β-hydroxybutrate) which can cross the blood brain barrier and are oxidised by the brain. In prolonged starvation this allows brain to reduce its carbohydrate oxidation to around 25 $g.day^{-1}$ (Owen, Morgan et al. 1967).

Progressing from the postprandial to postabsorptive state, plasma insulin concentration decreases and glucose disposal and oxidation by the periphery is diminished. As starvation progresses maximal rates of peripheral proteolysis must be avoided so circulating insulin concentrations are maintained at a low but physiologically effective concentration (Mortimore and Poso 1987; Fryburg, Barrett et al. 1990; Klein, Sakurai et al. 1993). However, insulin stimulation of muscle glucose uptake must also be avoided. These dual objectives are achieved via a reduction in the amount of glucose taken up by skeletal muscle in response to a given level of insulin (Defronzo, Soman et al. 1978; Mansell and Macdonald 1990; Horowitz, Coppack et al. 2001; Johnson, Stannard et al. 2006), without any diminution of the antiproteolytic effect of insulin (Fryburg, Barrett et al. 1990). This selective reduction in insulin sensitivity was initially referred to as

"starvation diabetes" (Peters 1945), but has now been recognised as an important part of the body's survival response during starvation (Reaven 1998).

When liver and skeletal muscle glucose oxidation is reduced, these tissues instead obtain energy by oxidation of lipids (Owen, Felig et al. 1969; Owen and Reichard 1971). The reduction in glucose oxidation itself provides a metabolic signal for increased lipid oxidation via the malonyl-CoA pathway. For glucose to be aerobically metabolised, the pyruvate produced by glycolysis must enter the mitochondria and be converted into acetyl-CoA for entry into the TCA cycle. Similarly, complete oxidation of lipids occurs via β-oxidation, which produces acetyl-CoA for further oxidation in the TCA cycle. A small proportion of mitochondrial acetyl-CoA is exported from the mitochondria as citrate via the citrate-malate shuttle, and is converted back to acetyl-CoA by ATP-citrate lyase (Saha, Vavvas et al. 1997). Cytosolic acetyl-CoA exists in equilibrium with malonyl-CoA, with the forwards reaction catalysed by acetyl-CoA carboxylase (ACC) (Brady, Gurin et al. 1952) and the reverse reaction catalysed by malonyl-CoA decarboxylase (MCD) (Alberts, Goldman et al. 1963; Saha, Vavvas et al. 1997). Cytosolic malonyl-CoA allosterically interacts with the cytosolic domain of carnitinine palmitoyl transferase-1 (CPT-1), increasing its K_m for LCACoA (Saggerson and Carpenter 1981; Saggerson and Carpenter 1981; McGarry, Mills et al. 1983). The reaction catalysed by CPT-1 is the rate limiting step for fatty acid transport into the mitochondria and for lipid β-oxidation, so increasing the K_m of CPT-1for LCACoA reduces the proportion of energy demands met by lipid oxidation (Saggerson and Carpenter 1981).

In skeletal muscle, the cytosolic concentration of malonyl-CoA can be altered by the concentration of its precursor, acetyl-CoA, or by the reaction rates of ACC and MCD. The flux through ACC and MCD is high (Reszko, Kasumov et al. 2004), so the cytosolic

concentration of malonyl-CoA responds rapidly (within minutes) to changes in the activity of these enzymes (Reszko, Kasumov et al. 2001). The ACC enzyme is activated allosterically by cytosolic citrate (Vavvas, Apazidis et al. 1997) and is inhibited by phosphorylation by AMPK (Chen, McConell et al. 2000), and by LCACoA via AMPK-dependent and as yet unidentified AMPK-independent phosphorylation (Fediuc, Gaidhu et al. 2006). As discussed in the previous paragraph, cytosolic citrate arises primarily by export from the mitochondria, and is a precursor of acetyl-CoA, so the activation of ACC by citrate helps to convert citrate to malonyl-CoA. LCACoAs are the substrate of CPT-1, and the form which lipids must take to enter the mitochondria for oxidation. The inhibition of ACC by LCACoA serves to decrease malonyl-CoA concentration when intracellular LCACoA is high, thus reducing inhibition of CPT-1, increasing removal of LCACoA from the cytosol, and increasing the proportion of energy demands met by lipid oxidation. Skeletal muscle AMPK activity and ACC phosphorylation are reported to be increased by LCACoA (Fediuc, Gaidhu et al. 2006), glycogen depletion (Wojtaszewski, MacDonald et al. 2003), and contraction (Winder and Hardie 1996; Vavvas, Apazidis et al. 1997; Wojtaszewski, Nielsen et al. 2000; Wojtaszewski, MacDonald et al. 2003). In turn, contraction increases AMPK activity via a reduced level of allosteric inhibition by PCR (Ponticos, Lu et al. 1998), increased allosteric activation by AMP (Ponticos, Lu et al. 1998; Stein, Woods et al. 2000), and phosphorylation by the AMP activated AMPK kinase (AMPKK) (Stein, Woods et al. 2000). Thus, AMPK is activated when cellular lipid supply is increased or ATP supply is reduced, and alters cell metabolism to increase ATP supply by, amongst other actions, inhibiting ACC, reducing malonyl-CoA concentration and increasing the rate of β-oxidation.

In human skeletal muscle during starvation, lipid oxidation is increased (Owen and Reichard 1971), probably primarily due to a decrease in the concentration of malonyl-

CoA (McGarry, Mills et al. 1983). However, there is some evidence suggesting that starvation may also reduce the effect of malonyl-CoA on lipid oxidation.. In rats, hepatic lipid oxidation during starvation is further increased by a reduction in the sensitivity of the hepatic isoform of CPT-1 (CPT-1a) to malonyl-CoA inhibition (Bremer 1981; Boon and Zammit 1988; Drynan, Quant et al. 1996). However, the same reduction of inhibition does not occur in rat cardiac muscle (Cook 1984; Mynatt, Lappi et al. 1992), which has the same isoform of CPT-1 as skeletal muscle (CPT1-b), and the reduction of inhibition has not been investigated in skeletal muscle or in humans. In any case, the malonyl-CoA pathway clearly plays an important role in matching fuel oxidation to intracellular fuel supply and energy demands.

Mechanism of decreased insulin sensitivity during starvation

The complete chain of physiological and biochemical events responsible for the reduction in whole body insulin sensitivity during starvation has not been determined. However, an important link in the chain is the intracellular FFA concentration in insulin sensitive tissues, particularly skeletal muscle. Many other signals have been suggested to reduce insulin sensitivity during obesity [e.g. plasma leptin (Shimomura and Hammer 1999), plasma adiponectin (Yamauchi, Kamon et al. 2001), adipose tissue inflammation (Xu, Barnes et al. 2003), and skeletal muscle blood flow (Baron, Laakso et al. 1991)], but have not been closely examined in the case of starvation.

Starvation causes a dramatic rise in plasma FFA concentration as the rate of adipose tissue lipolysis is increased (Horowitz, Coppack et al. 1999; Stannard, Thompson et al. 2002). Many studies have demonstrated a negative effect of plasma FFA concentration on insulin sensitivity. These studies are summarised in Table 4-1.

Table 4-1: Summary of human research linking blood plasma FFA concentration to whole body insulin sensitivity

Study	n	Subjects	Design	Results
(Perseghin, Scifo et al. 1999)	28	Sedentary first degree relatives of T2D patients and healthy controls	Cross section	-ve relationship $R^2 = 0.21$
(Perseghin, Scifo et al. 2002)	28	Young, healthy non-obese men and women	Cross section	-ve relationship $R^2 = 0.41$
(Paolisso, Tagliamonte et al. 1998)	50	First degree relatives of T2D patients	FFA lowered by acipimox or maintained by acipimox + lipid infusion	Insulin sensitivity higher when FFA were lowered ($p < 0.01$)
(Dresner, Laurent et al. 1999)	7	Young healthy	5 hour lipid + heparin infusion vs. saline, repeated measures	50% reduction in insulin sensitivity after lipid infusion
(Santomauro, Boden et al. 1999)	43	Lean healthy and obese healthy, impaired glucose tolerance and NIDDM	FFA lowered by overnight infusion of acipimox	Insulin sensitivity increased in all groups ($p < 0.001$)
(Boden and Chen 1995)	28	Older NIDDM patients	4 hour lipid infusion group vs. saline infusion group	40% reduction in insulin sensitivity after lipid infusion
(Boden, Chen et al. 1994)	14	Young healthy	6 hour lipid infusion group vs. saline infusion group	Dose dependent reduction in insulin sensitivity
(Kelley, Mokan et al. 1993)	10	Young healthy	4 hour lipid infusion vs. saline, repeated measures	Reduced whole body insulin sensitivity and single leg insulin sensitivity
(Roden, Price et al. 1996)	9	Young healthy	6 hour lipid infusion vs. saline, repeated measures	46% reduction in whole body insulin sensitivity

In skeletal muscle, FFAs are esterified to form triacylglycerols which are integrated into intramyocellular lipid (IMCL) droplets for storage (Watt 2009). A close relationship between the concentration of lipid within skeletal muscle and the extent of insulin resistance in overweight and obese humans was first observed in 1996 (Phillips, Caddy et al. 1996). During the following decade, encouraged by the development of NMR techniques to non-invasively measure IMCL (Boesch, Slotboom et al. 1997), researchers devoted a great deal of attention to IMCL as a potential mechanism explaining insulin resistance. This research showed that within populations with homogeneous levels of physical activity, people with elevated IMCL have lower insulin sensitivity (Phillips, Caddy et al. 1996; Goodpaster, Thaete et al. 1997; Pan, Lillioja et al. 1997; Krssak, Falk Petersen et al. 1999; Perseghin, Scifo et al. 1999; Ellis, Poynten et al. 2000; Levin, Daa Schroeder et al. 2001; Virkamaki, Korsheninnikova et al. 2001; Perseghin, Scifo et al. 2002; van Loon, Koopman et al. 2004; Salgin, Sleigh et al. 2009). Similarly, dietary interventions that increase IMCL concentrations also reduce insulin sensitivity (Bachmann, Dahl et al. 2001; Johnson, Stannard et al. 2006; van Loon and Goodpaster 2006). However, the reciprocal relationship between insulin sensitivity and IMCL content breaks down when differing levels of physical fitness are considered (Goodpaster, He et al. 2001; Stannard, Thompson et al. 2002; He, Goodpaster et al. 2004; Pruchnic, Katsiaras et al. 2004) and when exercise is undertaken simultaneously to IMCLs being elevated by dietary intervention (Schenk, Cook et al. 2005). This dissociation combined with the chemically inert nature of triglyceride molecules suggests that IMCL are not directly involved with a reduction in insulin sensitivity, but may be associated with the activity of other second-messengers.

The first stage of IMCL formation is the esterification of the FFA with coenzyme A to form a long chain acyl-CoA (LCACoA, Soupene and Kuypers 2008). LCACoA may

then be esterified with glycerol to form diacylglycerol (DAG, Koya and King 1998) or converted to ceramide at the endoplasmic reticulum (Cutler and Mattson 2001). All these molecules are potential second-messengers that may alter the insulin signalling cascade. Biochemical studies suggesting that intramyocellular FFA or its metabolites affect insulin sensitivity are summarised in Table 4-2.

Table 4-2: Summary of research linking intracellular FFA or its metabolites to decreased insulin sensitivity in whole organisms or skeletal muscle tissue.

Study	n	Subjects	Design	Results
(Turinsky, O'Sullivan et al. 1990)	12	Lean vs. insulin resistant obese Zuker rats	Metabolites measured in hind-limb muscles	Increased concentrations of ceramide and DAG in insulin resistant rat muscle
(Turinsky, O'Sullivan et al. 1990)	12	Lean Sprague-Dawley rats	Hind-limb muscle metabolites before and during hind-limb denervation	Increased ceramide and DAG in insulin-resistant soleus muscle, but not in insulin sensitive plantaris muscle
(Yu, Chen et al. 2002)	30	Male Wistar rats	1, 3 or 5 hour lipid infusion vs. saline (5 groups of 6)	LCACoA increased simultaneously with increased IRS-1 Ser phosphorylation and decreased IRS-1 activation by insulin. No change in ceramide, transient increase in DAG
(Chalkley, Hettiarachchi et al. 1998)	12	Male Wistar rats	5 hour lipid infusion vs. saline	Insulin sensitivity decreased and IMCL and muscle LCACoA increased
(Ellis, Poynten et al. 2000)	17	Men of varying insulin sensitivity undergoing elective knee surgery	Cross section	Intramyocellular LCACoA was significantly correlated with whole body insulin sensitivity ($r = -0.58$, $p = 0.01$)
(Thompson, Lim-Fraser et al. 2000)	9	*In vitro* soleus muscle strips from male Wistar rats	Muscles incubated with various free fatty acids	Intramyocellular LCACoA was significantly correlated with muscle insulin sensitivity ($r = -0.92$, $p = 0.03$)

Study	n	Subjects	Design	Results
(Schmitz-Peiffer, Craig et al. 1999)	6	C2C12 myotubes	Myotubes incubated with or without free fatty acids or ceramide	Incubation with either palmitate or ceramide produced similar changes in intramyotube ceramide and glucose uptake
(Saha, Kurowski et al. 1994)	12	6 lean and 6 obese mice	Cross section	Obese mice had elevated DAG, malonyl-CoA, plasma insulin and plasma glucose
(Itani, Ruderman et al. 2002)	12	Healthy men, 6 in lipid group and 6 in no-lipid group	Hyperinsulinaemic-euglycaemic clamp with or without lipid-heparin infusion	Intramyocellular DAG was elevated and insulin sensitivity was reduced at 6 hours of infusion but not at 2 hours.
(Kraegen, Saha et al. 2006)	15	Male Wistar rats, 3 groups of 5-7	3 or 5 hour glucose infusion to reduce insulin sensitivity	Intramyocellular DAG was elevated and insulin sensitivity was reduced at 5 hours of infusion but not at 3 hours.
(Yu, Chen et al. 2002)	30	Male Wistar rats, 5 groups of 6-8	5 hour lipid or glycerol infusion	Lipid infusion elevated DAG at 3-4 hours and LCACoA by 5 hours, with concomitant decreases in insulin stimulated IRS1 tyrosine phosphorylation

Intramyocellular FFA balance

The concentration of intramyocellular FFA is ultimately controlled by the balance between FFA transport into the cell and FFA oxidation in the mitochondria, although there is also buffering of FFA concentration by IMCL esterification and lipolysis (van Loon and Goodpaster 2006).

The mechanism of FFA transport across the cell membrane has traditionally been assumed to be passive diffusion requiring no carrier, consistent with the observation that the concentration of FFA in the cytoplasm of muscle cells is much lower than in blood plasma (Van der Vusse and Roemen 1995; Kiens, Roemen et al. 1999). However, in the late 1990s improved methodologies demonstrated that diffusion through a plasma membrane is too slow to account for observed rates of FFA uptake (Massey, Bick et al. 1997). In fact, the rate of FFA uptake into rat skeletal muscle plasmalemmal giant vesicles is saturable (Bonen, Luiken et al. 1998; Turcotte, Swenberger et al. 2000), and the rate is reduced by chemical disruption of protein structure (Bonen, Luiken et al. 1998) or by antibodies to putative carrier proteins (Turcotte, Swenberger et al. 2000), indicating that FFA diffusion is mediated by membrane proteins. The similar rates of membrane transport observed in giant vesicles with no ability to dispose of FFA, compared to intact cells that can quickly sequester FFA, demonstrate that it is membrane transport rather than metabolism or esterification of FFA that is rate limiting in FFA absorption (Bonen, Luiken et al. 1998; Turcotte, Swenberger et al. 2000). This information has led some authors to suggest that FFA uptake into muscle cells may be rate limiting in FFA metabolism (Bonen, Chabowski et al. 2007; Glatz, Luiken et al. 2010).

During starvation, plasma unbound FFA concentration increases to approximately the level required to saturate skeletal muscle FFA uptake (see Appendix 1 for calculation) (Bonen, Luiken et al. 1998; Turcotte, Swenberger et al. 2000) resulting in elevated IMCL content during starvation (Stannard, Thompson et al. 2002) with concomitant increases in intramyocellular LCACoA, ceramide and diacylglycerol concentrations.

Glucose-Fatty Acid Cycle (The Randle Cycle)

First proposed by Phillip Randle (Randle, Garland et al. 1963), the glucose-fatty acid cycle was the most prominent early theory to explain the association between plasma FFA concentration and insulin sensitivity. According to the cycle, elevated intracellular FFA concentration leads, by mass action, to increased FFA oxidation which in turn increases the acetyl-CoA/CoA ratio and mitochondrial concentration of the tri-citric acid (TCA) cycle intermediate citrate. Acetyl-CoA inhibits pyruvate dehydrogenase while citrate inhibits phosphofructokinase, leading to accumulation of glucose-6-phosphate (G6P). In turn, hexokinase is inhibited by G6P leading to an accumulation of glucose in the cytoplasm, decreased concentration gradient across the cell membrane and decreased flux through GLUT4 transporters (Randle, Priestman et al. 1994). While the existence of the enzymatic interactions of the cycle are well established, it seems unlikely that the cycle is quantitatively important in physiological insulin resistance. Modern techniques have allowed the measurement of decreased intracellular concentrations of glucose (Dresner, Laurent et al. 1999) and G6P (Roden, Price et al. 1996; Dresner, Laurent et al. 1999) when insulin sensitivity is reduced by lipid infusion, contrary to the prediction of the cycle. Furthermore, the demonstration of defective GLUT4 translocation in human insulin resistance (Garvey, Maianu et al. 1998) provides a mechanism that better fits these more recent observations. However, the glucose-fatty acid cycle may still be an

important determinant of the fate of glucose (glycolysis or glycogen synthesis) once it has entered the cell.

LCACoA

There is strong evidence for an association between intramyocellular LCACoA concentration and insulin sensitivity, primarily from rodent studies. In humans with a variety of different levels of insulin sensitivity there is a significant correlation between intramyocellular LCACoA concentration and insulin sensitivity ($r = -0.58$, $p = 0.01$, Ellis, Poynten et al. 2000). In obese rats, when insulin sensitivity was acutely increased by low-fat diet, exercise or starvation, changes in insulin sensitivity were significantly correlated with changes in intramyocellular LCACoA concentration ($r = -0.7$, $P = 0.001$, Oakes, Bell et al. 1997). When rat soleus muscle was incubated with various different FFAs, the reduction in insulin sensitivity was highly correlated with increases in LCACoA concentration ($r = -0.91$, $p < 0.03$, Thompson, Lim-Fraser et al. 2000). In addition, cross sectional rodent studies have reported elevated LCACoA in the muscle of insulin resistant rats (Oakes, Cooney et al. 1997; Chalkley, Hettiarachchi et al. 1998; Hoy, Brandon et al. 2009).

There have been few studies examining the molecular mechanisms behind the association between LCACoA concentration and insulin sensitivity. One potential mechanism is activation by LCACoA of the serine kinase protein kinase C (PKC), which in turn inhibits IRS1 by phosphorylation at serine^{307} (Orellana, Hidalgo et al. 1990; Majumdar, Rossi et al. 1991). However, another study suggests that that LCACoA can only activate PKC when diacylglycerol is also present (Bronfman, Morales et al. 1988). Study of rat skeletal muscle *in vivo* shows that lipid infusion increases intramyocellular LCACoA

concentration with simultaneous increases in PKC activation and IRS1 ser^{307} phosphorylation and decreases in IRS1 associated PI3-kinase (Yu, Chen et al. 2002). However, while there is strong evidence for an association between intramyocellular PKC activation and insulin sensitivity an rats, there is as yet no evidence demonstrating a direct action of LCACoA on the insulin signalling cascade in humans.

Ceramide

Like LCACoA, the intramyocellular concentration of ceramide has been shown to be related to insulin sensitivity in rats (Turinsky, O'Sullivan et al. 1990; Schmitz-Peiffer, Craig et al. 1999). However, unlike LCACoA there is also good evidence that ceramide directly interferes with insulin signalling. This is primarily because the ready availability of cell membrane permeable ceramide analogues (C_2- and C_6-ceramide) allows for simple experimental manipulation of intracellular ceramide concentrations. Incubation of cells with these analogues has been the methodology used in most studies to date, and studies using C2C12 myotubes in particular are likely to reflect the metabolic pathways of intact skeletal muscle. Incubating 3T3-L1 adipocytes, myeloid 32D cells and rat hepatoma FAO cells with C_2- or C_6-ceramide caused a decrease in insulin stimulated IRS1 tyrosine phosphorylation (Kanety, Hemi et al. 1996; Peraldi, Hotamisligil et al. 1996), although in one study this effect was only transient (Kanety, Hemi et al. 1996). In 3T3-L1 adipocytes, C2C12 myotubes and HMN1 motor neurons, incubation with C_2-ceramide inhibited insulin stimulated PKB phosphorylation and glucose uptake with no effect on IRS1 phosphorylation or PI3-K activity (Summers, Garza et al. 1998; Wang, O'Brien et al. 1998; Zhou, Summers et al. 1998). Interestingly, incubation of C2C12 myotubes with C_2-ceramide also increased basal (non-insulin stimulated) glucose absorption (Summers, Garza et al. 1998) and p38-MAPK activity (Wang, O'Brien et al. 1998), as is observed in

the skeletal muscle of diabetic humans (Kelley and Mandarino 2000). Incubation of C2C12 myotubes with C_2-ceramide induced similar changes in both AKT2 phosphorylation and ERK-MAPK activity (see Figure 4-1) as incubation with palmitate, with no change in IRS1 phosphorylation (Schmitz-Peiffer, Craig et al. 1999). This evidence suggests that increases in intramyocellular ceramide concentration act to decrease insulin sensitivity by interfering with MAPK and PKB signalling, although this phenomenon has not been confirmed *in vivo*.

Diacylglycerol

Evidence from both human and animal studies indicates that intramyocellular diacylglycerol is associated with decreased insulin sensitivity. Early research demonstrated that increased levels of intracellular LCACoA leads to increased diacylglycerol which in turn activates protein kinase C (PKC, Bronfman, Morales et al. 1988) which then phosphorylates and disables IRS1 via phosphorylation at serine^{307} (Cortright, Azevedo et al. 2000). Increased levels of diacylglycerol have been observed in the skeletal muscle of insulin resistant mice (Saha, Kurowski et al. 1994; Hiramatsu, Sekiguchi et al. 2002), rats (Schmitz-Peiffer, Browne et al. 1997) and in the insulin resistant denervated muscles of otherwise insulin sensitive rats (Turinsky, O'Sullivan et al. 1990). Furthermore, the development of insulin resistance is temporally related to the increase in DAG (Kraegen, Saha et al. 2006). In humans, intramyocellular diacylglycerol concentration was doubled and PKC activity was quadrupled when insulin resistance was induced by six hours of lipid-plus-heparin infusion (Itani, Ruderman et al. 2002).

It is possible that the association between LCACoA concentration and insulin sensitivity is an indirect effect via DAG. As DAG is synthesised from LCACoA, elevations in

LCACoA are likely to produce similar elevations in DAG. Also, the putative mechanism of DAG action is via PKC activation, and LCACoA concentration is also associated with PKC activation. However, lipid infusion in rats has been reported to reduce insulin sensitivity and elevate intramyocellular LCACoA concentration and PKC activation with no change in DAG concentrations (Yu, Chen et al. 2002), so it is likely that there is some alternative indirect pathway or an as yet undiscovered direct effect of LCACoA on insulin signalling.

Malonyl-CoA

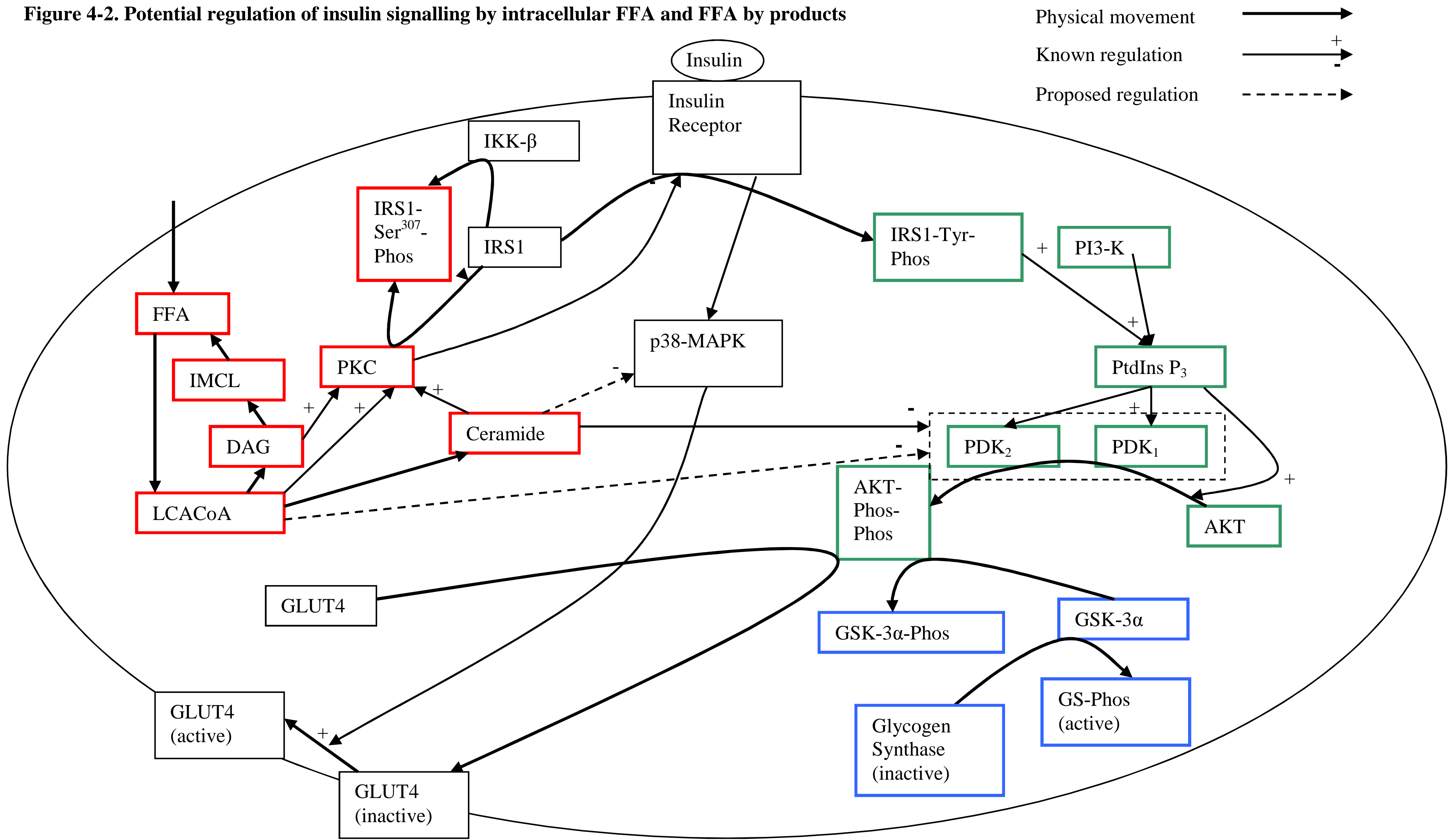
There is also a possibility that malonyl-CoA may participate in the attenuation of skeletal muscle insulin sensitivity during starvation (Ruderman, Saha et al. 1999; Ruderman and Prentki 2004; Patil, Minteer et al. 2007). There is a well established negative association between intracellular malonyl-CoA concentration and insulin sensitivity (Saha, Kurowski et al. 1994; Saha, Kurowski et al. 1995; Ruderman, Saha et al. 1998). Ruderman et al. (1999) suggested that this association could be accounted for by an over-supply of lipid, leading to increased production of acetyl-CoA, malonyl-CoA, and lipid products such as LCACoA, DAG and ceramide. Patil et al. (2007) found that incubation of the myotubes with the CPT-1 inhibitor 2-bromopalmitate had the same effect as increasing intracellular [malonyl-CoA], suggesting that reduced oxidation of lipids is sufficient to account for the association between [malonyl-CoA] and insulin sensitivity. However, when Patil et al. (2007) altered [malonyl-CoA] in myotubes by polyamine-mediated delivery of malonyl CoA or incubation with dichloroacetate, diclofop, clethodim, or Pfizer CP-640186, they found that insulin sensitivity changed in a negative relationship with [malonyl-CoA] but that no treatments produced the change in AKT phosphorylation predicted by the intracellular lipid product mechanism (Figure 4-2). These data suggest that malonyl-CoA is able to affect insulin sensitivity by some other pathway. With the evidence published

to date, the intracellular concentration of lipid products seems the most likely explanation for the association between [malonyl-CoA] and insulin sensitivity, but the Patil et al. (2007) study sheds some doubt on this mechanism.

Summary of metabolism during starvation

Starvation presents a challenge to the body's glucose homeostasis system. To meet this challenge, there is a decrease in circulating insulin concentration and simultaneous decrease in skeletal muscle insulin sensitivity, minimising the oxidation of glucose and catabolism of protein, and prolonging survival. In turn, skeletal muscle lipid oxidation is increased via a decrease in the intracellular concentration of malonyl-CoA. The mechanisms responsible for the decrease in skeletal muscle insulin sensitivity during starvation have not been resolved, but a likely signal is an increase in intramyocellular FFA concentration. Three potential second-messenger molecules mediating the effect of intramyocellular FFA on insulin sensitivity are LCACoA, ceramide and DAG, as summarized in Figure 4-2.

Figure 4-2. Potential regulation of insulin signalling by intracellular FFA and FFA by products



4.3.2 Carbohydrate deprivation

As described in the previous section, adaptation to starvation is geared towards preserving the limited remaining carbohydrate stores. It follows that other situations in which carbohydrate is scarce should produce a similar metabolic response, as blood glucose must be maintained in the face of low carbohydrate intake regardless of total dietary energy. This principle was first suggested by Fery *et al.* (1982) and has been supported Johnson *et al.* (2006). Both studies exposed lean, healthy subjects to mixed diet, isocaloric very-low-carbohydrate high-fat diet, or starvation, and found almost identical metabolic adaptations to starvation and the low-carbohydrate diet (Fery, Bourdoux et al. 1982; Johnson, Stannard et al. 2006). Furthermore, infusion of palmitate to maintain energy balance throughout an 84 hour fast did not alter the metabolic profile nor adipose lipolysis compared to 84 hours of starvation (Klein and Wolfe 1992). Conversely, infusion of glucose to maintain euglycaemia (~27 $g.day^{-1}$ or 10 % of resting energy requirements) partially ameliorated the increase in adipose lipolysis usually observed with 84 hours of starvation despite containing only a fraction of the energy of the lipid infusion (Klein, Holland et al. 1990).

The low-carbohydrate diet used by Johnson *et al.* (2006) was not able to completely eliminate dietary carbohydrate, but average residual daily carbohydrate intake was only 14 g, an order of magnitude less than the 130 g of glucose consumed each day by the average human brain when carbohydrates are plentiful (Reinmuth, Scheinberg et al. 1965) and approximately half the amount required to maintain euglycaemia (Klein, Holland et al. 1990). As the dietary carbohydrate intake equivalent was not sufficient to meet normal daily carbohydrate requirements, the authors suggested that the body activated the same responses as during starvation to preserve carbohydrate stores and functional tissue.

4.3.3 Lipid oversupply

Decreased insulin sensitivity is also observed in response to lipid infusion (Dresner, Laurent et al. 1999; Bachmann, Dahl et al. 2001; Kim, Kim et al. 2001; Itani, Ruderman et al. 2002; Yu, Chen et al. 2002; Schenk and Horowitz 2007; Hoy, Brandon et al. 2009) and high dietary fat intake (Oakes, Bell et al. 1997; Oakes, Cooney et al. 1997; Meugnier, Bossu et al. 2007) regardless of total energy intake, and in response to excess energy intake even in the form of carbohydrate (Schwarz, Neese et al. 1995; Faeh, Minehira et al. 2005). The common factor among all the dietary interventions known to decrease insulin sensitivity is elevated FFA availability in insulin sensitive tissues.

The supply of FFA is not necessarily reflected by the circulating FFA concentration. FFA can also be delivered to insulin sensitive tissues as circulating triglycerides (Miles, Park et al. 2004). Starvation (Stannard, Thompson et al. 2002), carbohydrate deprivation (Johnson, Stannard et al. 2006) and lipid infusion (Bachmann, Dahl et al. 2001) all increase FFA availability by increasing circulating FFA directly. High dietary fat intake decreases circulating FFA but increases circulating triglycerides primarily in the form of chylomicrons released by the gut (Oakes, Bell et al. 1997; Oakes, Cooney et al. 1997; Meugnier, Bossu et al. 2007). Energy excess, even when it is supplied only by carbohydrate, increases plasma triglyceride by up-regulating hepatic de-novo lipogenesis and very low density lipoprotein release and by down-regulating whole body lipid oxidation (Schwarz, Neese et al. 1995; Faeh, Minehira et al. 2005). Elevated triglycerides will not be detected when plasma samples are analysed for FFA, but insulin sensitive tissues contain lipases that release FFA from the circulating triglyceride droplets and thereby increase the local availability of FFA in the immediate extracellular environment

(Kiens, Essen-Gustavsson et al. 1987) so intracellular FFA concentration may be elevated even if sampled plasma FFA concentration is decreased (Oakes, Cooney et al. 1997; Miles, Park et al. 2004).

The ability of a high fat diet to increase intracellular FFA load and decrease insulin sensitivity raises the possibility that the changes observed by Johnson *et al.* (2006) in response to the low carbohydrate diet were due to high fat intake rather than carbohydrate scarcity. This possibility is further supported by lower plasma FFA concentrations in the low carbohydrate diet compared to starvation (Johnson, Stannard et al. 2006), suggesting decreased adipose tissue lipolysis and increased reliance on dietary lipid in the low carbohydrate diet compared to starvation. However, plasma FFA concentration was still significantly elevated in the low carbohydrate condition compared to the mixed diet, indicating increased adipose lipolysis. Furthermore, the residual carbohydrate in the low carbohydrate diet could also explain the difference in lipolysis between low carbohydrate and starvation conditions (Klein, Holland et al. 1990). The increase in FFA supply to insulin sensitive tissues in the Johnson *et al.* (2006) study was probably due to a combination of both carbohydrate deprivation (resulting in increased plasma FFA) and high dietary fat intake (resulting in increased plasma triglycerides). As the IMCL concentrations were equally elevated in the low carbohydrate and starvation conditions, it seems likely that the FFA availability to skeletal muscle was also similar between conditions despite the differing routes of FFA supply. However, experiments to date, which all replace dietary carbohydrate with isocaloric fat intake (Fery, Bourdoux et al. 1982; Klein and Wolfe 1992; Johnson, Stannard et al. 2006) cannot conclusively demonstrate that carbohydrate scarcity, independent of dietary fat content, is the trigger for the decrease in insulin sensitivity.

4.4 Exercise and insulin sensitivity

Skeletal muscle is a unique tissue in that it can rapidly increase its ATP production in response to contraction. During sustained exercise, replenishing ATP for repeated contractions requires the oxidation of large quantities of glucose, FFA, or both. The depletion of energy substrates in muscle cells has a profound effect on the uptake of those substrates.

4.4.1 Acute metabolic effect of exercise

A single exercise session is able to elevate glucose disposal above sedentary levels (Heath, Gavin et al. 1983; Richter, Ploug et al. 1985; Mikines, Sonne et al. 1989), primarily due to increased insulin sensitivity in skeletal muscle (Richter, Mikines et al. 1989). While the magnitude and duration of this effect are likely to change depending on the mode, duration and intensity of the exercise, the effect of acute endurance exercise resulting in marked depletion of intramuscular glycogen stores is well characterised and causes increases in both insulin stimulated and insulin independent glucose disposal.

Insulin independent glucose disposal, often called the mass action of glucose, is increased during and up to two hours after endurance exercise (Borghouts and Keizer 2000). Glucose disposal at very low insulin concentrations (~ 7 $mmol.l^{-1}$) has been observed immediately after glycogen depleting exercise in healthy humans (Minuk, Vranic et al. 1981; Richter, Mikines et al. 1989), rats (Richter, Ploug et al. 1985), and rat muscle after insulin was completely removed by immunoprecipitation (Wallberg-Henriksson and Holloszy 1985). This increase in mass action can be attributed to an increase in membrane associated GLUT-4 transporter (Goodyear, King et al. 1990; Goodyear,

Hirshman et al. 1991) which persists for at least 30 minutes but not two hours after exercise (Goodyear, King et al. 1990). While the increase in mass action is important for providing fuel for exercising muscle, the effect is transient and is not observed beyond two hours after exercise (Devlin, Hirshman et al. 1987), so is not likely to have an important effect on glucose disposal under resting conditions in daily living.

The elevation of insulin sensitivity after a single exercise session persists much longer than the elevation of glucose mass action. There are numerous studies demonstrating increased insulin sensitivity both immediately (Maehlum, Felig et al. 1978; Stephens, Sautter et al. 2007) and 12 to 16 hours after glycogen depleting exercise (Maehlum, Felig et al. 1978; Bogardus, Thuillez et al. 1983; Devlin, Hirshman et al. 1987; Mikines, Sonne et al. 1989). One study has reported that insulin sensitivity and the maximum rate of glucose disposal were both elevated 48 hours after a 90 minute endurance exercise session in untrained subjects (n = 7), but that this effect did not persist for five days (n = 3, Mikines, Sonne et al. 1988). There has been no reported attempt to identify the time course of changes in insulin sensitivity between two and five days after exercise. Although the maximum duration of this effect is unknown, it persists for at least 48 hours after the last exercise session, long enough for it to have a major impact on glucose disposal under resting conditions.

The site of increased insulin sensitivity after exercise is the exercised muscle itself. Using a one legged exercise protocol, Richter *et al.* (1989) were able to demonstrate an increase in post-exercise glucose disposal in the exercised leg compared to the rested leg despite the same blood supply to both legs. Similarly, Maehlum *et al.* (1978) showed that skeletal muscle accounts for a greater proportion of whole body glucose disposal after exercise than in rested conditions. An early study by Richter *et al.* (1982) used perfused

rat hind-limbs to demonstrate that within a single muscle group, insulin stimulated glycogen repletion was only increased in high-oxidative muscle where glycogen was depleted, not in low-oxidative muscle where glycogen was preserved. Methodological difficulties have prevented the demonstration of fibre type specific effects of acute exercise on insulin sensitivity in human muscle which contains a mixture of fibre types. However, muscle glycogen depletion occurs in a fibre type specific manner with depletion occurring only in type I and IIa fibres at low intensities (Tesch, Ploutz-Snyder et al. 1998) but in all muscle fibres at high intensities (Tesch, Ploutz-Snyder et al. 1998; Koopman, Manders et al. 2006). Moreover, endurance training causes adaptations associated with increased insulin sensitivity only in type 1 fibres (Daugaard, Nielsen et al. 2000). These data suggest that the effect of exercise on insulin sensitivity may follow the size principle in human skeletal muscle. That is, that contraction only affects insulin sensitivity in those muscle fibres which are recruited, so changes in insulin sensitivity due to low intensity contractions will be specific to small, type 1 muscle fibres (Cope and Pinter 1995; Henneman and Mendell 2011).

There is considerable evidence suggesting that the acute increase in insulin sensitivity after exercise may be due to negative carbohydrate balance. In response to a single exercise session, energy replacement with carbohydrate feeding directly after exercise abolishes the improvement in insulin sensitivity (Bogardus, Thuillez et al. 1983; Holtz, Stephens et al. 2008; Newsom, Schenk et al. 2010) whereas energy replacement with fat feeding does not (Holtz, Stephens et al. 2008; Newsom, Schenk et al. 2010). Similarly, glycogen supercompensation reverses the exercise induced increase in muscle GLUT4 translocation in rats (Kawanaka, Han et al. 1999), whereas maintenance of low muscle glycogen stores prolongs it (Garcia-Roves, Han et al. 2003). More difficult to interpret are the results of Hagobian and Braun (2006). They found that insulin sensitivity was

reduced by three days of energy surplus, but was partially restored by one day of exercise with increased energy intake to maintain the same energy surplus. While this study controlled energy surplus, it did not control carbohydrate surplus, so a difference in carbohydrate balance may still be responsible for the differences in insulin sensitivity between conditions.

It seems paradoxical that *increased* insulin sensitivity with exercise can be attributed to negative carbohydrate balance, while *decreased* insulin sensitivity with starvation is associated with a lack of dietary carbohydrate. It is possible that there are different metabolic responses to a decrease in carbohydrate supply with normal demand (i.e. starvation) and an increase in carbohydrate demand with normal supply (i.e. exercise), perhaps mediated by differences in muscle glycogen concentration. However these apparently conflicting results have not yet been reconciled.

4.4.2 Chronic metabolic effect of exercise

It is not clear whether there is a true chronic metabolic effect of exercise on glucose disposal (i.e. an effect of physical fitness on glucose disposal), or whether apparent chronic effects are merely a summation of the acute effects of recent bouts of exercise.

There is an abundance of evidence demonstrating improvements in insulin sensitivity when sedentary people undertake a training program (Kahn, Larson et al. 1990; Dela, Larsen et al. 1995; Duncan, Perri et al. 2003; Nishida, Tokuyama et al. 2004; Cauza, Hanusch-Enserer et al. 2005; Nassis, Papantakou et al. 2005). Moreover, these improvements follow a dose response relationship with exercise frequency and intensity

(Duncan, Perri et al. 2003). However, these results could be interpreted as the sum of the acute effects of the individual exercise bouts performed during training.

The lack of clarity as to whether there is a true chronic metabolic effect of exercise on insulin action is primarily due to the difficulty of separating chronic from acute effects. As the acute effect of a single exercise session has not been detected beyond five days after exercise, one approach to this problem has been to test trained people six or more days after the last exercise session. In sedentary young people who undertook six weeks of endurance training with no change in body mass or body composition, glucose effectiveness (i.e. the ability of glucose to promote its own disposal from the bloodstream) and insulin sensitivity were elevated after training and remained elevated one week after the last exercise session (Nishida, Higaki et al. 2001). However, when the same training protocol was used for twelve weeks, only elevated glucose effectiveness, not insulin sensitivity, was maintained after seven days of inactivity (Nishida, Tokuyama et al. 2004). Another study found that ten weeks of one legged cycling training in healthy sedentary subjects significantly improved insulin sensitivity in the trained vs. untrained leg, and this effect was still present six days after the final training session (Dela, Larsen et al. 1995). While the persistence of a training effect at six or seven days suggests a chronic effect of exercise, it is likely that the cumulative acute effects of multiple recent training sessions are detectable for longer than the effect of a single exercise session, so these results are not conclusive. Evidence of a more persistent effect has been found in sedentary overweight subjects who underwent an eight month training program of either 125 or 200 min.week^{-1} aerobic exercise. After training insulin sensitivity was increased 16 hours after the last exercise session, and in the 200 min.week^{-1} group insulin sensitivity remained elevated at 15 days after the last exercise session (Bajpeyi, Tanner et al. 2009). However, subjects in this study lost an average of 1.6 kg and, although it was

not measured, probably increased or maintained their lean body mass (Pavlou, Steffee et al. 1985). Thus, it is possible that increases in insulin sensitivity with training were not the result of alterations in tissue metabolism, but were due to reduced adiposity and increased volume of muscle to participate in glucose disposal.

An alternative approach to attempt to identify metabolic effects of chronic exercise is to compare the metabolism of trained subjects with that of sedentary subjects who have undergone acute exercise. Using this method Mikines *et al.* (1989) found greater insulin sensitivity and maximum rate of glucose disposal in trained subjects 16 hours after their last exercise session compared to age and weight matched rested, untrained subjects. However, a single exercise session was able to increase the untrained subjects' insulin sensitivity, but not maximum rate of glucose disposal, to equal to that of the trained subjects. That is, acute effects of a single exercise session could account for the decreased amount of insulin required to elicit 50 % of maximal glucose disposal in trained subjects, but acute effects could not account for the increased maximal glucose disposal in trained subjects. Differences in body composition between subject groups were also insufficient to account for the difference in maximum rate of glucose disposal (Mikines, Sonne et al. 1989). Unfortunately, the repeatability of these results needs to be confirmed as another study using a similar subject pool found that compared to sedentary subjects, trained subjects had elevated insulin sensitivity but not maximum rate of glucose disposal (King, Dalsky et al. 1987), in direct conflict with the results of Mikines *et al.* (1989).

Like the metabolic effects of acute exercise, the effects of exercise training on insulin sensitivity appear to be mediated by negative carbohydrate balance. When sedentary people were trained for eight days those who continued on their original diet increased insulin sensitivity, whereas those who received additional carbohydrates to replace the

energy expended showed no change in insulin sensitivity (Black, Mitchell et al. 2005). Similarly, ten weeks of training with energy replacement in lean, obese or diabetic men did not alter insulin sensitivity (Segal, Edano et al. 1991).

It remains uncertain whether the chronic metabolic effects of exercise on insulin sensitivity are due to some fundamental change in muscle metabolism with increased physical fitness, or merely the summation of the acute effects of recent bouts. However, this question is to some extent academic. Increased physical fitness increases exercise capacity, which in turn allows exercise to be performed more frequently, for a longer duration and at a higher intensity, thus increases the acute effects of exercise. Similarly, performing repeated bouts of exercise is likely to increase lean body mass and thus increase the amount of skeletal muscle participating in glucose disposal. As such, increased physical fitness can indirectly increase insulin sensitivity even though it may not alter tissue metabolism directly.

4.4.3 Intramyocellular FFA balance during exercise

As discussed in a previous section (4.3.1 Starvation), intramyocellular FFA concentration is a likely mediator of changes in insulin sensitivity. As the intramyocellular FFA concentration is always lower than the blood plasma concentration (Martin, Dalsky et al. 1993) and there is no active transport of FFA out of muscle cells, the only way that FFA may exit muscle cells is by oxidation (Martin, Dalsky et al. 1993). As such, a massive increase in substrate oxidation with exercise has a profound effect on intramyocellular FFA balance.

At rest, the metabolic rate of skeletal muscle is low, as is its rate of FFA oxidation. However, FFA oxidation is greatly increased during exercise (Martin, Dalsky et al. 1993; van Loon, Greenhaff et al. 2001; Watt, Heigenhauser et al. 2003). Although the proportions of carbohydrate, protein and FFAs oxidised by skeletal muscle depend on the available substrate supply (Staudacher, Carey et al. 2001) (also see the discussion of malonyl-CoA signalling in section 4.3.1) and the training status (van Loon, Jeukendrup et al. 1999) of the individual, the maximum absolute rate of FFA oxidation occurs during moderate intensity exercise, usually around 55 to 65% of VO_{2Max} (Essen, Hagenfeldt et al. 1977; van Loon, Greenhaff et al. 2001). At higher exercise intensities the rate of FFA oxidation falls and carbohydrate oxidation increases until carbohydrate accounts for almost all of the energy requirements (Essen, Hagenfeldt et al. 1977).

At the onset of exercise in the fed or postabsorptive state, circulating FFA availability and myocellular FFA uptake are low. During the first two hours of moderate intensity exercise, myocellular FFA uptake does not match oxidation and there are significant decreases in IMCL concentration (Watt, Heigenhauser et al. 2002; Koopman, Manders et al. 2006) and intramyocellular FFA concentration (Kiens, Roemen et al. 1999). During the first few minutes of moderate intensity exercise, plasma FFA concentration falls as adipose lipolysis fails the match the increased skeletal muscle FFA uptake (van Loon, Jeukendrup et al. 1999; Watt, Heigenhauser et al. 2003). However, the rate adipose lipolysis gradually increases and beyond 30 minutes of exercise plasma FFA concentration begins to rise (Romijn, Coyle et al. 1993; van Loon, Jeukendrup et al. 1999). By two hours of exercise, plasma FFA concentration is significantly elevated compared to rest (Romijn, Coyle et al. 1993; van Loon, Jeukendrup et al. 1999), and plasma FFAs continue to rise steadily until at least four hours of exercise (Watt, Heigenhauser et al. 2002). Simultaneously, and assuming no carbohydrate ingestion, the

rate of lipid oxidation rises and carbohydrate oxidation falls (Romijn, Coyle et al. 1993; Watt, Heigenhauser et al. 2002). Despite the increasing rate of lipid oxidation, IMCL stores remain stable between two and four hours of exercise at 57 % of VO_{2Max} (Watt, Heigenhauser et al. 2002), indicating that myocellular FFA uptake is equal to FFA oxidation during this time.

4.4.4 Exercise during starvation

During starvation the conditions at the onset of exercise resemble the conditions at the end of a prolonged exercise session. That is, carbohydrate stores are reduced and gluconeogenesis and plasma FFAs are elevated to a similar extent after both starvation and prolonged exercise (Ahlborg and Felig 1982; Knapik, Meredith et al. 1988; Bonen, Luiken et al. 1998; Turcotte, Swenberger et al. 2000; Watt, Heigenhauser et al. 2002). However, unlike a prolonged exercise session, starvation decreases insulin sensitivity and increases IMCL concentration (Johnson, Stannard et al. 2006). It is clear that skeletal muscle lipid oxidation must increase with exercise during starvation to meet metabolic demand, presumably via ACC inhibition and malonyl-CoA signalling (section 4.3.1). However, because plasma FFA are elevated even at the onset of exercise during starvation, its effects on intramyocellular FFA balance and insulin sensitivity are unknown.

Two studies have examined the effect of starvation on fuel selection during exercise. In both studies, healthy soldiers exercised to exhaustion at 45 % of VO_{2Max} after either an overnight fast or 3.5 days of starvation either with (Henschel, Taylor et al. 1954), or without (Knapik, Meredith et al. 1988) daily exercise. During starvation, resting plasma FFA concentration was elevated to a similar level as after two hours of exercise in the

postabsorptive state, and was further increased by exercise (Henschel, Taylor et al. 1954; Knapik, Meredith et al. 1988). Fat oxidation was increased and carbohydrate oxidation decreased during starvation, with significantly lower RER throughout exercise (Henschel, Taylor et al. 1954; Knapik, Meredith et al. 1988). Although muscle glycogen concentration was initially lower during starvation, exercise resulted in much greater glycogenolysis in the postabsorptive state so that post exercise muscle glycogen concentration was greater in starvation than in the postabsorptive state (Knapik, Meredith et al. 1988). Unfortunately, neither study attempted to measure muscle FFA uptake or IMCL content.

Studies which include exercise during periods of low dietary energy intake show qualitatively similar effects to total starvation. During Ramadan, the requirement to fast during daylight results in daily fasting of ~15 hours. One week of daily fasting significantly increased fat oxidation during exercise at 45 % of VO_{2Max}, although this effect was partially reversed after three weeks of daily fasting (Stannard and Thompson 2008). Similarly, in soldiers undergoing five days of heavy physical activity and low energy diet producing an energy deficit of approximately 9600 $kJ.day^{-1}$, plasma FFA were increased and RER was decreased both at rest and during exercise (Guezennec, Satabin et al. 1994).

When IMCL has been depleted by exercise without fasting, increasing myocellular FFA availability by high fat feeding results in IMCL supercompensation within 24 hours (Starling, Trappe et al. 1997; Décombaz, Fleith et al. 2000). As myocellular FFA availability is also increased during starvation, it seems likely that IMCL supercompensation may also occur after exercise during starvation.

4.4.5 Summary of exercise and insulin sensitivity

Acute exercise causes transient increases in both glucose effectiveness (~ two hours) and insulin sensitivity (two to five days). Exercise training also increases both glucose effectiveness and insulin sensitivity, but these increases are quickly lost after cessation of training. It is not clear whether the metabolic effects of chronic exercise on insulin sensitivity are genuine training effects or merely the sum of the acute effects of recent exercise sessions. Exercise increases the rate of FFA oxidation and endurance exercise results in negative myocellular FFA balance and decreased IMCL concentrations. As exercise progresses, the rate of carbohydrate oxidation decreases and FFA oxidation increases, but plasma FFA concentrations also rise and myocellular FFA uptake eventually increases to match FFA oxidation.

During starvation, insulin sensitivity and carbohydrate stores are reduced and plasma FFA concentration is elevated before exercise begins. During exercise, plasma FFA concentration is increased still further while carbohydrate oxidation is lower and FFA oxidation is higher than during exercise after an overnight fast. However, it is not clear what effect exercise has on insulin sensitivity or intramyocellular FFA balance during starvation.

4.5 Overfeeding, body composition and insulin sensitivity

It is no surprise that over-eating causes weight gain, increased body fatness and decreased insulin sensitivity. This common knowledge is confirmed by research demonstrating increased body mass and decreased insulin sensitivity when energy intake is increased above normal levels (summarised in Table 4-3), by cross-sectional observations that obese people have lower insulin sensitivity than lean people despite normal glucose tolerance (Bogardus, Lillioja et al. 1985; Karter, Mayer-Davis et al. 1996; Goodpaster, Thaete et al. 2000), and that thigh-section adipose tissue area is negatively related to insulin sensitivity (Goodpaster, Thaete et al. 1997).

4.5.1 Mechanisms of decreased insulin sensitivity during obesity

There are numerous potential means by which increased adiposity might decrease insulin sensitivity, but the complex nature of biological signalling has prevented the clear identification of any one mechanism. However, many authors (Kahn and Flier 2000; Kim, Gavrilova et al. 2000; Shulman 2000; Boden and Shulman 2002; Guilherme, Virbasius et al. 2008) have suggested that important information may be gleaned by also considering lipodystrophy (i.e. an absence of adipose tissue), which has an identical effect on insulin sensitivity, IMCL and hepatic triglyceride concentration (Carr, Samaras et al. 1998; Kim, Gavrilova et al. 2000). This section reviews the various proposed mechanisms by which obesity may induce insulin resistance with reference to their ability to explain insulin resistance in lipodystrophy.

Elevated circulating lipid availability is the most well described mechanism for obesity induced insulin resistance. In both obesity (Campbell, Carlson et al. 1994) and

lipodystrophy (Kim, Gavrilova et al. 2000) adipose tissue cannot maintain normal postprandial net lipid absorption so there is an increase in plasma lipid availability. Elevated lipid availability may then lead to decreased insulin sensitivity as described in section 4.3.1. As the inability to dispose of lipids is a major effect of lipodystrophy (Kim, Gavrilova et al. 2000), and in healthy mice overexpression of adipose lipid storage proteins increases adipose lipid balance and is protective against insulin resistance and but not obesity (Jong, Voshol et al. 2001), it is likely that changes in adipose lipid disposal are a primary defect in the insulin resistance of obesity.

Another potential signal for decreased insulin sensitivity in obesity is reduced secretion of adiponectin, an adipose specific hormone whose production is decreased in adipocytes from obese mice, rats and humans (Hu, Liang et al. 1996). Plasma adiponectin concentration is negatively correlated with adiposity and positively correlated with insulin sensitivity in humans (Weyer, Funahashi et al. 2001). Furthermore, myocellular adiponectin receptor content is positively related to insulin sensitivity and negatively related to family history of type-2 diabetes (Civitarese, Jenkinson et al. 2004), and adiponectin activation of AMP-kinase is positively related to insulin sensitivity in cultured myocytes obtained from lean, obese and type-2 diabetic humans (Chen, McAinch et al. 2005). In obese or lipodystrophic insulin resistant mice, adiponectin supplementation normalises insulin sensitivity via an increase in peripheral β-oxidation of lipids and a decrease in IMCL and hepatic triglyceride content (Yamauchi, Kamon et al. 2001). Similarly, adiponectin knockout mice exhibit decreased insulin sensitivity which is reversed by adiponectin supplementation (Maeda, Shimomura et al. 2002). However, despite the clear evidence in mouse models, there is no published data demonstrating that an increase in plasma adiponectin concentration improves insulin sensitivity in insulin resistant humans, so the importance of this mechanism remains unknown.

Adipose tissue in obese humans (Curat, Wegner et al. 2006) and mice (Xu, Barnes et al. 2003) contains an increased number of cytokine producing macrophage cells and has increased release of inflammatory cytokines such as TNF-α (Hotamisligil, Arner et al. 1995; Kern, Ranganathan et al. 2001) interlukin-6 (IL-6, Kern, Ranganathan et al. 2001), resistin (Curat, Wegner et al. 2006) and visfatin (Curat, Wegner et al. 2006), of which TNF-α (Hotamisligil, Peraldi et al. 1996; Kern, Ranganathan et al. 2001) has been proven to directly reduce insulin sensitivity in isolated skeletal muscle fibres. Increased macrophage content has also been associated with increased adipocyte death and greater adipose FFA release (Cinti, Mitchell et al. 2005). Lipodystrophic mice have very high macrophage content in their residual adipose tissue (Herrero, Shapiro et al. 2010) and are more susceptible to TNF-α induced liver damage (Sennello, Fayad et al. 2005) which could also contribute to the whole body insulin resistance. However, although IL-6 is elevated in the plasma of insulin resistant humans (Bastard, Jardel et al. 2000), infusion of IL-6 increases whole body insulin sensitivity in humans (Carey, Steinberg et al. 2006) and incubation of skeletal muscle with IL-6 increases insulin stimulated glucose disposal (Glund, Deshmukh et al. 2007). Thus, while there is very strong evidence for an association between insulin resistance, macrophage infiltration of adipose tissue and increased release of inflammatory cytokines, it is unclear whether increased inflammation is causal of or coincidental with insulin resistance.

Table 4-3: Summary of overfeeding research in humans

Study	n	Subjects	Physical activity	Estimated energy excess (MJ)	Mass gain (%)	Portion of mass gained as lean (%)	Mass gain efficiency ($g.MJ^{-1}$)	Effect on insulin sensitivity
(Ohannesian, Marco et al. 1999)	6	Young, lean, mixed gender, active	Instructed to continue normal activity	162-190	7.3	29	25-29	↔ Clamp
(Bouchard, Tremblay et al. 1990; Oppert, Nadeau et al. 1995)	24	Young, lean, sedentary, male, twin pairs	Sedentary	353	13	33	23	↓ OGTT
(Meugnier, Bossu et al. 2007)	8	Young, lean, male	Instructed to continue normal activity	61	1.5	80	16	↔ Fasting measures
(Erdmann, Kallabis et al. 2008)	10	Young, lean, male	Instructed to continue normal activity	-	8.7	48	-	↓ Clamp, OGTT and fasting measures
(Sun, Bishop et al. 2007)	61	Young, healthy, 24 lean, 16 overweight, 23 obese	Normal activity verified within 15 % by accelerometer	70	2.7	70	41	↓ Fasting measures

Study	n	Subjects	Physical activity	Estimated energy excess (MJ)	Mass gain (%)	Portion of mass gained as lean (%)	Mass gain efficiency (g.MJ^{-1})	Effect on insulin sensitivity
(Diaz, Prentice et al. 1992)	9	Young, 6 lean and 3 obese, male, variable activity	2 hours.day^{-1} controlled light exercise	260	10	42	29	-
(Forbes, Brown et al. 2007)	15	Young, lean, mixed gender	40 min.day^{-1} controlled light exercise	79-159	6.0	51	36	-
(Levitsky, Obarzanek et al. 2005)	12	Young, lean, mixed gender, active	Instructed to continue normal activity	56	2.3	48	31	-
(Norgan and Durnin 1980)	6	Young, lean, male	Sedentary	273	10	38	22	-
(Levine, Eberhardt et al. 1999)	16	Healthy, mixed gender	Sedentary	216	7.1	49	22	-
(Joosen, Bakker et al. 2005)	14	Young, lean, female	Instructed to continue normal activity	62	2.2	31	23	-

Leptin is another adipose hormone proposed to be a mediator of insulin resistance. Leptin supplementation improves insulin sensitivity in insulin resistant mice (Muoio, Dohm et al. 1997; Shimomura and Hammer 1999; Yamauchi, Kamon et al. 2001) via an increase in lipid oxidation (Muoio, Dohm et al. 1997; Minokoshi, Kim et al. 2002) and decrease in energy intake (Halaas, Gajiwala et al. 1995). Paradoxically, leptin concentration in humans is increased in obesity and is negatively correlated with insulin sensitivity (Kennedy, Gettys et al. 1997). The discord between the metabolic effects of leptin and its association with insulin resistance led to the proposal that leptin resistance might be an important component of obesity induced insulin resistance (Unger 1997). However, more recent evidence shows that leptin is able to stimulate a dose dependent increase in the expression of activated monocytes (Santos-Alvarez, Goberna et al. 1999) and macrophages (Loffreda, Yang et al. 1998) and the TNF-α and IL-6 production by those leucocytes (Loffreda, Yang et al. 1998; Santos-Alvarez, Goberna et al. 1999), which may in turn reduce differentiation and increase phagocytosis of adipocytes (Heilbronn and Campbell 2008). Thus, although leptin is insulin sensitizing via its whole body effects (i.e. decreased energy intake and increased lipid oxidation), it may also have insulin de-sensitizing effects via increased inflammation in adipose tissue. Which effect predominates probably varies depending on both genetic and environmental factors, but the current literature does not have enough data to confirm these effects in humans.

4.5.2 Experimental overfeeding and insulin sensitivity

As obesity is one of the most important risk factors for the development of insulin resistance, it does not seem surprising that interventions that increase adiposity also decrease insulin sensitivity. Unfortunately, the majority of interventions used to increase adiposity have altered or not adequately controlled physical activity (Table 4-3), making

it difficult to distinguish the effects of energy excess from those of changes in the pattern of exercise. A notable exception to this generalisation is the study by Sun *et al*. (2007) where subjects' regular physical activity levels were held constant and were verified by accelerometer. Although the data of Sun *et al*. (2007) suggested a reduction in insulin sensitivity, the study was not designed to investigate insulin sensitivity specifically so used only fasting insulin and glucose measurements. Studies that have used more reliable estimates of insulin sensitivity have found either no change (Ohannesian, Marco et al. 1999) or decreased insulin sensitivity (Bouchard, Tremblay et al. 1996; Erdmann, Kallabis et al. 2008), but have lacked strict control of physical activity.

A potentially important difference between the study which found no change in insulin sensitivity (Ohannesian, Marco et al. 1999), and those that observed decreased insulin sensitivity with overfeeding (Bouchard, Tremblay et al. 1996; Sun, Bishop et al. 2007; Erdmann, Kallabis et al. 2008), is the habitual physical activity levels of the subjects. Ohannesian *et al.* (1999) specifically recruited physically active subjects, whereas the other studies had no physical activity inclusion criteria. The greater habitual physical activity of the subjects used by Ohannesian *et al.* (1999) may have limited the change in insulin sensitivity.

4.5.3 Experimental overfeeding causes variable weight gain

While it is clear that overfeeding leads to an increase in body mass in healthy individuals, there is striking variability in the efficiency of mass gain and in the proportions of lean and fat mass gain. As shown in Table 4-3, reported mean efficiency of mass gain in overfeeding studies ranges from 16 to 36 $g.MJ^{-1}$ of energy excess (42 to 95 % efficient storage of excess energy provided, Diaz, Prentice et al. 1992). While variability of

energy storage is undoubtedly due to error in the estimation of energy intake or expenditure, it appears that a portion of these errors are systematic and due to real differences between the responses of individuals to overfeeding. That is, the change in energy expenditure when individuals are overfed may be able to be predicted, even though most studies have failed to measure it. The high variability in mass gain efficiency remains even between subjects within the same study with strict control of diet and physical exercise (Sims, Goldman et al. 1968; Diaz, Prentice et al. 1992; Levine, Eberhardt et al. 1999). Importantly, Bouchard *et al.* (1990) overfed twelve pairs of twins by 4.2 $MJ.day^{-1}$ in a live-in dormitory situation for 84 days and found that variability of mass gain was significantly smaller within twin pairs than between twin pairs ($p < 0.05$), suggesting that genetic or lifestyle factors influence mass gain efficiency. Similarly, there was significantly less variability in changes in regional adiposity within twin pairs than between twin pairs ($p < 0.01$, Bouchard, Tremblay et al. 1990).

Despite the large variability in mass gain in response to overfeeding, all energy consumed must somehow be accounted for. Differences in macronutrient absorption between subjects and diets are not large enough to explain the variability in efficiency (Saavedra and Brown 1991), so there must be some increase in energy expenditure in the low-efficiency subjects. One potential mechanism to achieve this is facultative thermogenesis or 'luxuskonsumption' (Grafe and Koch 1912), a putative phenomenon that allows some individuals to avoid obesity by increasing substrate cycling so as to offload excess energy as heat. Facultative thermogenesis has been the subject of countless experiments but has never been identified in well controlled human experiments (Sims, Goldman et al. 1968; Sims and Danforth 1987). A more likely mechanism is a change in "non-exercise activity thermogenesis" as proposed by Levine *et al.* (1999). According to this mechanism, some individuals may increase their energy expenditure through changes in activities such as

"fidgeting, spontaneous muscle contraction, and maintaining posture" (Levine, Eberhardt et al. 1999) without any conscious alteration of physical activity.

There is a surprising lack of research attempting to identify factors that might predict the effect of imposed energy surplus on changes in body composition. A comprehensive literature search revealed only one intervention study (Sun, Ukkola et al. 2002) that attempted to identify factors which can predict the change in body composition in response to a given energy surplus. Sun *et al.* (2002) overfed 24 twins (12 identical pairs) by 1000 $kcal.day^{-1}$ every day except Sunday for 100 days. They found that the proportion of type 1 fibres in *vastus lateralis* muscle was a significant predictor of the increase in body fat % with overfeeding. As an individual's proportion of type 1 muscle fibres is associated with the amount and type of physical activity they perform, the data of Sun *et al.* (2002) suggest that habitual exercise may modulate the effect of overfeeding on body composition.

In addition to Sun's (2002) intervention study, there is also epidemiological evidence which identifies factors which may modulate the effect of overfeeding. In particular, insulin sensitivity and circulating leptin concentration may affect subsequent weight gain in populations that are prone to weight gain. Subjects with initially higher insulin sensitivity subsequently gained more weight than those with initially lower insulin sensitivity in long term follow-up studies of Pima Indians (Swinburn, Nyomba et al. 1991; Pannacciulli, Ortega et al. 2007), older Dutch people (Wedick, Snijder et al. 2009), older American people (Wedick, Mayer-Davis et al. 2001), postmenopausal American women (Howard, Adams-Campbell et al. 2004), and Quebec adults (Boule, Chaput et al. 2008; Chaput, Tremblay et al. 2008). While this effect may be due to many factors, one possible cause is that poor insulin sensitivity may reduce the efficiency of weight gain in

response to overfeeding. There have also been reports of both positive (Lissner, Karlsson et al. 1999; Wedick, Snijder et al. 2009) and negative (Ravussin, Pratley et al. 1997; Monteleone, Fabrazzo et al. 2002) relationships between fasting plasma leptin concentrations and subsequent weight gain within adult populations prone to weight gain. Both fasting leptin concentration and insulin sensitivity could conceivably predict the efficiency of energy storage in response to overfeeding or the proportion of energy stored in different tissues in response to overfeeding, but neither possibility has been examined experimentally.

4.5.4 Summary of overfeeding

Overfeeding results in increased body mass and adiposity. The efficiency of mass gain in response to overfeeding appears to be highly variable. There has been little research attempting to identify factors that can predict the efficiency of weight gain in response to subsequent overfeeding. Numerous studies have examined the effect of acute overfeeding on insulin sensitivity but these studies have either used inadequate measures of insulin sensitivity or have had poor control of physical activity, and have produced conflicting results. The effects of overfeeding on weight gain and insulin sensitivity deserve further investigation.

5 Hypotheses

Study 1

Compared to the moderate carbohydrate diet, starvation would induce reductions in blood glucose concentration and insulin sensitivity and associated increases in IMCL and circulating FFA concentration, whereas HPLC diet would not.

Study 2

Endurance exercise during short term starvation will prevent the changes in insulin sensitivity, glucose effectiveness, plasma FFA concentration and IMCL normally observed during starvation without exercise.

Study 3

Endurance training will prevent the changes in insulin sensitivity usually observed with excess energy intake.

Insulin sensitivity, fasting leptin concentration or relative energy expenditure will predict the magnitude of body mass and fat gain in response to a given level of overfeeding

6 Research Design

This section gives a very brief out line of the design of each experiment. Full details and justification of each design are given in the review of techniques and each experimental chapter.

Study 1: The effect of high-protein, very-low-carbohydrate diet on insulin sensitivity and IMCL concentration.
Insulin sensitivity and IMCL concentration measured after each of three dietary interventions: three days of controlled mixed diet, three days of starvation and three days of high-protein, very-low-carbohydrate diet.

Study 2: The effect of endurance exercise on the changes in insulin sensitivity and IMCL that usually occur with starvation.
Insulin sensitivity and IMCL concentration measured after each of three dietary and exercise interventions: three days of controlled mixed diet with no exercise, three days of starvation with no exercise and three days of high-protein, very-low-carbohydrate diet with two endurance exercise sessions.

Study 3: The response to overfeeding in humans undergoing endurance training
Insulin sensitivity and body composition measured before and after four weeks of overfeeding in endurance trained athletes with normal training maintained throughout the study.

7 Review of techniques

This review describes the various measurement techniques considered for use in the experiments described in this thesis. For each technique there is a brief description of the development and methodology, then a discussion of the advantages, disadvantages and appropriate usage. The actual techniques chosen are described in each experimental chapter.

7.1 To determine insulin sensitivity

7.1.1 Direct Measures of Insulin Sensitivity

Hyperinsulinaemic-Euglycaemic Clamp

Development: The glucose clamp technique was formally described by DeFronzo *et al.* in 1979 (DeFronzo, Tobin et al. 1979) after being first developed at Yale Medical School in the 1960's (Andres, Swerdloff et al. 1966).

Methodology: With the subject in the fasted state, a cannula is inserted into a vein in each arm. The original protocol used an arterial cannula for blood sampling but more recent work has shown that venous sampling is sufficient as long as the blood is "arterialised" by warming the hand (Morris, Ueda et al. 1997). Plasma insulin concentration is raised by infusion of insulin at a constant rate for 120 minutes. To account for differences in body size, the insulin infusion rate is usually set in units of $mU.min^{-1}.m^{-2}$ body surface area (DeFronzo, Tobin et al. 1979; Beysen, Murphy et al. 2007). The increased plasma insulin concentration suppresses hepatic glucose production so that only infused glucose is entering the bloodstream. Glucose is then infused at a rate

sufficient to "clamp" blood glucose at a predefined concentration approximately equal to fasting blood glucose concentration. Blood glucose concentration is sampled every five minutes and the glucose infusion rate is adjusted to maintain blood glucose at basal levels (Furler, Zelenka et al. 1986). The rate of whole body glucose disposal in response to the set concentration of plasma insulin is taken to be equal to the rate of glucose infusion once a steady state is reached (DeFronzo, Tobin et al. 1979), usually defined as the final 30 minutes of a three hour protocol.

If within subject comparisons are of primary interest then the glucose disposal rate is often compared directly. Otherwise, an index of insulin sensitivity can be computed by normalising glucose disposal to body mass, body water, plasma insulin or some combination of these factors. One commonly used index is the insulin sensitivity index (S_I) which is defined as $S_I = M/(G \times \Delta I)$ where M is the steady state glucose disposal rate ($mg.min^{-1}$), G is the blood glucose concentration ($mg.dL^{-1}$) and ΔI is the difference between fasting and steady state plasma insulin concentrations ($\mu U.mL^{-1}$) (Katz, Nambi et al. 2000).

Assumptions: As the rate of glucose disposal is taken to be equal to the rate of glucose infusion, it is assumed that the steady state plasma insulin concentration is sufficient to completely suppress hepatic glucose production. This is a reasonable assumption in healthy subjects at plasma insulin concentrations > 60 $\mu U.mL^{-1}$ (Rizza, Mandarino et al. 1981; Soop, Nygren et al. 2000), which most hyperinsulinaemic-euglycaemic clamp protocols achieve. However, the assumption may not hold in subjects exhibiting hepatic insulin resistance (Defronzo, Soman et al. 1978). If it is suspected that hepatic glucose production may not be fully suppressed, it can be quantified by infusing a radio-labelled glucose tracer (Defronzo, Soman et al. 1978).

Many hyperinsulinaemic-euglycaemic clamp protocols assume a steady state will be maintained during a fixed time period (E.g. From 90-120 minutes of infusion) (DeFronzo, Tobin et al. 1979; Rizza, Mandarino et al. 1981). However, a steady state is often not attained until five hours of infusion, especially in less insulin sensitive subjects (Soop, Nygren et al. 2000). This assumption can be avoided by defining steady state as a period of 30 minutes or more where the coefficient of variation of plasma glucose, plasma insulin and glucose infusion rate are all < 5 % (Katz, Nambi et al. 2000; Chen, Sullivan et al. 2003).

It is often assumed that the fixed rate of insulin infusion per body surface area results in the same plasma insulin concentration in all subjects (DeFronzo, Tobin et al. 1979). This assumption may be confirmed by post-hoc analysis of blood samples for insulin concentration, although this extra analysis significantly increases the cost of the protocol.

Advantages: The hyperinsulinaemic-euglycaemic clamp is the most direct measure of whole body glucose disposal at a given insulin concentration. It has good repeatability (Morris, Ueda et al. 1997) with within subject coefficient of variation < 10 % (Mattias, Nygren et al. 2000; Mather, Hunt et al. 2001) and requires relatively few assumptions compared to other methods. As a result this test is the reference standard measure of insulin sensitivity.

Disadvantages: The hyperinsulinaemic-euglycaemic clamp is a very expensive and labour intensive procedure that requires a highly skilled operator. As such it is only

feasible in relatively small scale studies. For epidemiological studies or routine clinical monitoring a less labour intensive technique must be used.

The most frequently performed protocol for the hyperinsulinaemic-euglycaemic clamp measures glucose disposal at only one plasma insulin concentration. If this concentration happens to fall in a low-gain region of the glucose disposal – insulin concentration curve then it will render the test insensitive to differences in insulin sensitivity (**Figure 7-1**). In a homogenous population an insulin infusion rate can be chosen to match plasma insulin concentration to the high gain region of the curve, but in a heterogeneous population this approach may not be possible. In such situations sequential hyperinsulinaemic-euglycaemic clamps at increasing insulin infusion rates allow the determination of the entire curve (Rizza, Mandarino et al. 1981). When this approach is used, the outcome is divided into two parts: "insulin responsiveness", which is the maximum rate of glucose disposal at greater than physiological insulin concentrations; and "insulin sensitivity" which in this case is defined as the concentration of insulin required to elicit half maximal glucose disposal (Rizza, Mandarino et al. 1981). However, despite the extra information provided by sequential clamps, logistics and expense usually prevent their use.

Appropriate usage: The hyperinsulinaemic-euglycaemic clamp is the preferred technique for smaller studies involving a relatively homogenous group of subjects where insulin sensitivity is of primary interest. If a highly inhomogeneous group of subjects is used then repeated euglycaemic clamps with varying levels of hyperinsulinaemia are indicated.

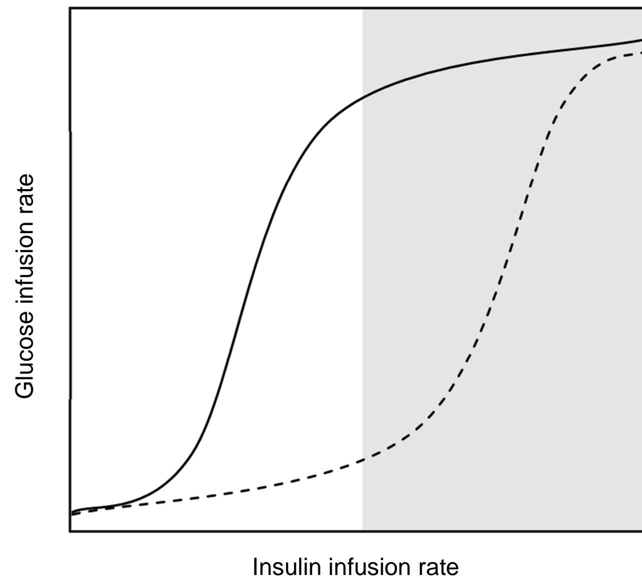


Figure 7-1. **Glucose response to different levels of insulin infusion during a euglycaemic clamp.** The white area represents a range of insulin infusion rates that will produce little change in the glucose infusion rate for insulin resistant individuals. The gray area represents a range of insulin infusion rates that will produce little change in the glucose infusion rate for insulin sensitive individuals. The hyperinsulinaemic-euglycaemic clamp may prove insensitive to changes in insulin sensitivity if the insulin infusion rate falls in a low gain region of any individual insulin-glucose curve.

Insulin Suppression Test

Development: Developed by a group at Stanford University during the 1960's (Shen, Reaven et al. 1970) and modified to its current form by Harano (Harano, Hidaka et al. 1978), the insulin suppression test is another technique that provides a direct measurement of whole body glucose disposal for a given rate of insulin infusion.

Methodology: After an overnight fast, cannulae are inserted as for the euglycaemic-hyperinsulinaemic clamp. Endogenous insulin and glucose production are suppressed by infusion of somatostatin [125 μg initial bolus followed by 250 μg.hour^{-1} (Harano, Hidaka et al. 1978)] or the somatostatin analogue octreotide [25 μg initial bolus followed by 30

μg.hour^{-1} (Pei, Jones et al. 1994)]. Insulin (25 mU.min^{-1}.m^{-2} body surface area) and glucose (240 mg.min^{-1}.m^{-2} body surface area) are infused simultaneously with the somatostatin for 180 minutes. Blood samples for plasma glucose and plasma insulin analysis are taken every 30 minutes until 150 minutes and then every ten minutes until 180 minutes. The mean of the last four samples is taken to be a steady state value (Ginsberg, Kimmerling et al. 1975). As the steady state plasma insulin concentrations are generally similar between subjects, the steady state plasma glucose (SSPG) concentration gives a direct estimate of insulin sensitivity. I.e. greater SSPG indicates poorer insulin sensitivity while a lower SSPG indicates better insulin sensitivity (Shen, Reaven et al. 1970; Pei, Jones et al. 1994).

Assumptions: As with the euglycaemic-hyperinsulinaemic clamp, the insulin suppression test assumes that endogenous glucose production is completely suppressed. This is a safe assumption in most subjects given the high levels of plasma insulin and glucose attained (Pei, Jones et al. 1994) plus the infusion of somatostatin (Wahren, Efendic et al. 1977; Bergman, Ader et al. 1984; Moller, Bagger et al. 1995). However it may be invalid in extremely insulin resistant subjects.

The insulin suppression test assumes there is no urinary glucose loss. While this is not likely to be a problem in healthy subjects, insulin resistant subjects develop very high plasma glucose concentrations during the test, which may cause significant glycosuria and underestimation of the severity of insulin resistance.

The period from 150 to 180 minutes of infusion is assumed to represent steady state conditions. Similarly to the hyperinsulinaemic-euglycaemic clamp protocol, this assumption can be avoided by defining steady state as a period of 30 minutes or more

where the coefficient of variation of plasma glucose and plasma insulin are both < 5 % (Katz, Nambi et al. 2000; Chen, Sullivan et al. 2003).

It is also assumed that similar steady state plasma insulin concentrations are obtained in all trials. As with the hyperinsulinaemic-euglycaemic clamp, the standardisation of insulin infusion rate to body surface area will usually achieve this aim, and the assumption can be verified by post-hoc analysis. Furthermore, a steady state is more readily achieved in the insulin suppression test than in the hyperinsulinaemic-euglycaemic clamp as there is no requirement to achieve a specific SSPG (Ginsberg, Kimmerling et al. 1975).

Perhaps the most tenuous assumption required for the insulin suppression test is that that somatostatin does not affect the response to insulin. Indeed, this effect is the reason that somatostatin or octreotide are used in the modern test and not the combination of epinephrine and propanolol used in the original protocol (Shen, Reaven et al. 1970; Harano, Hidaka et al. 1978). There is some evidence suggesting this assumption is false. When somatostatin is infused and insulin and glucagon are infused to maintain basal concentrations, whole body glucose disposal is increased 65% above basal (Bergman, Ader et al. 1984). Similarly, when somatostatin is infused directly into the forearm, local forearm glucose disposal is increased 55% compared to when a systemic somatostatin infusion is used (Moller, Bagger et al. 1995). As the same absolute rate of somatostatin infusion is used for all subjects regardless of body size, there may be a relative overestimation of insulin sensitivity in smaller subjects vs. larger subjects.

Advantages: The insulin suppression test provides a highly reproducible direct measure of insulin sensitivity that requires less operator skill than the hyperinsulinaemic-

euglycaemic clamp (Pei, Jones et al. 1994). It has been used to determine insulin sensitivity in studies involving up to 490 subjects (Yeni-Komshian, Carantoni et al. 2000). Steady state plasma glucose during the insulin suppression test correlates very well (r > 0.93 in healthy subjects, r = 0.91 in diabetic subjects) with steady state glucose disposal during the hyperinsulinaemic-euglycaemic clamp (Harano, Hidaka et al. 1978; Greenfield, Doberne et al. 1981; Beysen, Murphy et al. 2007).

Disadvantages: The insulin suppression test has similar disadvantages to the hyperinsulinaemic-euglycaemic clamp. While the insulin suppression test may be performed by a less skilled operator than the hyperinsulinaemic-euglycaemic clamp, it is still a labour intensive process requiring close monitoring of the subject for greater than three hours. Like the hyperinsulinaemic-euglycaemic clamp, the insulin suppression test uses only a single steady state insulin concentration, so care must be taken to ensure that this concentration falls in a high gain region of the insulin response curve.

The insulin suppression test exposes subjects to infusion of octreotide or other somatostatin analogue. Long-term medication with octreotide is associated with the development of gallstones and rare cases of anaphylaxis, bradycardia, pituitary and gastro-entero-pancreatic tumours, and thyroid disfunction, as well as more common but less serious side effects such as diarrhoea, abdomincal pain, nausea, flatulence, headache, cholelithiasis, hyperglycaemia and constipation (Medsafe New Zealand 2011). Although these drugs have low risk when used in an acute clinical setting with medical supervision, exposure may be avoided altogether by use of the hyperinsulinaemic-euglycaemic clamp.

While the insulin suppression test has been validated in a normal healthy population and an insulin resistant population, its performance has not been tested in an extremely insulin

sensitive population such as well trained endurance athletes. There may be a danger of these subjects becoming hypoglycaemic during the protocol. The insulin suppression test therefore requires further testing before being applied to this population in a research setting.

Appropriate usage: The insulin suppression test provides a reliable alternative to the hyperinsulinaemic-euglycaemic clamp when the latter technique proves unfeasible. However, care should be taken in applying the insulin suppression test to highly insulin sensitive or highly inhomogeneous populations.

7.1.2 Frequently Sampled Intravenous Glucose Tolerance Tests

Frequently sampled intravenous glucose tolerance test (FSIVGTT) with minimal model analysis

Development: While the intravenous glucose tolerance test was first performed in the late 1930's (Tunbridge and Allibone 1940), algorithms to estimate insulin sensitivity were not developed until after the advent of direct measures that could be used to validate the model. Even after these techniques became available many published models gave inaccurate results (Corte, Romano et al. 1970; Segre, Turco et al. 1973) or involved greater logistical challenges than direct methods (Insel, Liljenquist et al. 1975). The current FSIVGTT protocol with minimal model analysis was developed by Richard Bergman in 1979 by selecting the best of seven proposed models of glucose disposal (Bergman, Ider et al. 1979).

Methodology: After an overnight fast cannulae are inserted as for the euglycaemic-hyperinsulinaemic clamp. A single bolus of glucose (usually 300 $mg.kg^{-1}$ body mass) is infused into one cannula over a two minute period beginning at time 0. Blood samples for plasma glucose and insulin analysis are drawn from the sampling cannula at frequent intervals over three hours. A typical sampling schedule might take blood samples at -10, 0, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180 minutes (Cobelli, Ruggeri et al. 1985). The plasma glucose and insulin data are then used to determine the parameters of the minimal model, which in turn can be used to determine the insulin sensitivity index, S_i (Bergman, Ider et al. 1979). A computer program, MINMOD, is available to perform the minimal model calculations (Pacini and Bergman 1986).

The minimal model consists of two simultaneous differential equations. Equation 7-1 describes the rate of glucose disappearance [dG/dt] in terms of measured plasma glucose concentration [G(t)] and the insulin concentration in a hypothetical insulin signalling compartment separate from plasma insulin [X(t)]. Equation 7-2 describes the rate of insulin appearance or disappearance in the insulin signalling compartment in terms of plasma insulin [I(t)]. Parameters G_b and I_b are basal plasma insulin and glucose respectively, G_0 is the hypothetical glucose concentration at time 0 extrapolated from the modelled glucose measurements, and p_1, p_2 and p_3 are fitted to the data using nonlinear least squares estimation. The physiological relevance of each of the terms in the equations is given in Figure 7-2.

Equation 7-1 $$dG/dt = -G(t).[\ p_1 + X(t)\ ] + p_1.G_b$$

$$G(0) = G_0$$

Equation 7-2 $$dX/dt = -p_2.X(t) + p_3.[\ I(t) - I_b\ ]$$

$$X(0) = 0$$

The insulin sensitivity index is calculated as the ratio of p_3 to p_2 ($S_I = p_3/p_2$). The minimal model can also provide an estimate of glucose effectiveness (the tendency of plasma glucose to promote glucose disappearance) which is given by p_1.

Radzuik (2000) argues that the minimal model is mathematically equivalent to the hyperinsulinaemic-euglycaemic clamp, as well as the HOMA and QUICKI fasting indices (see below), and that this explains the good correlation between these methods. However, the mathematical equivalence of different empirically derived models could also be viewed as conceptual validation of these models.

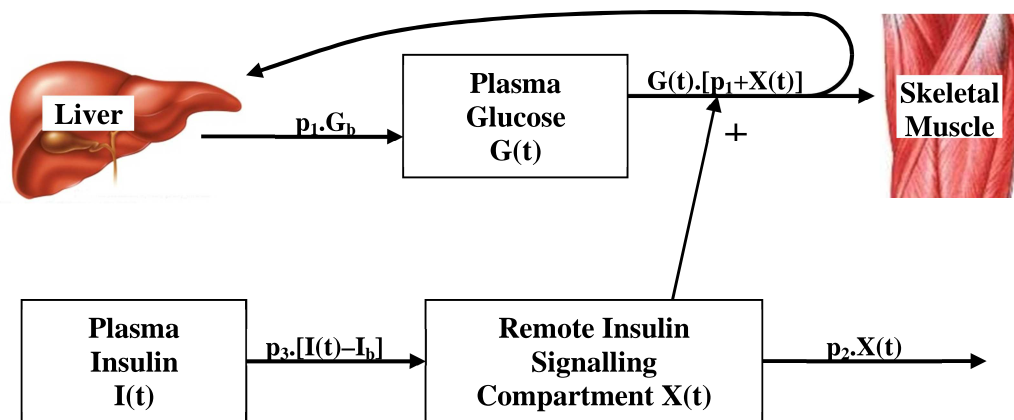


Figure 7-2. Schematic diagram of the minimal model of glucose disposal. Glucose appearance in the blood is given by $p_1.G_b$. Glucose disappearance is given by $G(t).[p_1+X(t)]$. The model does not adjust liver glucose production for changes in insulin concentration; instead these changes are modelled by a greater rate of glucose absorption by the liver and skeletal muscle. The movement of insulin from the blood into the insulin signalling compartment is given by $p_3.[I(t)–I_b]$ and the disappearance of insulin from the signalling compartment is given by $p_2.X(t)$. Explanations of the symbols used are given in the text.

Assumptions: While the FSIVGTT avoids the need for steady state conditions required for direct measurement of insulin sensitivity, the minimal model requires a number of additional assumptions:

- The infused glucose is assumed to diffuse instantaneously throughout the body. In reality the infused glucose can take up to eight minutes to diffuse (Yang, Youn et al. 1987) requiring that data collected during this time be excluded from the fitting of the minimal model. This approach may be problematic in very insulin sensitive populations in which the majority of the insulin response may occur during the mixing period.
- Glucose is assumed to be distributed in and act from a single homogenous glucose space, resulting in artificially inflated estimates of glucose effectiveness, particularly when there is a small insulin response (Finegood and Tzur 1996; Cobelli, Bettini et al. 1998).
- Net glucose disposal is assumed to occur at a mono-exponential rate, suggesting a non-saturable passive diffusion mechanism.
- The insulin response to the glucose load is assumed to be large enough and prolonged enough to allow meaningful estimation of the effect of insulin (Yang, Youn et al. 1987). This assumption is often violated in diabetic patients who may have only a minimal insulin response.
- Plasma glucose is assumed to return to resting levels by the end of the FSIVGTT protocol.

Advantages: The FSIVGTT with minimal model analysis provides an estimate of insulin sensitivity that in healthy subjects that correlates well with [r = 0.89 (Bergman, Prager et al. 1987), r = 0.72 (Steil, Hwu et al. 2004)] and has a similar coefficient of

variation (Ferrari, Alleman et al. 1991; Steil, Murray et al. 1994) to the hyperinsulinaemic-euglycaemic clamp.

Compared to direct methods for measuring insulin sensitivity, the FSIVGTT requires less skill to perform as it uses only one infusion at the start of the protocol and does not require steady state conditions. As such it to be used in larger studies than would be feasible with a direct method (Howard, O'Leary et al. 1996). The use of only a single glucose infusion also reduces the risk to the subject.

The FSIVGTT provides more information than direct methods to measure insulin sensitivity. As it is a dynamic test, it combines information about insulin sensitivity over a range of physiological insulin concentrations, rather than just a single steady state insulin concentration. As it utilises endogenous insulin production, the FSIVGTT can provide estimates of glucose effectiveness and pancreatic β-cell response as well as insulin sensitivity.

Disadvantages: While a FSIVGTT is simpler to perform than direct methods for measuring insulin sensitivity, it is still requires intravenous infusion and three hours of blood sampling, precluding its use in larger studies for all but the most well resourced researchers.

Due to its oversimplification of glucose homeostasis, the minimal model involves many assumptions. While the minimal model provides good estimates in populations with normal insulin secretion, it can provide very poor estimates in diabetic populations (Yang, Youn et al. 1987; Saad, Anderson et al. 1994; Katz, Nambi et al. 2000). Modified

FSIVGTT protocols (see below) are available that improve the performance of the minimal model in these populations.

Appropriate usage: The FSIVGTT with minimal model analysis is a good method for estimating insulin sensitivity in a healthy population if estimates of glucose effectiveness and/or β-cell responsiveness are of interest in addition to insulin sensitivity. The FSIVGTT may also be used if insulin sensitivity is of primary interest but technical or ethical considerations preclude the use of a direct method. When subjects with impaired insulin secretion are to be tested then a modified FSIVGTT protocol is preferred to improve the performance of the minimal model.

Insulin modified FSIVGTT with minimal model analysis

Development: Due to the poor performance of the standard FSIVGTT with minimal model when used with diabetic subjects, modified FSIVGTT protocols that increase the plasma insulin response have been established. The same laboratory that developed the original minimal model modified the FSIVGTT by introducing a tolbutamide infusion to stimulate a second insulin peak (Yang, Youn et al. 1987). To allow for diabetic subjects in whom the tolbutamide infusion was ineffective, the protocol was further modified to use an insulin infusion (Finegood, Hramiak et al. 1990).

Methodology: The modified FSIVGTT is performed as for the standard FSIVGTT with the additional infusion of 300 mg of tolbutamide over two minutes beginning at 20 minutes (Bergman, Prager et al. 1987; Yang, Youn et al. 1987; Saad, Anderson et al. 1994). The tolbutamide infusion stimulates pancreatic insulin release resulting in a second insulin peak well after full mixing of the glucose has occurred, improving the

performance of the minimal model. Alternatively, the tolbutamide infusion may be replaced by a 0.03 U.kg^{-1} insulin infusion (Finegood, Hramiak et al. 1990; Saad, Steil et al. 1997), which also results in a second insulin peak with the added advantage that it can be used with type-1 diabetic subjects who have no endogenous insulin production.

Assumptions: By artificially elevating insulin, the modified FSIVGTT avoids the assumption that the insulin response will be large enough to allow efficient minimal model estimation. The secondary insulin peak also reduces the importance of rapid mixing of the infused glucose. Otherwise, the modified FSIVGTT requires the same assumptions as the standard FSIVGTT.

Advantages: The modified FSIVGTT allows for greater accuracy of the minimal model in diabetic subjects (Saad, Steil et al. 1997).

Disadvantages: The increased levels of plasma insulin induced by the modified FSIVGTT have the potential to result in hypoglycaemia in very insulin sensitive individuals, while the addition of another infusion adds to the risk of complications. The modified FSIVGTT therefore entails a greater level of risk than a standard FSIVGTT, particularly when used in very insulin sensitive subjects.

Appropriate usage: The modified FSIVGTT is indicated when the standard FSIVGTT is to be used on diabetic patients, but is contraindicated for very insulin sensitive subjects. Given the added complication and risk of insulin infusion, direct methods to estimate insulin sensitivity should also be considered whenever the modified FSIVGTT is indicated.

"Hot" FSIVGTT with minimal model analysis

Development: The hot FSIVGTT was developed by Claudio Cobelli (Cobelli, Pacini et al. 1986) to allow estimation of the rate of glucose appearance and disappearance during an FSIVGTT.

Methodology: The hot FSIVGTT is performed as for the standard or modified FSIVGTT with the exception that the infused glucose contains a small amount of radio-labelled or stable-labelled tracer. A modified minimal model is then used which separates net glucose balance into rates of peripheral glucose disposal, hepatic glucose disposal and hepatic glucose production. Both single glucose compartment (Cobelli, Pacini et al. 1986) and two glucose compartment (Caumo and Cobelli 1993) models have been developed.

Assumptions: As the hot FSIVGTT allows estimation of separate rates of glucose appearance and disappearance, it avoids the assumption that the liver and skeletal muscle act in concert as a one-dimensional glucose balance mechanism. In the two-glucose compartment minimal model, glucose is assumed to rapidly distribute throughout an "inaccessible compartment" which slowly equilibrates with an "accessible compartment". Insulin is then assumed to act only on glucose in the accessible compartment.

Advantages: The primary advantage of the hot FSIVGTT is that it allows the use of a more realistic physiological model. The improved model allows estimation of individual rates of glucose appearance and hepatic and peripheral glucose disposal, dramatically

reducing the coefficient of variation of S_i in diabetic subjects (hot FSIVGTT 12 %, standard FSIVGTT 105 %, Avogaro, Vicini et al. 1996).

Disadvantages: While the hot FSIVGTT with two compartment minimal model is undoubtedly a better representation of the physiology of glucose disposal, the use of labelled glucose makes it a much more expensive and difficult protocol than the standard FSIVGTT. The use of radio-labelled tracer allows cheaper analysis of glucose kinetics than does stable-labelled tracer, but also exposes subjects and researchers to radiation.

Appropriate usage: The hot FSIVGTT with stable-labelled tracer is appropriate for well resourced studies where insulin sensitivity is of primary interest but the insulin infusion required by direct methods is considered too great a medical risk (e.g. in children or pregnant women). The hot FSIVGTT is also indicated if the individual insulin sensitivities of liver and skeletal muscle are required.

7.1.3 Oral Glucose Tolerance Tests

The standard OGTT (measurement of blood glucose two hours after a 75 g glucose drink) is widely used in clinical settings for the diagnosis of all forms of diabetes (American Diabetes Association 2007). However, it is a test of glucose tolerance and the results are affected not only by insulin sensitivity but also β-cell sensitivity, rate of glucose absorption from the gut and other metabolic factors (Matsuda and DeFronzo 1999), so will not be further considered in this review.

Frequently Sampled OGTT with minimal model analysis

Development: To obtain an estimate of insulin sensitivity from the OGTT, a research team lead by Claudio Cobelli developed another version of the minimal model that accounts for the variable rate of ingested glucose appearance in different subjects (Caumo, Bergman et al. 2000; Breda, Cavaghan et al. 2001).

Methodology: With the subject in the fasted state, a sampling cannula is inserted into a vein in the arm. At time 0, the subject ingests a meal or glucose solution containing 75 g of glucose. Blood samples for analysis of plasma glucose and insulin are taken at 0, 10, 20, 30, 60, 90 and 120 minutes (Breda, Cavaghan et al. 2001). An estimate of insulin sensitivity is then obtained using a modified minimal model.

The OGTT minimal model differs from the FSIVGTT minimal model in that it includes an extra term to represent the slow appearance of ingested glucose into the blood stream (Equation 7-3). This term includes two additional parameters that must be determined: ‘a’ describes the mass action effect of glucose on glucose disappearance and ‘b’ is a scale factor for the relationship between the rate of glucose absorption and changes in blood glucose concentration.

Equation 7-3 $dG/dt = -G(t).[\, p_1 + X(t)\,] + p_1.G_b + [a\Delta g(t) + \Delta dG/dt] / b.V$

$G(0) = G_0$

Where $\Delta g(t)$ is the excursion of glucose above basal levels, $\Delta dG/dt$ is the rate of change of $\Delta g(t)$ and V is the volume throughout which glucose is distributed. As there are now two extra parameters that must be estimated this model is not uniquely identifiable unless

values are assumed for glucose effectiveness and the fraction of total glucose absorbed from the gut (Dalla Man, Campioni et al. 2005).

Assumptions: The primary assumption of the minimal model used with the OGTT is that the rate of absorption of glucose into the bloodstream is directly proportional to the blood glucose concentration and the rate of change of blood glucose concentration. This assumption has been validated by comparing glucose appearance calculated using the model with glucose appearance determined using a dual tracer method and shows no significant differences between individual data points and no systematic deviation of residuals from zero (Dalla Man, Caumo et al. 2004).

To make the model parameters uniquely identifiable, values for glucose effectiveness and the fraction of glucose absorbed from the gut are assumed to be equal to standard values from literature. While changes in glucose effectiveness have little effect on estimates of insulin sensitivity, changes in the fraction of glucose absorbed have a profound effect so violation of this assumption (e.g. by testing subjects after dietary manipulation) will produce in poor results (Caumo, Bergman et al. 2000).

Advantages: As the OGTT involves no infusions, it is much safer, less invasive and easier to perform than the FSIVGTT. It also has slower insulin and glucose kinetics than the FSIVGTT so may be a better representation of the glucose challenges encountered in daily living. Like the FSIVGTT, the OGTT allows estimation of β-cell sensitivity as well as insulin sensitivity.

In validation studies using populations with normal and impaired insulin sensitivity, S_i from the OGTT correlates well with S_i from the modified FSIVGTT [r = 0.9 (Caumo, Bergman et al. 2000) and r = 0.72 (Basu, Breda et al. 2003)], dual tracer OGTT [r = 0.86 (Dalla Man, Caumo et al. 2004) and hyperinsulinaemic-euglycaemic clamp [r = 0.76 (Steil, Hwu et al. 2004)]. These results show that the OGTT compares very favourably with, and in some situations out-performs, the FSIVGTT.

Unlike other OGTT estimates of insulin sensitivity, the oral minimal model uses the observed glucose data to inform the model of glucose rate of appearance so is able to perform well even in subjects with slow glucose absorption, provided the fraction of glucose absorbed is correctly estimated. This is evidenced by the validation of the model using either a rapidly absorbed glucose solution (Dalla Man, Caumo et al. 2004) or a more slowly absorbed mixed meal (Caumo, Bergman et al. 2000; Steil, Hwu et al. 2004) and by including elderly subjects with slower glucose absorption (Dalla Man, Caumo et al. 2004).

Disadvantages: The primary disadvantage of the OGTT is the potential for it to be influenced by changes in the total absorption of glucose from the gut. While it has been validated using different carbohydrate sources, it has not been determined what effect starvation or changes in diet prior to the test may have on the estimation of insulin sensitivity. Similarly, the OGTT has only been validated in populations with normal insulin secretion. In diabetic populations it is likely to suffer from the same high variability that affects the FSIVGTT, and it has never been validated in very insulin sensitive populations.

Appropriate usage: The OGTT with minimal model is ideal for use as a diagnostic tool in a clinical setting. In a research context, it is appropriate for large studies where insulin sensitivity is of primary interest and no major changes in diet prior to the test are expected. It may also be suitable for smaller studies where logistical or expense issues preclude the use of the FISVGTT.

Other OGTT estimates

Various mathematical techniques have been developed to estimate insulin sensitivity from OGTT data (Matsuda and DeFronzo 1999; Albareda, Rodríguez-Espinosa et al. 2000; Stumvoll, Mitrakou et al. 2000; Mari, Pacini et al. 2001; Yeckel, Weiss et al. 2004). However, while these techniques show reasonable correlation with direct methods in a healthy population they do not attempt to model the physiology of glucose absorption and disposal. As such, they are really indices of glucose tolerance, not insulin sensitivity.

7.1.4 Fasting Measurements

HOMA and QUICKI

While these two techniques were developed independently, they are mathematically (Radziuk 2000) and empirically (Katz, Nambi et al. 2000) very closely related so they will be considered together in this review.

Development (HOMA): The homeostasis model assessment (HOMA) is based on a model of the glucose-insulin feedback loop that was developed at Oxford University in 1979 (Turner, Holman et al. 1979). When this model is applied to steady-state fasting

conditions it can be approximated by a very simple formula to estimate insulin resistance (Matthews, Hosker et al. 1985).

Methodology (HOMA): After an overnight fast, a single resting blood sample is analysed for glucose and insulin concentrations. An approximate estimate of insulin resistance ($HOMA_{IR}$) is then determined by applying Equation 7-4 (Matthews, Hosker et al. 1985), or a slightly more accurate estimate can be made by using a free computer program (Levy, Matthews et al. 1998).

Equation 7-4 $$HOMA_R = (I_0 \times G_0) / 22.5$$

Where I_0 and G_0 are fasting plasma insulin and glucose concentrations respectively. The scale factor of 22.5 is the product of the "normal" fasting plasma concentrations of insulin (5 $\mu U.mL^{-1}$) and glucose (4.5 $mmol.L^{-1}$).

If HOMA is to be used to provide an estimate of insulin sensitivity rather than insulin resistance then $\log(1/HOMA_{IR})$ should be used (Radziuk 2000). The log transformation has the advantage that it gives an estimate that is linearly related to the other estimates of insulin sensitivity covered in this review (Emoto, Nishizawa et al. 1999).

Development (QUICKI): The quantitative insulin sensitivity check index (QUICKI) was developed using a different approach from the other techniques covered in this review. Rather than working from a physiological model, QUICKI was developed empirically by finding the function that best fit the data generated by hyperinsulinaemic-

euglycaemic clamps performed on 28 healthy non-obese, 13 healthy obese and 15 diabetic subjects (Katz, Nambi et al. 2000).

Methodology (QUICKI): After an overnight fast, a single resting blood sample is analysed for glucose and insulin concentrations. An estimate of insulin resistance (QUICKI) is then determined by applying **Equation 7-5** (Katz, Nambi et al. 2000).

Equation 7-5 $$QUICKI = 1 / \log(I_0 \times G_0)$$

The reader will notice that the equations for $\log(1/HOMA_R)$ and QUICKI differ only by the inclusion of a constant.

Assumptions: The primary assumption of both methods is that steady state fasting conditions are present. If the subject is strictly fasted and well rested then conditions approaching a steady state should exist (Matthews, Lang et al. 1983). However, as there is some time delay in the glucose-insulin feedback loop some “hunting” does occur. Hunting can be compensated for by taking the average of a number of samples over the space of an hour.

Fasting measurements also assume that there is the same rate of appearance relative to body mass of glucose into the bloodstream in all cases. While an overnight fast will ensure this assumption is met in most cases, it may be violated in situations where there are abnormally high or low liver glycogen stores, such as after starvation, carbohydrate loading, or endurance exercise.

Advantages: As HOMA and QUICKI can each be calculated from a single blood sample, they present much less logistical difficulty and expense than other techniques. They can easily be added to any study where a fasting blood sample is drawn without placing extra burden on subjects, or may even be retrospectively calculated from old data.

Both methods correlate well with the hyperinsulinaemic-euglycaemic clamp in study cohorts with a wide range of insulin and β-cell sensitivities (Matthews, Hosker et al. 1985; Emoto, Nishizawa et al. 1999; Bonora, Targher et al. 2000; Katz, Nambi et al. 2000; Bastard, Robert et al. 2001; Uwaifo, Fallon et al. 2002; Chen, Sullivan et al. 2003; Skrha, Haas et al. 2004) and show good power to predict metabolic disorders (Katsuki, Sumida et al. 2002; Yokoyama, Emoto et al. 2003; Skrha, Haas et al. 2004). Studies comparing the hyperinsulinaemic-euglycaemic clamp, the FSIVGTT and fasting measurements show that in healthy sedentary populations HOMA and QUICKI are more highly correlated with the clamp than is the FSIVGTT (Katz, Nambi et al. 2000; Chen, Sullivan et al. 2005).

The use of a resting sample with no infusions means that a physiological steady state can be achieved. This means that the coefficient of variation is lower than with the FISVGTT or OGTT, yet the non-physiological situations that occur in the hyperinsulinaemic-euglycaemic clamp and insulin suppression test are avoided.

Disadvantages: While QUICKI and HOMA perform well in healthy sedentary study cohorts, they perform less well in populations that are either very insulin sensitive or insulin resistant (Rabasa-Lhoret, Bastard et al. 2003). At these extremes in insulin sensitivity, non-linearities in the insulin-glucose curve expose the over-simplification

necessary for a single sample estimate, resulting in inaccurate estimation of insulin sensitivity. In addition, these techniques have not been validated in unusual conditions such as prolonged fasting or overfeeding.

Appropriate usage: HOMA and QUICKI are useful initial screening tools for clinical diagnosis of insulin resistance, or for longitudinal monitoring of changes in insulin sensitivity in a single patient. They are also suitable for very large epidemiological studies or for adding to projects where insulin sensitivity is not of primary interest. However, the lack of accuracy at extremes of insulin sensitivity precludes their use in physiological investigations where insulin sensitivity is of prime importance.

1/Insulin and Glucose/Insulin

The reciprocal of plasma insulin concentration and the plasma glucose concentration divided by the plasma insulin concentration have both been used in a research setting as estimates of insulin sensitivity (Legro, Finegood et al. 1998; Silfen, Manibo et al. 2001; Vuguin, Saenger et al. 2001). However, both estimators are conceptually flawed. In a healthy population they perform reasonably well as the pancreas will increase its insulin secretion to maintain plasma glucose at a constant level. In this situation both measures are essentially identical as the fasting plasma glucose concentration does not vary much within a healthy population. However, in a diabetic population with impaired insulin secretion both measures will indicate good insulin sensitivity, a clear fallacy. It is always preferable to use HOMA or QUICKI instead of 1/insulin or glucose/insulin.

7.1.5 Methods Used

The FSIVGTT with minimal model analysis was chosen to measure insulin sensitivity in all the studies presented in this thesis. The FSIVGTT was chosen because it allowed an estimate of glucose effectiveness in addition to insulin sensitivity, and because the insulin infusion required by direct measures creates a risk of hypoglycaemia in the highly insulin sensitive subjects used. Similarly, the standard rather than the insulin modified FSIVGTT was chosen to avoid the risk of hypoglycaemia. Although the ability to obtain individual insulin sensitivities of liver and skeletal muscle would have been beneficial, the hot FSIVGTT was not used due to financial constraints. Other methods to measure insulin sensitivity were not considered reliable enough for research with low subject numbers.

7.2 To determine intramyocellular lipid concentration

Biochemical analysis

Development: Biochemical analysis of muscle tissue for determination of lipid content has been in use for over 100 years (as cited by Greene and Nelson 1921). The technique was first applied to muscle biopsy samples from living humans by Morgan (1969).

Methodology: A muscle sample is obtained by biopsy. Visible fat is manually removed, the sample is freeze dried and the lipid portion is separated by chloroform-methanol extraction. The triacylglycerols are hydrolysed using a strong alkali and then quantified by using a standard assay for either glycerol or FFA (Morgan, Short et al. 1969). If different FFA species are quantified individually then the makeup of the fatty-acyl chains in IMCL stores can be determined.

Assumptions: As biochemical analysis cannot distinguish between extracellular lipids (EMCL) and IMCL, this technique assumes that no EMCL remains in the sample after the visible fat has been removed. As there is a low between-sample coefficient of variation in IMCL determined biochemically in very lean subjects (Watt, Heigenhauser et al. 2002), but a large coefficient of variation in normal subjects (Wendling, Peters et al. 1996), it seems likely that this assumption will be valid only for very lean populations.

All techniques that rely on muscle biopsies assume that the portion of muscle sampled is representative of the entire muscle under examination. Different types of muscle fibres are generally distributed randomly throughout human muscles. While some non-random grouping of fibres does occur, these groups are very small except in severe disease cases

(Lexell, Downham et al. 1983), so a muscle sample large enough for biochemical analysis should encompass enough fibres to satisfy statistical assumptions of randomness.

Advantages: Biochemical analysis is the only technique that allows the determination of the composition of different fatty-acyl chains in IMCL triglycerides. The technique requires no expensive equipment so may be accomplished with little capital outlay. While medical expertise is required to perform the muscle biopsy, the analysis procedure is relatively simple. As biochemical analysis was the predominant technique until the late 1990s it is easier to compare biochemically determined IMCL content with historical data. However, this advantage is becoming less relevant as other techniques become more established.

Disadvantages: The primary disadvantage of biochemical analysis is that the sample may be easily contaminated with EMCL, particularly in subjects with high adiposity. Biochemical analysis provides no information regarding the size or distribution of IMCL droplets. This technique shows poor correlation with IMCL content determined by electron microscopy ($r = 0.475$, Howald, Boesch et al. 2002). As with all muscle biopsy techniques, biochemical analysis requires an invasive medical procedure, relies on a small sample volume, and does not allow for repeated measurements on the same sample volume.

Appropriate usage: Biochemical analysis is appropriate when the fatty-acyl composition of IMCL triglycerides needs to be determined. If the fatty-acyl composition is not required then other techniques that are less prone to contamination by EMCL should be used.

Quantitative histochemistry

Development: Oil Red O staining for neutral lipids is a long established histochemical technique. It was first combined with digital quantitative histochemical analysis to determine IMCL concentrations at the University of Pittsburgh by Goodpaster *et al.* (2000). The technique was further developed by the same research group to enable the determination of the size and location of IMCL droplets within muscle fibres (He, Goodpaster et al. 2004).

Methodology: Muscle biopsy samples are obtained and immediately frozen by immersion in pentane cooled to -160º C by liquid nitrogen. Cross-section slices are cut by cryostat and stained with Oil Red O neutral lipid stain. Stained sections are digitally photographed at high magnification and the area of stain is automatically calculated in user defined regions (Goodpaster, Theriault et al. 2000). Alternatively, manual analysis allows the determination of the distribution of lipid droplets and the area of each individual lipid droplet stain. These areas can then be converted to lipid droplet volumes by comparison with known standard lipid spheres (He, Goodpaster et al. 2004).

Assumptions: This technique requires the same assumption of fibre type distribution as other biopsy techniques, but as only a small part of the biopsy sample is directly examined this assumption is even more important. Multiple slices are obtained from each biopsy sample to increase the chances that a representative sample of fibres will be analysed.

Advantages: Quantitative histochemistry allows for the determination of the size and location of the lipid droplets within muscle cells. It also allows other histochemical techniques to be performed on the same slide (e.g. fibre typing) so differences between individual muscle cells can be investigated. As the lipid droplets are directly visualised there is no risk of contamination by adipose tissue. The equipment is relatively cheap and can be found in any histochemistry laboratory, so this technique may be performed with little capital investment.

Disadvantages: This technique is very labour intensive so cannot be used for large studies. As a muscle biopsy is required this technique is very invasive and the same sample volume cannot be measured again in repeated measurements.

Appropriate usage: Quantitative histochemistry is an appropriate technique for studies with small subject numbers where IMCL concentration is of primary interest. It is the only technique that allows comparison between IMCL concentration and other histochemically determined characteristics of individual muscle cells.

Electron microscopy

Development: Electron microscopy has been used to image skeletal muscle since the late 1930s (Scott and Packer 1939). The first report of imaging of IMCL droplets by electron microscopy was published in 1967 (Prineas and Y. Ng 1967), and these images were converted to IMCL content in 1973 (Hoppeler, Lüthi et al. 1973)

Methodology: Muscle biopsy samples are obtained and thin slices (50-100 nm) are prepared similarly to quantitative histochemistry. These slices are then imaged at

between 20,000 × and 30,000 × magnification and the area of lipid droplets measured (Howald, Boesch et al. 2002).

Assumptions: The images produced by electron microscopy images an even smaller area than those produced by quantitative histochemistry, and still must be assumed to be representative of the entire muscle. Many images are taken from each biopsy sample, but the total image area remains small.

Advantages: Like quantitative histochemistry, electron microscopy directly images the lipid droplets so their location within muscle cells can be determined and contamination with EMCL is not an issue. Electron microscopy also allows the determination of other ultrastructural characteristics of the individual muscle cells under examination.

Disadvantages: This technique is very labour intensive so cannot be used for large studies. As a muscle biopsy is required this technique is very invasive and the same sample volume cannot be measured again in repeated measurements. The equipment required is expensive, so this technique will usually be limited to research institutions that already have electron microscopy capabilities.

Appropriate usage: Electron microscopy is appropriate for studies with a small number of samples where the relationship between IMCL and cell ultrastructure is of primary interest. If the IMCL content of the whole muscle is of primary interest then cheaper techniques will be more appropriate.

Nuclear magnetic resonance (NMR)

Development: A split in the -CH_2 lipid signal in a ^1H NMR spectrum obtained *in vivo* from human skeletal muscle was first described by Schick *et al.* (1993). This phenomena was then used for the determination of muscle IMCL content by a group from Bern University (Boesch, Slotboom et al. 1997).

Methodology: Using a clinical whole body NMR magnet, ^1H spectra are obtained using double spin echo (PRESS) with long echo time from a voxel (usually between 1 and 3 cm^3) containing lean muscle tissue. In spectra obtained using a 1.5 Tesla magnet, the resonance peaks from IMCL and EMCL are fitted at 1.30 and 1.51 ppm respectively. The amplitudes of the lipid peaks are normalised to either the water or creatine peaks (Boesch, Slotboom et al. 1997). Values for within subject comparisons are usually left as simple ratios but absolute quantitation of lipid contents can be performed if standard values are assumed for the water or creatine content of the muscle.

Assumptions: The major assumption of ^1H NMR determination of IMCL content is that the amplitude of the IMCL and EMCL peaks can be uniquely determined by modelling the ^1H spectrum using mixed Gaussian and Lorentzian peaks. While this assumption holds well for lean subjects, the very large EMCL peak in obese subjects often obscures the IMCL peak making accurate separation impossible.

Advantages: The primary advantage of using NMR to measure IMCL content is that it is non-invasive, greatly reducing subject burden. The non-invasive measurement also allows the same muscle volume to be analysed repeatedly, especially useful for comparing pre vs. post intervention measurements. IMCL content determined by ^1H

NMR shows high correlation with IMCL content determined by electron microscopy (r = 0.934, Howald, Boesch et al. 2002) and a moderate correlation with that determined by quantitative histochemistry (r = 0.6, van Loon, Schrauwen-Hinderling et al. 2003).

As the NMR spectrum represents the average signal from a relatively large voxel volume, it is much more likely to be representative of the entire muscle than is the smaller volume of a muscle biopsy. Furthermore, even if the voxel is not representative of the whole muscle, the ability to take repeated measurements from the same volume means that relative changes will still be important.

Disadvantages: As the IMCL and EMCL peaks overlap, it is not possible to determine IMCL content in obese subjects using NMR, at least at 1.5 Tesla. Similarly, subjects with metallic implants cannot enter an NMR magnet so are not able to be measured using this technique. An NMR magnet is very expensive so the use of this technique is usually limited to institutions that already have whole body NMR capability. The technique is labour intensive and requires specially trained personnel, making it relatively expensive and precluding its use in very large studies. It is also prone to operator bias, necessitating blinding of the researcher performing the analysis.

Appropriate usage: ^{1}H NMR spectroscopy is appropriate for studies requiring a small or moderate number of samples where IMCL content is of primary interest. If obese subjects are to be studied, or if more information than gross IMCL content is required, then either quantitative histochemistry or electron microscopy should be considered.

7.2.1 Methods Used

IMCL content was measured in studies 1 and 2 (Chapters 8 and 9) and NMR spectroscopy was used to perform these measurements. NMR spectroscopy was chosen because it is non-invasive and captures a large volume of tissue. Biochemical analysis and quantitative histochemistry were not used because they require invasive muscle biopsy. Electron microscopy was not used because it captures only a very small volume of tissue, and is more expensive than NMR spectroscopy.

7.3 To determine free living energy expenditure

Direct calorimetry

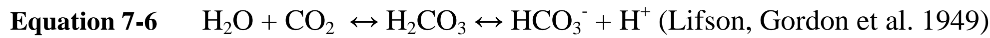
The most well established and reliable technique to determine energy expenditure is direct calorimetry (i.e. measurement of the total heat production of the body). However, direct calorimetry requires the subject to remain within a whole body calorimeter for the duration of the study, precluding its use to measure free living energy expenditure. The technique is mentioned here only as it is the gold standard for the measurement of energy expenditure so has been used to validate the free living techniques.

Doubly labelled water

Development: The doubly labelled water method arose from the observation by Lifson, Gordon and McClintock that the oxygen in body water rapidly equilibrated with oxygen in carbon dioxide via bicarbonate buffering (Lifson, Gordon et al. 1949). Over the subsequent six years they developed and validated the technique in mice (Lifson, Gordon et al. 1955), but it was not applied to humans until Schoeller and van Santen a quarter century later (Schoeller and van Santen 1982).

Methodology: Subjects consume a bolus of water containing known quantities of ^{18}O and deuterium. At intervals throughout the measurement period, subjects provide urine and expired-gas samples. These samples are analysed by mass spectroscopy to determine the fractions of ^{18}O and deuterium. The fractions of labelled atoms expired are used to determine the total body water volume and the CO_2 production as described below.

The labelled ^{18}O and ^{2}H disperse throughout the total body water pool and participate in the bicarbonate buffering equilibrium (Equation 7-6).



Equation 7-6 $H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$ (Lifson, Gordon et al. 1949)

Via this reaction, the ^{18}O rapidly equilibrates throughout the entire pool of oxygen in water, bicarbonate and carbon dioxide, whereas the ^{2}H is distributed only within water and bicarbonate. Thus, ^{2}H will be excreted only as water (urine, sweat, saliva, breath moisture and stool) while ^{18}O is excreted as both water and breath carbon dioxide (Lifson, Gordon et al. 1949).

The first measurement after ingestion of the labelled water determines the initial dilution of ^{18}O and ^{2}H to estimate total body water. Subsequent measurements allow the calculation of the amount of ^{18}O and ^{2}H excreted, the difference of which gives the amount of ^{18}O excreted in CO_2. This value allows the calculation of the total CO_2 production between the two measurements according to Equation 7-7.

Equation 7-7 $T_{CO2} = N\ (f_{18O} - f_{2H})\ /\ 2$ (Lifson, Gordon et al. 1955)

Where N is total body water in mol and f_{18O} and f_{2H} are the fractional reductions of ^{18}O and ^{2}H concentrations respectively. N is assumed to remain constant throughout the measurement period (Lifson, Gordon et al. 1955).

Once T_{CO2} is determined an estimate of the total oxygen consumption (T_{O2}) is required to estimate energy expenditure. This is obtained by (a) assuming a value for the respiratory exchange ratio (RER) based on a typical diet for the population being studied, (b) having

the subject keep a food diary and taking RER equal to the combustion ratio of the foods consumed during the study period, or (c) measuring the RER for a short period using expired gas collection and applying this value to the entire study period (Ainslie, Reilly et al. 2003). The total energy expenditure may then be calculated according to Equation 7-8.

Equation 7-8 $T_{EE} = 14.61T_{O2} + 4.63T_{CO2} - 9.08T_{N}$ (Weir 1949)

Where T_{EE} is the total energy expenditure that occurred between the two urine and breath measurements and T_N is the urinary nitrogen excretion between the two measurements (usually taken from literature values).

Assumptions: The primary assumption required for the doubly labelled water technique is the RER value. While this assumption can be avoided by having the subjects keep a detailed food diary, such recording places a large burden on the subject which will inevitably alter the subjects' behaviour, so may not be a true free living measurement. Estimating long-term average RER from acute expired gas measurements is problematic as RER is influenced by diet and physical activity, so acute measurements are not likely to be representative of a free living situation where diet and physical activity are unknown and changeable. Thus, estimating RER from acute expired gas measurements may not be an improvement over simply using an average population value.

The doubly labelled water technique also assumes that total body water remains stable throughout the measurement period. This is a reasonable assumption as long as the subjects remain weight stable and well hydrated (Westerterp 1999).

Urinary nitrogen excretion is usually assumed based on standard values for the population under examination. This assumption may be avoided by collecting all urine during the study period, and in any case normal variation in total nitrogen excretion has only a small effect on the final estimation of energy expenditure.

The standard equations for estimating energy expenditure assume that no alcohol is oxidised. If the subjects consume a large amount of alcohol then this assumption may introduce significant error.

Advantages: Doubly labelled water provides the only reliable measure of free living energy expenditure. It allows subjects to continue their lives with minimal alteration. The coefficient of variation of measurements is low (6.6%, Schoeller and van Santen 1982)

Disadvantages: The doubly labelled water technique requires a number of assumptions. These assumptions hold reasonably well for "average" adults in western society, but may be violated in people who consume abnormal diets, are alcoholic, or are undergoing rapid changes in body mass. While these assumptions may be avoided by abstaining from alcohol and meticulous recording of diet and collection of urine, these actions will alter subject behaviour so true free living energy expenditure will not be obtained.

Doubly labelled water provides the total energy expenditure over a period of between 4 and 20 days (Ainslie, Reilly et al. 2003), but cannot measure acute changes in energy expenditure as occur between periods of physical activity and rest.

^{18}O and ^{2}H labelled water is very expensive, precluding the use of doubly labelled water to measure energy expenditure in all but the most well resourced studies.

Appropriate usage: When financial resources allow, doubly labelled water is the best method to measure free living energy expenditure over a number of days. However, if acute changes in energy expenditure are of primary interest then a technique that allows real time monitoring will be more suitable.

Heart rate interpolation

Development: Heart rate interpolation to determine free living energy expenditure was first developed by Bradfield (1971). The technique was further improved by Ceesay *et al.* (1989) who introduced the 'FLEX' threshold heart rate.

Methodology: Energy expenditure (indirect calorimetry) and heart rate are measured simultaneously with the subject lying at rest, sitting, standing, and performing exercise at a variety of intensities, and these data are used to generate an energy expenditure vs. heart rate curve for each subject. A 'FLEX' threshold heart rate is calculated as the average of the highest resting heart rate and the lowest exercising heart rate. The subject then wears a heart rate monitor for the entire period of interest. When the heart rate is below the FLEX threshold, energy expenditure is assumed to be equal to the average of the resting energy expenditures, and when it is above the FLEX threshold energy expenditure is calculated according to the energy expenditure vs. heart rate curve (Ceesay, Prentice et al. 1989).

Assumptions: Heart rate interpolation makes two major assumptions: (1) that energy expenditure when heart rate is below the FLEX threshold will, on average, be equal to the average of the energy expenditures when lying down, sitting and standing, and (2) that energy expenditure when heart rate is above the FLEX threshold will follow the same energy expenditure vs. heart rate relationship observed in the laboratory.

The first assumption holds reasonably well. While heart rate may be elevated by a variety of stimuli (e.g. physical activity, stress, adverse temperature and posture), heart rate is always increased by physical activity. Thus, while the heart rate may increase above the FLEX threshold despite energy expenditure remaining low, significant increases in energy expenditure cannot occur without heart rate exceeding the threshold. This assumption can be further strengthened by measuring the subjects sleeping energy expenditure in a whole body calorimeter and applying this rate to periods when the subject is asleep.

The same logic that was applied to the first assumption also demonstrates that the second assumption is less robust as elevated heart rate may be achieved without significant elevation of energy expenditure. In addition, different exercise modalities can produce different heart rates for the same energy expenditure. The latter violation of this assumption can be avoided by generating individual energy expenditure vs. heart rate curves for each of the different exercise modalities the subject performs during the observation period, but this additional testing markedly increases laboratory time and requires the subject to keep an activity diary, which may influence activity patterns.

Advantages: Like doubly labelled water, heart rate interpolation allows unbiased monitoring of free living energy expenditure with minimal impact on subjects' normal activity patterns. Importantly, it is also able to provide information on minute by minute changes in energy expenditure. Heart rate interpolation is very much cheaper than the doubly labelled water technique so has the potential to be used in larger and less well resourced studies.

Disadvantages: The inevitable violations of the assumptions required for heart rate interpolation lead to greater variability than the doubly labelled water technique (standard deviation of ± 6.2 %, range -11.4 to +10.6 % vs. whole body calorimetry, Ceesay, Prentice et al. 1989). Also, some subjects find the heart rate monitor uncomfortable to wear for extended periods.

Appropriate usage: Heart rate interpolation provides a cheaper alternative to doubly labelled water for the assessment of free living energy expenditure. However, there is markedly more variability introduced, so doubly labelled water is preferred if resources allow. If minute by minute changes in free living energy expenditure are of interest then simultaneous energy expenditure assessment by heart rate interpolation and doubly labelled water should be considered.

Activity diary and activity recall questionnaires

Development: There have been many different diary and recall instruments developed and validated against doubly labelled water. It is beyond to the scope of this review to describe the development of all the various instruments.

Methodology: Both activity diaries and activity recall questionnaires attempt to quantify the amount of time the subject spends performing different physical activities. The energy expenditure is then calculated based on the subject's body mass and standard tables of the energy cost of common activities (Ainsworth, Haskell et al. 1993; Ainsworth, Haskell et al. 2000).

An activity diary requires the subject to record all their physical activity throughout their waking hours. There are two approaches: either the subject records the activity they were engaged in for each 15 minute portion of the day (Bratteby and Sandhagen 1997), or the subject records the start and finish times and intensity of all structured physical activity (Ekelund, Yngve et al. 2000; Cunniffe, Griffiths et al. 2009).

An activity recall questionnaire asks a series of questions of the subject, the answers of which are used to estimate the amount of time spent doing each physical activity (Bonnefoy, Normand et al. 2001).

Assumptions: Both diaries and recall questionnaires assume that the subject is honest, unbiased, and capable of reporting of their physical activity. These assumptions are often violated, especially in populations with cognitive impairment (Hale, Pal et al. 2008) or where subjects are motivated to increase or decrease energy expenditure (Cunniffe, Griffiths et al. 2009). If the recall questionnaire or training diary techniques are used then it must be assumed that any time not classified as "physical activity" can be represented by an average sedentary energy expenditure. As energy expenditure is determined with the aid of published data, these techniques assume that all physical activity performed can be adequately represented by one of the documented activities. This assumption may be

violated in people with a physical disability, people who perform a unique activity, or people who perform an activity at an unusually high or low intensity (Ainsworth, Haskell et al. 1993; Ainsworth, Haskell et al. 2000). Physical activity diaries also require the assumption that subjects' normal activity patterns are not changed by the act of keeping a diary.

Advantages: Diary and recall methods require no special equipment and can be performed cheaply on a large scale. While they tend to have large random errors, they provide a good representation of the average physical activity of a population (Wolf, Hunter et al. 1994; Ainslie, Reilly et al. 2003). An important advantage of physical activity diaries is that they allow the nature of the physical activity to be described for publication, or for the same activities to be performed again at a later date.

Disadvantages: Physical activity diary or recall are not sufficiently reliable to quantify an individual subject's energy expenditure (Wolf, Hunter et al. 1994). They cannot be used for subjects with cognitive impairment or for activities where average energy expenditure has not been documented (Ainsworth, Haskell et al. 1993; Ainsworth, Haskell et al. 2000). They are also prone to bias in populations where there is a real or perceived incentive to modify energy expenditure (Cunniffe, Griffiths et al. 2009). A physical activity diary places a large recording burden on the subject. This is likely to decrease compliance and to alter the behaviour of the subject so the recording period will not be representative of normal free living.

Appropriate usage: Physical activity diaries and recall questionnaires are useful for quantifying the average physical activity of a population in large epidemiological studies.

They may also be used to describe the physical activity performed, or to ensure that the same physical activity patterns are maintained in future interventions.

Accelerometers and pedometers

Development: The use of pedometers and accelerometers to quantify energy expenditure is a new technique that is still being developed. While the equipment has been in use since the 1980s (Montoye, Washburn et al. 1983; Kashiwazaki, Inaoka et al. 1986), early accuracy and reliability of energy expenditure estimation was poor. More complex algorithms have improved the estimates in recent years.

Methodology: The subject wears either a pedometer or accelerometer that provides an estimate of the movement of the subject. Regression equations are used to convert the total movement into an estimate of energy expenditure. These equations may be standard equations with additional independent variables such as height and weight (Washburn, Cook et al. 1989), or they may be individually developed for each subject similarly to heart rate interpolation (Levine, Baukol et al. 2001).

A pedometer is a small unit, about the size of a large wrist watch, containing an inertia switch that is activated whenever there is a large acceleration along the vertical axis, such as when a step is taken. The pedometer is worn on the hip or ankle and keeps a count of the acceleration events, which approximates the step count (Storti, Pettee et al. 2008).

An accelerometer unit for estimation of energy expenditure has similar appearance to a pedometer but records total acceleration rather than counting events over a threshold. Older uni-axial accelerometer units contained only one accelerometer so could only

measure acceleration along a single axis, but modern tri-axial accelerometer units contain three accelerometers so are able to record acceleration in all directions (Bouten, Westerterp et al. 1994). The total acceleration along each axis may then be quantified either as a count of events above a threshold (similar to a pedometer) or as a sum of vector magnitudes. Accelerometers have been worn on the hip, ankle or wrist, but the results from different locations are not directly comparable as the accuracy of accelerometers small enough to wear is not yet sufficient for meaningful integration (Swartz, Strath et al. 2000).

Recently, systems incorporating multiple accelerometers plus global positioning system and altimeter data have been produced (Fahd, Stephen et al. 2010; Kahn and Kinsolving 2010; Reed, Mosqueda et al. 2010). While these systems are still developing, they have already been used to detect the type of activity being performed and adjust the energy expenditure estimate accordingly (Fahd, Stephen et al. 2010). These systems are likely to improve further and may replace heart rate interpolation as a highly portable means of determining an individual subject's instantaneous energy expenditure.

Assumptions: The primary assumptions required by the use of accelerometers and pedometers are that all physical activity energy expenditure is used to produce acceleration, and all acceleration is the result of energy expenditure. The first assumption is violated when performing non-weight bearing activity (e.g. swimming or cycling) and when gaining or losing altitude. The second assumption is violated when travelling on non-human powered transport, although the magnitude of the accelerations in this case is not likely to be quantitatively important.

Finally, accelerometers also require that the magnitude of acceleration is proportional to the energy expended to produce that acceleration. This assumption may be violated when the motion of the whole body is not related to the motion of the limbs (e.g. when playing tennis or doing press ups) or when the mass of the body is artificially inflated (e.g. when carrying a heavy load).

Advantages: Accelerometers and pedometers provide an objective quantification of movement that is not affected by subject bias or recording skill. Accelerometers and pedometers create very little subject burden so have high compliance and are not likely to influence subject behaviour. They are also cheap enough to be used in very large studies.

Disadvantages: Estimates of energy expenditure from acceleration require a number of assumptions that are easily violated. These violations result in large random errors in energy expenditure estimate (R^2 = 0.32 vs. indirect calorimetry, Swartz, Strath et al. 2000), although these tend to average out over large sample sizes. Systematic error may also be introduced by the inability of accelerometers and pedometers to detect the work associated with non-weight bearing exercise or with gaining altitude, thus creating a bias in populations who perform unusually large or small amounts of these activities.

Appropriate usage: Accelerometers and pedometers are appropriate for estimating average energy expenditure in a population, but at the time of writing are not yet suitable for determining energy expenditure in individual subjects. When data is expressed as a count or sum of accelerations rather than as an estimate of energy expenditure, accelerometers and pedometers are very useful for determining the effectiveness of interventions designed to alter physical activity levels within subjects.

7.3.1 Methods Used

Free living energy expenditure was estimated in study 3 (Chapter 10) using heart rate interpolation. Heart rate interpolation was chosen because it is a low-cost method and energy expenditure was not a variable of primary interest. Doubly labelled water was not used because of financial constraints. Accelerometers were not used because most subjects performed large amounts of non-weight bearing exercise which is not well captured by these methods. Activity diaries were not used because they are not suitable for determining energy expenditure in individual subjects.

7.4 To determine free living energy intake

Development: The three general methods described in this section have been independently developed in slightly different forms by many different researchers. It is beyond the scope of this review to describe the development of each individual form.

Complete dietary provision

Methodology: Complete dietary provision is usually achieved by having subjects reside in special accommodation throughout the observation period. Subjects are provided with ready access to a wide range of food options at all times, and with packaged food options for time spent outside of the special accommodation (e.g. lunch to take to work). Subjects are requested to live and eat as normally as possible, but to only eat food provided by the researchers. Any food provided to the subjects but not consumed is returned to the laboratory for analysis. Dietary intake is quantified either by using prior knowledge of the food provided, or by chemical analysis of duplicate meals (Buchet, Lauwerys et al. 1983).

Assumptions: The primary assumption of complete dietary provision is that subjects do not consume any food not provided to them by the researchers. This assumption is fairly robust, as demonstrated by the very small differences between estimates of energy intake by complete dietary provision and energy expenditure by doubly labelled water in weight stable populations (± 5%, Prentice, Leavesley et al. 1989; Black, Prentice et al. 1993).

If this technique is to be used to determine free living energy expenditure then it must be assumed that the provision of food and the altered living arrangements do not affect the

subjects' eating or activity habits. This assumption will likely be violated for subjects not already living in a suitably controlled setting (e.g. hospital ward or university dormitory).

Advantages: Complete dietary provision is the only technique that can accurately and reliably quantify the energy intake of individual subjects.

Disadvantages: Complete dietary provision affects the eating and activity patterns of most subjects. As such, observed dietary intake may not reflect the subject's true free living energy intake. This technique is also extremely expensive and labour intensive. Apart from the provision of live-in accommodation, many hours of work by well trained staff are required to analyse the intake of each subject.

Appropriate usage: Due to the expense and disruption to normal life that is occasioned by living away from home, complete dietary provision is usually only performed on populations that are already living in an appropriately controlled setting. In these special cases, complete dietary provision is the most appropriate technique for well resourced studies to assess individual subjects' energy intakes. However, if the population of interest does not already live in an appropriately controlled setting then a less disruptive dietary assessment technique is required to examine free living energy intake.

Prospective self reporting: food diaries

Methodology: Subjects are provided with a food diary and scales or other measuring equipment. They are then required to record all the food they eat throughout the observation period, usually 3-14 days (Trabulsi and Schoeller 2001). Subjects are provided with careful instruction on techniques for estimation or measurement of food

quantities, and techniques for describing the food consumed. The energy content of each food item is then determined using food tables specific to the region and culture of the subjects (Buzzard, Price et al. 1991).

Assumptions: The primary assumption of this technique is that subjects will record their normal diet without bias. There are two likely sources of bias that will affect the estimate of energy intake in subjects from Western cultures: under-reporting of foods consumed and a reduction in energy intake during the recording period. That either one or both of these assumptions is violated is demonstrated by reported energy intakes being consistently and significantly lower than energy expenditure in weight stable populations (Livingstone, Prentice et al. 1990; Livingstone, Prentice et al. 1990; Martin, Su et al. 1996; Seale and Rumpler 1997). As the duration of food diary data tends to be too short for a moderate energy deficit to produce a statistically significant change in body mass it is difficult to determine the relative contributions of each assumption to the overall bias.

As food diaries rely on food databases to generate energy intake data, it must be assumed that the recorded foods can be adequately represented by foods in the database. Unprocessed and some processed foods will be subject to seasonal and regional variation that may affect their energy content and is often not accounted for in databases. However, the expected error due to violation of this assumption is low, especially in Western cultures where a smaller portion of total energy comes from fresh foods.

Advantages: A food diary provides an estimate of energy intake that allows subjects true free choice of dietary intake and is not affected by subjects' ability to recall their

food intake. The diary quantifies each individual food item so it allows the same dietary intake to be replicated in the future.

Disadvantages: Like all self-reporting techniques, food diaries are biased towards under-reporting and have poor reliability. The bias cannot be accurately corrected for, even on a population level, as degree of bias is specific to the population under study and changes with time within a single homogeneous population (Trabulsi and Schoeller 2001). Food diaries represent a large reporting burden for the subjects, with a large portion of incomplete records reported (Gersovitz, Madden et al. 1978), so subjects need careful instruction and monitoring to ensure compliance.

Appropriate usage: Food diaries are useful for quantifying energy intake when a detailed record of individual foods is required by the study design (such as when the same diet must be repeated in the future). If a record of individual foods is not required then a retrospective self reporting technique may be more appropriate.

Retrospective self reporting: 24-hour recall, food frequency questionnaire and diet history

Methodology: There are various forms of retrospective reporting, but in all forms the subject answers a questionnaire, usually administered by a trained researcher, that is designed to evaluate normal dietary habits. A 24 hour recall is the simplest form of retrospective reporting and requires the subject to list all the foods and portion sizes consumed in the last 24 hours. The recall is usually repeated over multiple days including both week and weekend days. A food frequency questionnaire presents the subject with a comprehensive list of foods and asks them to rate their frequency of consumption (from

never to multiple times per day) and normal portion size for each food. A diet history is a hybrid method with questions similar to the food frequency questionnaire plus a food recall section designed to evaluate a "typical" meal pattern (Thomson, Giuliano et al. 2003). The energy content of the diet is then determined using food tables specific to the region and culture of the subject (Buzzard, Price et al. 1991).

Assumptions: The primary assumptions of all retrospective methods are that the subject's recall of their diet is truthful, complete and unbiased. That these assumptions are routinely violated in normal populations as demonstrated by consistent under-reporting of energy intake assessed by retrospective methods compared to doubly labelled water energy expenditure in weight stable populations (Gersovitz, Madden et al. 1978; Bingham, Cassidy et al. 1995; Trabulsi and Schoeller 2001; Thomson, Giuliano et al. 2003). These assumptions are even more likely to be violated in children or people with cognitive impairment.

Like food diaries, retrospective questionnaire methods assume that the foods consumed are adequately represented by the food database used. This is particularly important for the food frequency questionnaire method where subjects may not report foods that are not listed on the questionnaire. As such it is very important that the questionnaire be appropriately designed for the location and culture being investigated.

Advantages: Retrospective self reporting techniques estimate energy expenditure with a bias similar to that of food diaries (Sawaya, Tucker et al. 1996; Bathalon, Tucker et al. 2000) yet with much lower recording burden placed on the subject. As the questionnaires

are administered retrospectively, the subject can be blinded to when the recording days will occur, thus ensuring that the act of recording will not influence eating patterns.

Disadvantages: Like food diaries, retrospective questionnaires are subject to under-reporting bias that cannot be accurately corrected for (Trabulsi and Schoeller 2001). While the degree of bias is similar to food diaries, individual variation is greater in retrospective techniques (Thomson, Giuliano et al. 2003), making them unsuitable for assessing individual energy intake. Retrospective questionnaire techniques do not allow for accurate reconstruction of individual meals so will not enable the subjects to repeat an identical diet in the future.

Appropriate usage: Retrospective questionnaires to assess energy intake are appropriate for large studies where the energy intake of the population, rather than individual subjects, is of primary interest.

7.4.1 Methods Used

Free living energy intake was estimated in study 3 (Chapter 10). A food diary was used because it allows the same diet to be replicated in the future. Complete dietary provision was not used because it would cause too great a disruption of normal diet. Retrospective self-reporting methods were not used because they do not allow the diet to be replicated in the future.

7.5 To determine body composition

Hydrodensitometry

Development: The first use of hydrodensitometry to determine the proportions of two components of different and known densities within a single object is generally attributed to Archimedes following his famous eureka moment. That there were differences in the density of humans, and these differences were associated with degree of obesity, was first explicitly stated by Welham and Behnke (1942), but the Archimedes principle was not mathematically applied until a decade later by Keys and Brozek (1953).

Methodology: The subject is accurately weighed in air and while fully submerged. The residual air contained in the lungs during underwater weighing can be estimated based on anthropometric factors, or can be measured by mixing with a known volume of gas at a known concentration. For the latter method, upon surfacing subjects breath into a bladder containing known volume and composition of gas (van der Ploeg, Gunn et al. 2000). Once the subject has taken sufficient breaths to allow the gas in the lungs to mix with the gas in the bag, the remaining volume and fractions of carbon dioxide and oxygen are measured. The residual lung volume may then be calculated according to Equation 7-9:

Equation 7-9 $$V_{Latps} = [V_{Bag}(\%N_2 - \%Impure)] / [80 - (\%N_2 + 0.2)] - DS$$

Where V_{Latps} is the residual lung volume at ambient temperature and pressure of saturated air, V_{Bag} is the volume of gas in the bag before equilibrating with alveolar gas, $\%N_2$ is the percentage of nitrogen and other metabolically inert gasses in the equilibrated bag, DS is

the deadspace of the mouthpiece, %Impure is the percentage of nitrogen or other inert gasses in the bag before equilibrating with alveolar gas, and 0.2 is an estimate of the difference in %N_2 between the equilibrated bag and the alveoli, and 80 is an estimate of the percentage of nitrogen and other inert gases in the lungs before equilibration (van der Ploeg, Gunn et al. 2000). Underwater weight may then be corrected for the buoyancy of the residual air in the lungs, and body density (BD) calculated according to Archimedes principle Equation 7-10:

Equation 7-10 $$\mathbf{BD = (weight\ in\ air) \times (density\ of\ water) / (weight\ in\ water)}$$

Body composition may then be calculated according to the Brozek equation (Brozek, Grande et al. 1963) Equation 7-11:

Equation 7-11 $$\mathbf{\%BF = (497.1 / BD) - 451.9}$$

Assumptions: The determination of residual lung volume assumes that there is good mixing of the gas in the lungs with the known gas, and that the volume of nitrogen and other metabolically inert gasses in the lung-bladder system does not change during mixing. This assumption may be violated if the subject fails to hold their breath between surfacing and breathing into the bladder, but this possibility can be minimised by careful instruction and observation. A small volume of nitrogen will be released from the blood into the lungs during mixing, but this volume is estimated to be < 10 ml (van der Ploeg, Gunn et al. 2000), so is expected to alter the final estimation of body composition by < 0.1% body fat. There is also an assumption that the fraction of nitrogen and inert gasses in the lungs before mixing is 80%. While this assumption is hard to verify, substantial violation of ± 1% gas fraction would result in only ± 0.3% body fat.

The Brozek equation itself requires the assumption that the body can be modelled by a lean compartment with density of 1.1 $g.ml^{-1}$ and a fat compartment with density of 0.9 $g.ml^{-1}$ (Brozek, Grande et al. 1963). For the lean compartment, this assumption may be violated by variation in hydration status and bone density. While the variation in hydration status may be minimised by careful preparation by the subject, variation of up to ± 2% can still be expected due to variation in the density of the lean compartment (Siri 1956; Lohman 1981).

Advantages: Hydrodensitometry provides a relatively low-cost, non-invasive technique for determining body composition, with better reliability than bio-electrical impedance or anthropometry. As it has historically been considered the gold standard technique for determining body composition, hydrodensitometry allows ready comparison with published data. Many laboratories will already have equipment to perform hydrodensitometry.

Disadvantages: Although hydrodensitometry does not involve invasive sampling of bodily fluids or exposure to radiation, it does require the subject to endure the discomfort of underwater weighing and the possible embarrassment of wearing skimpy clothing. The underwater weighing procedure also requires a minimum level of physical aptitude on the part of the subject, so may not be possible in young children or the mentally or physically impaired. The assumptions required by the two-compartment model means that the reliability of hydrodensitometry is relatively low.

Appropriate usage: Hydrodensitometry is appropriate to estimate body composition when it is not a variable of primary interest, provided the laboratory already has the

equipment required. When body composition is of primary interest, hydrodensitometry may be appropriate for large studies where violations of the compartment density assumptions can be expected to average out. However, hydrodensitometry should be avoided in small studies and studies where comparison is made between groups which could be expected to have different compartment densities (e.g. between age groups).

Dual X-ray absorptiometry (DXA)

Development: The use of DXA in a medical contexts was first reported in the 1960s, but at that stage was only capable of producing a single spectrum from each scan (Jacobson 1964; Gustafsson, Jacobson et al. 1974). A prototype DXA imaging device was first produced by General Electric (Sartoris, Sommer et al. 1985). The first commercial device using the modern technique of K-edge filtration to produce the two energy levels was released by Hologic (Sartoris and Resnick 1989).

Methodology: DXA uses a three compartment model of human tissue consisting of bone, lean soft tissue, and fat. In a DXA analysis, a narrow beam of X-rays is projected through the subject and the intensity of the remaining beam measured to determine attenuation through the subject. Attenuation is dependent on the tissue passed through and the frequency of the radiation. Attenuation values are obtained for X-rays at two different frequencies, and the resultant attenuations used to solve simultaneous equations to calculate the mass per unit area of two tissue compartments within the beam. As the attenuation of X-rays passing through bone is much greater than the attenuation when passing through soft tissue, it is easy to determine whether the beam has passed through bone. When no bone is present, simultaneous equations are solved to determine the mass of fat and lean soft tissue within the beam. Where bone is present, the masses of bone

and soft tissue are determined, and the relative proportions of fat and lean soft tissue are estimated based on comparison with adjacent tissue. The procedure is repeated over the entire body so the mass of bone, lean soft tissue in the entire body may be obtained (Pietrobelli, Formica et al. 1996).

Assumptions: DXA analysis requires the use of literature values for the attenuation of X-rays at each of the frequencies used for each of the tissue compartments, and the assumption that the proportions of fat and lean tissue in beams containing bone can be estimated based on the composition of surrounding tissue. These assumptions can be easily verified by comparing the total mass of the subject calculated by DXA with a measured weight. DXA also assumes that differences in hydration state do not change the X-ray attenuation of lean tissue. This assumption is reasonably robust as the attenuation by fluid routinely consumed by humans is similar to that of human lean tissue. As such even severe hyper- or hypo-hydration of 5% of body mass is likely to result in only ± 1% error in body fat estimation (Pietrobelli, Wang et al. 1998). As DXA uses a three compartment model, the assumption of constant bone density is not required.

Advantages: DXA is an accurate and reliable method of determining body composition, with low standard deviation of repeated measures (Mazess, Barden et al. 1990; Pritchard, Nowson et al. 1993) and low standard error of estimation compared to computed tomography (Wang, Visser et al. 1996; Salamone, Fuerst et al. 2000). DXA is relatively cheap compared to direct methods such as computed tomography or nuclear magnetic resonance imaging. DXA does not require active participation by the subject, and does not involve physical discomfort, the need to wear skimpy clothing, or intimate personal

contact. In particular, DXA offers better validity compared to underwater weighing when there is a possibility differences in bone density between measurements.

Disadvantages: DXA exposes the subject to a low dose of X-ray radiation. While the dose is relatively small (similar to that received during a trans-pacific aeroplane flight, Lloyd, Eggli et al. 1998), it may still exclude vulnerable people such as young children, pregnant women, and people already exposed to high doses of radiation. Compared to other indirect methods, DXA incurs a high initial cost to buy the equipment, and can only be operated by a certified radiographer so requires highly trained staff.

Appropriate usage: DXA is appropriate for well-resourced studies where body composition is of primary interest, especially if there is a possibility of differences in bone density between measurements. However, DXA is contraindicated for studies involving subjects vulnerable to radiation injury.

7.5.1 Methods Used

Body composition was measured in studies 1 and 2 (Chapters 8 and 9) using hydrodensitometry and in study 3 (Chapter 10) using DXA. Hydrodensitometry was used in studies 1 and 2 because it is a low-cost technique and body composition was not a variable of primary interest. DXA was used in study 3 because body composition was a primary outcome measure of the study. Study 3 did not employ hydrodensitometry because the intervention had the potential to alter bone density and average subject hydration status.

8 Low-carbohydrate diet does not affect insulin sensitivity when protein intake is elevated

8.1 Introduction

Starvation challenges the body to maintain glucose homeostasis in the face of continuing glucose oxidation. Survival during starvation is optimised by minimising the use of carbohydrate by non-obligate tissues, most prominently skeletal muscle. This adaptation is achieved by simultaneously reducing circulating insulin concentration (Owen, Felig et al. 1969; Fryburg, Barrett et al. 1990) and skeletal muscle insulin sensitivity to glucose uptake (Mansell and Macdonald 1990; Webber, Taylor et al. 1994; Johnson, Stannard et al. 2006). The reduction in skeletal muscle insulin sensitivity avoids unnecessary glucose disposal despite the presence of some insulin, thereby allowing the starvation insulin concentration to be high enough to prevent excessive proteolysis and preserve functional tissue (Fryburg, Barrett et al. 1990).

The model of metabolism during starvation outlined here requires that a lack of glucose is able to trigger 'insulin resistance' (Stannard and Johnson 2006). Thus, other forms of dietary carbohydrate restriction should also be able to trigger a reduction in insulin sensitivity. Consistent with this prediction, lean, physically fit men experienced equivalent reductions in circulating glucose concentration, increases in *vastus lateralis* IMCL content and reductions in insulin sensitivity when exposed to either starvation or low-carbohydrate high-fat diet (Johnson, Stannard et al. 2006) despite vast differences in total energy intake. The common factors between starvation and low-carbohydrate high-

fat diet induced insulin resistance are reduced circulating glucose concentration and increased circulating FFA concentration.

It has been speculated that in lean healthy individuals dietary carbohydrate restriction independently mediates IMCL accumulation and insulin resistance (Stannard and Johnson 2006). However, previous research has not been able to minimise exogenous carbohydrate intake without also reducing circulating carbohydrate concentrations. As such, it is not clear whether endogenous or exogenous carbohydrate restriction may be the important factor triggering insulin resistance in lean healthy individuals.

High dietary protein intake provides an excess supply of gluconeogenic substrates and, in conjunction with minimal dietary carbohydrate (< 5% of energy), causes an upregulation in hepatic gluconeogenesis (Felig, Marliss et al. 1969; Felig, Marliss et al. 1970; Cowey, Knox et al. 1977; Kettelhut, Foss et al. 1980; Rossetti, Rothman et al. 1989). By ensuring high dietary protein intake, yet minimising carbohydrate intake, the delivery of hepatic gluconeogenic substrates is increased which presumably supports normal plasma glucose concentration despite minimal exogenous supply. By uncoupling exogenous and endogenous carbohydrate supply it is possible to determine the independent effects of each on IMCL accumulation and insulin sensitivity.

The aim of this study was to compare the effects on insulin sensitivity and *vastus lateralis* IMCL content of moderate carbohydrate diet versus two forms of carbohydrate restriction. Lean, physically fit men were exposed to two ~3 day dietary treatments that eliminated dietary carbohydrate but supplied differing amounts of gluconeogenic substrates: namely starvation and high-protein, very low-carbohydrate (HPLC) diet, as well as ~3 days of standardised moderate carbohydrate diet. It was hypothesized that,

compared to the moderate carbohydrate diet, starvation would induce reductions in blood glucose concentration and insulin sensitivity and associated increases in IMCL and circulating FFA concentration, whereas HPLC diet would not.

8.2 Materials and Methods

8.2.1 Subjects

Six healthy, physically fit males (age = 38.8 ± 12.7 years, body mass = 72.9 ± 8.8 kg) volunteered for this study. All subjects reported regularly undertaking exercise for more than 1.5 h daily, at least five days per week. Subjects were informed of the study protocol and risks before providing their written consent. The study was approved by the local institutional human ethics committee and conformed to the Declaration of Helsinki.

8.2.2 Preliminary testing

One week prior to participation in the dietary intervention, submaximal and maximal oxygen uptake tests were performed on an electronically braked cycle ergometer (Lode ergometer, Groningen, The Netherlands). Subjects' oxygen uptake was measured by indirect calorimetry while they cycled for four minutes at each of four constant workloads (100, 150, 200 and 250 W), and then while they cycled to volitional exhaustion at a workload beginning at 100 W and continuously increasing at a rate of 40 $W.min^{-1}$. External power output and VO_2 attained during the final minute of each submaximal workload were used to formulate regression equations ($y = mx + c$) relating oxygen consumption to external workload. These equations, in conjunction with the maximal oxygen consumption obtained from the ramp protocol, were used to derive each subject's workload for the control exercise bout.

On a separate occasion, subjects presented at the laboratory following a twelve hour overnight fast for measurement of resting metabolic rate (RMR) using respiratory gas analysis during the last 10 minutes of 30 minutes supine rest. Body density was assessed

via hydrodensitometry, and percentage body fat then calculated using a two-compartment model of Brozek *et al.* according to Equation 8-1 (Brozek, Grande et al. 1963):

Equation 8-1 $\%BF = (497.1 / BD) - 451.9$ (Brozek, Grande et al. 1963)

Where %BF is the percentage of body mass from fat and BD is the density of the body in $kg.L^{-1}$. Underwater weight was obtained by having the subject sit upon a harness in a weighing pool. Subjects were instructed to breathe out until they submersed, then to remain still until a weight was obtained. Upon surfacing, subjects breathed into a bag containing a mix of oxygen and carbon dioxide to determine residual lung volume by nitrogen dilution (van der Ploeg, Gunn et al. 2000). After equilibrating the gas in the bag with the gas in the lungs, the percentage of oxygen and carbon dioxide in the bag were measured and the remaining volume assumed to be nitrogen or other metabolically inactive gases. Residual lung volume was then calculated according to Equation 8-2.

Equation 8-2 $V_{Latps} = [V_{Bag} (\%N_2 - \%Impure)] / [80 - (\%N_2 + 0.2)] - DS$

(van der Ploeg, Gunn et al. 2000)

Where V_{Latps} is the residual lung volume at ambient temperature and pressure of saturated air, V_{Bag} is the volume of gas in the bag before equilibrating with alveolar gas, $\%N_2$ is the percentage of nitrogen and other inert gasses in the equilibrated bag, DS is the deadspace of the mouthpiece (in this case 50 mL), %Impure is the percentage of nitrogen or other inert gasses in the bag before equilibrating with alveolar gas (in this case 0.5 %) and 0.2 is an estimate of the difference in $\%N_2$ between the equilibrated bag and the alveoli.

8.2.3 Experimental protocol

All subjects underwent three supervised dietary interventions in random order. A minimum seven day washout was allowed after the mixed and HPLC diet interventions. A minimum of 25 days washout was allowed after the starvation intervention to allow body composition to return to pre-starvation levels. Each diet period was of 63 h duration and comprised: water-only starvation diet, HPLC diet, or mixed control diet. Beginning at midnight the day before initiation of the diet intervention (36 hours prior to initiation of the diet), subjects refrained from exercise and recorded all dietary intake. Prior to subsequent interventions subjects again refrained from exercise and consumed the same diet as before the first intervention. On the first day of intervention (between 67 hours before the intravenous glucose tolerance test), subjects consumed a standardized preconditioning meal containing 1.5 g carbohydrate.(kg body weight)$^{-1}$ and providing 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein. Four hours later (63 hours before the intravenous glucose tolerance test), participants performed a preconditioning exercise bout consisting of 60 minutes cycling at 65% of the power associated with VO_{2Max}. The combination of the recording of diet and exercise the day before the intervention period, plus the preconditioning meal and exercise, were designed to ensure subjects began all interventions in a similar metabolic state. These measures resulted in 4.5 days (108 hours) of diet and exercise control before each testing session. There were 63 and 15 hours between the last exercise session and the intravenous glucose tolerance test in the inactivity and exercise interventions respectively.

Immediately following the preconditioning exercise, subjects ingested one of: a carbohydrate snack, a protein snack, or nothing, according to their allocation to the mixed

diet, HPLC diet, or starvation respectively. This snack contained 1 g carbohydrate.(kg body weight)$^{-1}$ (100% energy from carbohydrate) in the mixed diet, equivalent energy but 1% of energy from carbohydrate and 99% from protein in the HPLC diet, or water only in the starvation diet. Except for the starvation diet, subjects ingested an evening meal two hours later. In the mixed diet this meal contained 1.5 g carbohydrate.(kg body weight)$^{-1}$ and supplied 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein. In the HPLC diet subject's consumed an isocaloric meal supplying 2% of energy from carbohydrate, 35% of energy from fat and 63% of energy from protein. Beginning the following morning, and continuing for the remainder of the dietary treatment (48 h), subjects in the mixed and HPLC treatments received diets which provided energy to match their estimated daily expenditure of 1.5×RMR. The mixed diet was designed to deliver 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein in while the HPLC diet was designed to deliver negligible carbohydrate, 35% of energy from fat and 65% of energy from protein. After pre-conditioning exercise in the starvation treatment, subjects continued a water-only diet until completion of the experimental treatment. Diet composition was quantified via Foodworks (Xyris Software®, Melbourne, Australia) using the New Zealand - Standard database. In all dietary interventions, subjects were instructed to maintain activities of daily living and avoid all forms of recreational exercise. Subjects were based in the university environment returning to their homes to sleep. During the intervention, the researchers met with the subjects at least once a day to ensure compliance with the dietary and exercise protocols.

8.2.4 Determination of intramyocellular lipid content

After 65 h of each diet, *vastus lateralis* proton magnetic resonance (^{1}H-MRS) spectra were obtained using a 1.5 Tesla Gyroscan NT whole-body system (Philips Medical Systems; Best). A 50 x 15 x 15 mm voxel was centred within the *vastus lateralis* at the level of the mid-femur. To allow accurate and repeatable localisation of the voxel, a vitamin E capsule was attached to the skin, and the location of the capsule marked between trials. The leg was fixed with the thigh orientated so that the muscle fibres of the *vastus lateralis* were aligned with the magnetic field of the magnet. Muscle spectra were acquired using the PRESS (point resolved spectroscopy) technique (TR = 5000 ms, TE = 32 ms, 32 measurements, 1024 sample points). Fully automated high-order shimming was performed on the volume of interest to ensure maximum field homogeneity. Excitation water suppression was used to suppress the water signal during data acquisition. Unsuppressed water spectra were also acquired for use as the internal standard. NMR spectroscopy was chosen to determine IMCL content to enable repeat measurements on the same region of muscle and to avoid requiring subjects to endure multiple muscle biopsies.

Spectral data were post-processed by magnetic resonance user interface software (jMRUI version 3.0, EU Project). *Vastus lateralis* IMCL was determined by the ratio of the methylene (—$[CH_2]_n$) resonance from IMCL at 1.3 ppm and intracellular water (Szczepaniak, Babcock et al. 1999). Muscle water signal amplitudes were measured from the non-water suppressed spectrum using HLSVD (Hankel Lanczos Squares Singular Values Decomposition). Water suppressed spectra were manually phased, the creatine CH_3 peak was set to 3.02 ppm, and the residual water peak was deleted using HLSVD. The IMCL methylene resonance was then determined by AMARES fitting using a nine

resonance model [IMCL-CH_3: 0.88 ppm, EMCL-CH_3: 1.19 ppm, IMCL-$(CH_2)_n$: 1.3 ppm, EMCL-$(CH_2)_n$: 1.51 ppm, L-acetate: 2.09 ppm, lipid C2 methylene: 2.24 ppm, creatine-CH_3: 3.02 ppm, trimethylacetate: 3.2 ppm, taurine-N-CH_3: 3.37 ppm]. ^{1}H-MRS processing was performed by an experimenter who was blinded to treatment allocation.

8.2.5 Intravenous glucose tolerance test

Following determination of intramyocellular lipid content, subjects reported to the laboratory where glucose tolerance was assessed by frequently sampled intravenous glucose tolerance test (IVGTT) without modification by insulin infusion (Pacini and Bergman 1986). Venous cannulas were inserted into the antecubital vein of each arm. One cannula was used for glucose infusion while the contralateral cannula was used for venous blood collection. Basal blood samples were collected after 15 min of rest, following which the IVGTT was initiated. Subjects rested in a supine position throughout the IVGTT. The subject was infused with 0.3 g.(kg body mass)$^{-1}$ of glucose (50% anhydrous glucose with sodium bicarbonate or hydrochloric acid for pH adjustment; AstraZeneca, Sydney, Australia) via syringe evenly over two min. Glucose infusion was immediately followed by infusion of 10ml of saline. Venous blood was sampled for determination of plasma glucose and insulin concentrations at 3, 4, 5, 6, 7, 8, 10, 14, 19, 22, 25, 30, 40, 60, 70, 80, 100, 140 and 180 min after the initiation of glucose infusion (Pacini and Bergman 1986). All samples were drawn within 15 s either side of the designated time. IVGTTs were undertaken after a twelve hour overnight fast (or 67 hour fast in the starvation intervention). Additional blood samples were collected at 15, 30, 60 and 120 min for determination of plasma FFA concentrations.

A glucose tolerance test was used to determine insulin sensitivity in order to avoid insulin infusion and the attendant risk of severe hypoglycaemia in these highly insulin sensitive subjects. Intravenous rather than oral glucose tolerance test was chosen in order to avoid potential confounding effects of diet (including starvation;` Corvilain, Abramowicz et al. 1995) on gastric emptying and subsequent glucose tolerance (Horowitz, Edelbroek et al. 1993).

8.2.6 Blood sampling

Prior to the IVGTT, 3ml of venous blood was sampled by syringe, transferred into EDTA, placed on ice and then centrifuged at 2000 g for eight minutes within 30 min of collection. Plasma was decanted off and stored at −85°C for later analysis of FFA concentration. An additional 2ml of venous blood was drawn into a lithium heparin-pelleted syringe. This sample was mixed and rested on ice for ~5 min, after which 1.3 ml was transferred into blood tubes, centrifuged at 2000 g for eight minutes, and the plasma frozen (−80°C) for later determination of plasma insulin concentration. Residual blood remained on ice in the lithium heparin pelleted syringe for later determination of plasma glucose concentration. Blood for glucose, insulin and FFA measurement was sampled according to these methods during the ensuing IVGTT according to the sampling schedule outlined above.

8.2.7 Analytical procedures and calculations

Plasma glucose concentration was measured by autoanalyser (EML 105, Radiometer, Copenhagen, Denmark). The plasma concentration of FFAs was determined using a Wako NEFA C Test kit (WAKO Chemical, Richmond, VA, USA) scaled for use in a micro-plate (Bio-Rad, Hercules, CA, USA). All measurements were made in duplicate

and the mean reported. Glucose tolerance was estimated by the slope of the least-squares linear regression of ln(glucose concentration) vs. time between 10 and 40 minutes of the IVGTT (K_g) as outlined by Galvin *et al.* (Galvin, Ward et al. 1992). A measure of glucose tolerance was also made by calculation of the incremental area under the curve (iAUC) for plasma glucose vs. time above the basal glucose concentration (Sparti and Decombaz 1992). Insulin sensitivity index (S_i) and glucose effectiveness (S_g) were determined via the Minimal Model analysis of the plasma glucose and insulin response to the IVGTT (Pacini and Bergman 1986) using MINMOD Millennium (version 6.02, MinMod, University of Southern California, Los Angeles, CA, USA). For these calculations, plasma insulin concentrations were converted from units of pmol.L^{-1} to microunits per litre (1 μU.L^{-1} = 6 pmol.L^{-1}) and glucose concentrations were converted from units of mmol.L^{-1} to mg.dl^{-1} (1 mmol.L^{-1} = 18 mg.dl^{-1}).

8.2.8 Analysis

Differences in nutritional intake, all basal measures (plasma glucose, insulin, FFA, and IMCL:water) and differences in indices of glucose tolerance, S_i and S_g between conditions were compared by one-way repeated measures ANOVA. Plasma glucose, insulin and FFA concentrations during IVGTT were compared by two-way repeated measures ANOVA for investigation of treatment and time (diet–time) interactions. When statistical significance was found, Tukey's honestly significant difference test was used to determine where the difference occurred. Pearson correlation coefficients (two-tailed) were used to express the relationship between IMCL:water and S_i. Statistical significance was accepted at $P < 0.05$. All values are expressed as mean ± standard deviation.

8.3 Results

8.3.1 Subject characteristics

Subjects were lean, with an average of 13.5 ± 2.1 % body fat. Their resting metabolic rate was 306 ± 35 (ml $O_2.min^{-1}$) and their VO_{2Max} was 75.5 ± 16.0 $ml.kg^{-1}.min^{-1}$.

8.3.2 Dietary intake

In the HPLC diet, carbohydrate intake was significantly less than in the mixed diet ($p < 0.001$), the diets providing 15 ± 4 and 389 ± 22 (g of carbohydrate)$.day^{-1}$ respectively. Dietary protein intake was significantly greater during the HPLC diet compared with the mixed diet ($p < 0.001$; **Table 8-1**), yet daily fat and energy intakes were not different between the HPLC and mixed diets. Despite efforts to abolish its intake, dietary carbohydrate consumption during the HPLC was significantly greater than during starvation ($p < 0.001$; **Table 8-1**), though by only 0.2 (± 0.1) $g.kg^{-1}.day^{-1}$.

8.3.3 Intramyocellular lipid content

The ratio of *v. lateralis* IMCL:water was significantly greater in the starvation condition ($25.6 \pm 5.9 \times 10^{-3}$) than in the mixed ($13.6 \pm 6.1 \times 10^{-3}$; $p < 0.001$) or HPLC ($13.6 \pm 3.3 \times 10^{-3}$; $p = 0.016$) conditions. The ratio of IMCL:water was not different between the mixed and HPLC conditions ($p > 0.99$; Figure 8-1). Intramyocellular lipid content demonstrated a significant within-subject correlation between the mixed and starvation conditions ($r = 0.82$, $p = 0.047$) but there were no significant correlations between the HPLC condition and either the mixed or starvation conditions.

Table 8-1 Forty-eight hour dietary analysis of group daily macronutrient intake.

	As a percentage of energy intake			Relative to body mass (kg)		
	Mixed	High-protein/low-carbohydrate	Starvation	Mixed	High-protein/low-carbohydrate	Starvation
CHO	49.5 ± 0.7 %	2.0 ± 0.4 %*	0 ± 0 %*†	5.2 ± 0.7 $g.kg^{-1}$	0.2 ± 0.1 $g.kg^{-1}$*	0 ± 0 $g.kg^{-1}$* †
Fat	34.3 ± 1.2 %	34.6 ± 1.4 %	0 ± 0 %* †	1.6 ± 0.2 $g.kg^{-1}$	1.6 ± 0.2 $g.kg^{-1}$	0 ± 0 $g.kg^{-1}$* †
Protein	15.8 ± 0.9 %	63.4 ± 1.1 %*	0 ± 0 %* †	1.7 ± 0.3 $g.kg^{-1}$	6.7 ± 1.0 $g.kg^{-1}$*	0 ± 0 $g.kg^{-1}$* †
Energy	12299 ± 843 $kJ.day^{-1}$	12314 ± 842 $kJ.day^{-1}$	0 ± 0 $kJ.day^{-1}$* †	168.9 ± 23.7 $kJ.kg^{-1}.day^{-1}$	169.1 ± 23.5 $kJ.kg^{-1}.day^{-1}$	0 ± 0 $kJ.kg^{-1}.day^{-1}$* †

Group daily macronutrient intake as percentage energy intake and relative to body mass in kg. Values are means (± S.D.), n = 7 subjects. *Significantly different versus mixed diet ($P < 0.001$); †significantly different versus high-protein/low-carbohydrate ($P < 0.001$).

8.3.4 Basal plasma metabolite and insulin concentrations

Basal plasma glucose concentrations were lower after starvation than after either the mixed ($p = 0.008$) or HPLC ($p = 0.019$) diets. There was no difference in basal glucose concentration between the mixed and HPLC diets ($p = 0.63$, Table 8-2). The basal plasma insulin concentration was significantly higher after the mixed diet than after either starvation ($p = 0.003$) or the HPLC diet ($p = 0.017$), but there was no significant difference in basal plasma insulin between starvation and the HPLC diet ($p = 0.10$, Table 8-2). The basal plasma FFA concentration was significantly higher after starvation than after either the mixed or HPLC diets ($p < 0.001$ for both) but there was no difference in basal plasma FFA between the mixed and HPLC diets (Table 8-2).

Table 8-2 Basal plasma substrate and insulin concentrations after dietary interventions.

	Mixed	High-protein/low-carbohydrate	Starvation
Plasma glucose ($mmol.l^{-1}$)	4.5 ± 0.3	4.2 ± 0.4	3.5 ± 0.3**‡
Plasma insulin ($pmol.l^{-1}$)	19.8 ± 8.3	12.7 ± 3.8*	8.5 ± 5.1**
Plasma free fatty acids ($\mu mol.l^{-1}$)	378 ± 120	387 ± 232	1179 ± 294**†

Values are means ± S.D., n = 6 subjects. **Significantly different vs. mixed (p < 0.01); *Significantly different vs. mixed (p < 0.05); ‡significantly different versus high-protein/low-carbohydrate (P < 0.01); †significantly different versus high-protein/low-carbohydrate (P < 0.05).

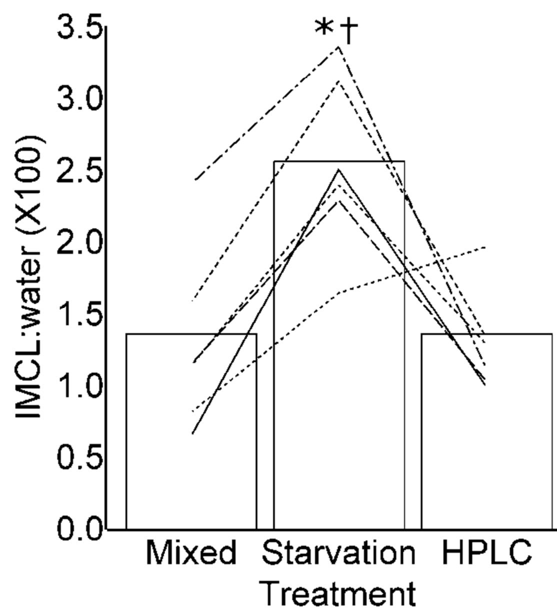


Figure 8-1 Effect of 67 h of mixed diet, high-protein/low-carbohydrate (HPLC) diet or starvation on intramyocellular lipid (IMCL):water ratio. n = 6 subjects. * Significantly different from mixed diet (p < 0.01). † Significantly different from HPLC diet (p < 0.05). Dashed lines indicate individual subject data. Reprinted from Metabolism Clinical and Experimental, 59, Green, J.G., Johnson, N. A., Sachinwalla, T., Cunningham, C.W., Thompson, M. W., and Stannard, S. R., *Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated*, 1633-1641, Copyright (2010), with permission from Elsevier.

8.3.5 Plasma metabolite and insulin responses to IVGTT

In all three treatments, the plasma glucose concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 14 minutes after initiation. Glucose concentration subsequently declined and was not different between treatments and not different from baseline by the last sample of the IVGTT at 180 minutes (Figure 8-2a). There was a significant treatment-time interaction effect ($p < 0.001$) with starvation resulting in greater plasma glucose concentrations compared to mixed from 30-140 minutes ($p < 0.05$) and compared to HPLC from 60-140 minutes ($p < 0.05$). There was also a significant within-subject main effect of treatment with the overall glucose concentration throughout the IVGTT being significantly lower after the mixed diet (7.6 ± 2.9 mM) than after either the HPLC diet (8.1 ± 3.4 mM, $p = 0.015$) or starvation (8.2 ± 2.5 mM, $p = 0.002$).

There were seven data points in which the plasma insulin concentration was below the detectable limit of the assay (6 pM) and a value of 5 pM was assumed for these points. Plasma insulin concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between three and eight minutes after initiation. Insulin concentration subsequently declined and was not different from baseline by 140 minutes and not different between treatments by 40 minutes after initiation of the IVGTT (Figure 8-2b). There was a significant treatment-time interaction effect ($p < 0.001$) with mixed diet resulting in greater plasma insulin concentrations compared to starvation at baseline and from 30-40 minutes ($p < 0.05$) and compared to HPLC diet at baseline and from 19-40 minutes ($p < 0.05$). Starvation also resulted in significantly greater plasma insulin concentration than HPLC diet at 3 and 19 minutes ($p < 0.05$, Figure 8-2b).

In all treatments, plasma FFA concentration declined after glucose infusion reaching a minimum at 60 minutes, after which time they began to rise again (Figure 8-2c). After starvation, there were significantly greater ($p < 0.001$) plasma FFA concentrations throughout the IVGTT compared to the mixed or HPLC diets. There was also a significant treatment-time interaction ($p = 0.008$) during the IVGTT, with plasma FFA concentrations declining more rapidly after starvation than after the mixed or HPLC diets (Figure 8-2c).

Table 8-3 Effect of 67 hours of mixed diet, high-protein/low-carbohydrate diet or starvation on indices of glucose tolerance and insulin sensitivity during an intravenous glucose tolerance test.

		Mixed	High-protein/low-carbohydrate	Starvation
Minimal Model				
	S_i (l.min^{-1}.mU^{-1})	16.5 ± 6.8	14.5 ± 4.8	5.7 ± 1.5**†
	S_g × 1000 (min^{-1})	4.4 ± 2.2	13.2 ± 2.2**	6.4 ± 2.3‡
Glucose tolerance				
	K_g × 10^{-2} (mmol.l^{-1}.min^{-1})	1.1 ± 0.4	1.4 ± 0.5	0.8 ± 0.2†
	Glucose iAUC (mmol.l^{-1}.min)	268 ± 91	339 ± 72	447 ± 50**‡

Values are means ± S.D., n = 6 subjects. **Significantly different vs. mixed ($p < 0.01$); *Significantly different vs. mixed ($p < 0.05$); ‡significantly different versus high-protein/low-carbohydrate ($P < 0.01$); †significantly different versus high-protein/low-carbohydrate ($P < 0.05$). S_i, Minimal Model insulin sensitivity index; S_g, Minimal Model glucose effectiveness index; K_g, rate of decline of ln(plasma glucose) between 10 and 40 min of IVGTT, higher values indicating better glucose tolerance; iAUC, incremental area under curve, higher values reflecting lower glucose tolerance.

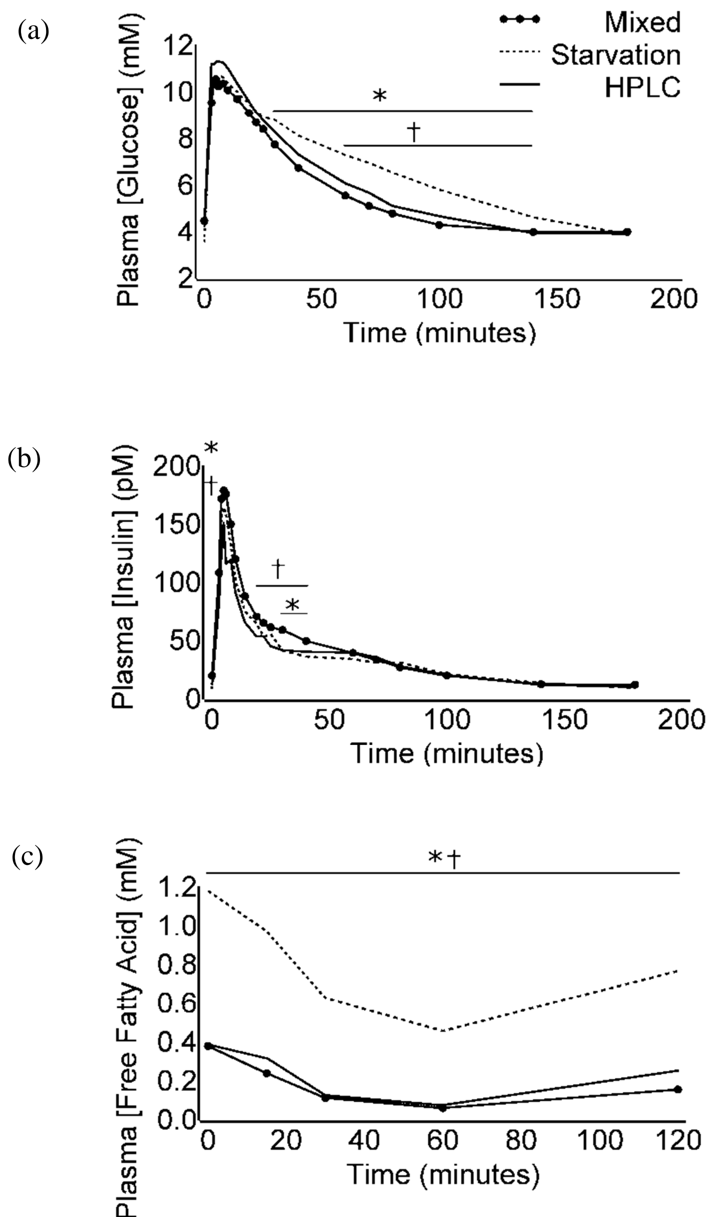


Figure 8-2 Effect of 67 h of mixed diet, high-protein/low-carbohydrate (HPLC) diet or starvation on plasma glucose, insulin and free fatty acid concentrations during an intravenous glucose tolerance test. n = 6 subjects. * Significantly different starvation vs. mixed diet ($p < 0.05$). † Significantly different starvation vs. HPLC diet ($p < 0.05$). Reprinted from Metabolism Clinical and Experimental, 59, Green, J.G., Johnson, N. A., Sachinwalla, T., Cunningham, C.W., Thompson, M. W., and Stannard, S. R., *Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated*, 1633-1641, Copyright (2010), with permission from Elsevier.

8.3.6 Minimal model analysis

Insulin sensitivity index (S_i) determined by the minimal model was significantly lower after starvation compared to after mixed diet ($p = 0.005$) or HPLC diet ($p = 0.016$). There was no difference in S_i between the mixed and HPLC diets ($p = 0.72$). Glucose effectiveness (S_g) was significantly greater after the HPLC diet than after either the mixed diet or starvation ($p < 0.001$ for both; Table 8-3).

8.3.7 Glucose tolerance

Glucose tolerance as estimated by K_g was significantly lower after starvation than after the HPLC diet ($p = 0.026$). K_g after the mixed diet was not significantly different from the other treatments. Glucose area-under-curve was significantly higher after starvation than after the mixed diet ($p < 0.001$) or HPLC diet ($p = 0.001$) but there was no difference in area-under-curve between the mixed and HPLC diets (Table 8-3).

8.4 Discussion

The primary finding of this study is that, in lean healthy men, carbohydrate restriction in the form of three days of HPLC diet does not cause any change in circulating FFA concentration, whole body insulin sensitivity or *v. lateralis* intramuscular lipid concentration compared to three days of mixed, carbohydrate containing diet. These results contrast with similar protocols that employ other forms of dietary carbohydrate restriction (starvation or very high-fat/low-carbohydrate diet) and have produced marked reductions in insulin sensitivity and increases in intramyocellular lipid concentrations (Bachmann, Dahl et al. 2001; Stannard, Thompson et al. 2002; Wietek, Machann et al. 2004; Schrauwen-Hinderling, Kooi et al. 2005; Johnson, Stannard et al. 2006).

The almost identical fasting plasma glucose and FFA concentrations after the HPLC and mixed diets, and the marked differences after starvation (Table 8-2), suggest that there was a continuing supply of glucose entering the blood throughout the HPLC condition. This could be accounted for either by a failure to sufficiently reduce dietary carbohydrate intake in the HPLC diet, or by an up-regulation of gluconeogenesis in the face of minimal carbohydrate intake and abundant supply of gluconeogenic substrates. The latter is more likely as basal insulin concentrations were significantly lower in HPLC than in the mixed condition. Furthermore, our laboratory has previously used diets containing ~2% of energy from carbohydrate to reduce fasting plasma glucose, increase fasting FFA and induce insulin resistance and intramyocellular lipid accumulation almost identically to starvation (Johnson, Stannard et al. 2006)

No study has measured the rate of gluconeogenesis or gluconeogenic enzyme activity in humans who are consuming a high-protein/very-low-carbohydrate diet (<5% of energy

from carbohydrate). However, where carbohydrate is not eliminated, increased protein intake up-regulates gluconeogenesis. Humans who routinely consume greater amounts of protein have increased overnight-fasted hepatic glucose output and an increased proportion of hepatic glucose output from gluconeogenesis (~65% vs. ~45%) compared to age, sex, body mass index and energy intake matched controls (Linn, Geyer et al. 1996; Linn, Santosa et al. 2000). Infusion of a physiological dose of gluconeogenic amino acids after an overnight fast doubles the rate of gluconeogenesis (Krebs, Brehm et al. 2003; Tremblay, Krebs et al. 2005). If somatostatin is also infused to prevent changes in insulin and glucagon concentrations, amino acid infusion results in increased gluconeogenesis and hyperglycaemia (Krebs, Brehm et al. 2003). Similarly, when carbohydrate intake was eliminated by 3-4 weeks of starvation in obese humans, infusion of alanine caused massive up-regulation of gluconeogenesis and hyperglycaemia (Felig, Marliss et al. 1969; Felig, Marliss et al. 1970).

In the present study the large amounts of protein consumed would be expected to increase hepatic gluconeogenesis. Subjects consumed an average of 109 g of protein per day, which is sufficient to produce about 60 g of glucose by gluconeogenesis (Owen, Morgan et al. 1967) in addition to the 15 g per day of residual carbohydrate in the HPLC diet. Thus, the HPLC diet provided a total daily intake equivalent to about 75 g of glucose, somewhat less than the 130 g of glucose consumed each day by the average human brain when carbohydrates are plentiful (Reinmuth, Scheinberg et al. 1965). This partial carbohydrate shortage is consistent with the reduced fasting insulin concentration after HPLC compared to mixed diet. However, the maintenance of blood glucose concentration after the HPLC diet compared to mixed diet demonstrates that the current study succeeded in maintaining an endogenous carbohydrate supply, albeit at a reduced rate.

It has been proposed that one physiological stimulus for IMCL accumulation and an associated insulin resistance in the skeletal muscle of lean, healthy men is an absence of dietary carbohydrate (Johnson, Stannard et al. 2006). According to this proposal dietary carbohydrate restriction, reduced blood glucose, and subsequent suppression of insulin and increased circulating FFA concentrations encourage elevations in IMCL and protect blood glucose concentration by blunting glucose disposal by skeletal muscle (Johnson, Stannard et al. 2006). In the current study, dietary carbohydrate restriction and replacement with protein did not cause a reduction in blood glucose and the concomitant alterations in insulin sensitivity, circulating FFA and IMCL were also absent. The present results demonstrate that exogenous carbohydrate intake does not influence circulating FFA concentration *per se*. However, it remains likely that total metabolic carbohydrate availability (i.e. the availability of glucose to peripheral tissues, irrespective of provenance) strongly influences circulating FFA concentration and insulin sensitivity.

Despite the teleological appeal of low carbohydrate status eliciting reduced insulin sensitivity, the possibility remains that the physiological stimulus for elevated circulating FFA in starvation conditions is dietary protein restriction or energy restriction *per se*, while the stimulus for elevated circulating FFA in low-carbohydrate, high-fat diet is the high exogenous fat intake. To conclusively demonstrate that circulating carbohydrate status is the stimulus for elevated circulating FFAs in low-energy conditions, it would be necessary to show elevated circulating FFAs in response to a normal fat, normal protein, low carbohydrate diet with reduced total energy intake, and normal circulating FFAs in response to an energy matched low fat, normal protein, moderate carbohydrate diet.

In the present study it is also important to consider the potential for other effects of a high protein intake on insulin sensitivity. Where dietary carbohydrate intake is not eliminated, high protein diet (Linn, Santosa et al. 2000) and intravenous infusion of amino acids (Tremblay, Krebs et al. 2005) both decrease insulin sensitivity. Conversely, high protein intake triggers an increase in myocellular IGF-1 concentration (Caverzasio, Shigematsu et al. 1995; Sanchez-Gomez, Malmlöf et al. 1999), which in turn could increase muscular insulin sensitivity (Moses, Young et al. 1996) or mask any decrease that would otherwise have occurred. However, as the well established relationship between IMCL and insulin sensitivity within a homogeneous population (He, Goodpaster et al. 2004) is maintained in the present study (Pearson coefficient $r = -0.68$, $p < 0.01$), it unlikely that other effects of dietary protein on insulin sensitivity are quantitatively important.

Another striking feature of the results of current study was the very high glucose effectiveness indicated by high S_g values in the HPLC condition. S_g is an estimate of the ability of circulating glucose to trigger its own removal from the blood. To conclusively determine the tissues responsible for the high S_g values would require tracer techniques that were not employed in the current study. Notwithstanding, it seems likely that the liver was the main site of altered non-insulin-stimulated glucose disposal during the IVGTT in the HPLC condition. As discussed above, hepatic glucose output is up-regulated by high-protein feeding. In rats, when protein is administered without simultaneous carbohydrate feeding, the increased rate of hepatic glucose output depletes liver glycogen despite increased gluconeogenesis (Moundras, Remesy et al. 1993; Gannon and Nuttall 1995). It is teleologically prudent that the liver should replenish its glycogen stores before skeletal muscle, as glycogen in skeletal muscle is difficult to recycle for use by glucose obligate tissues (Wagenmakers 1998), and in any case it seems likely that skeletal muscle glycogen stores were not depleted in the HPLC condition as

plasma glucose concentration did not change. Furthermore, a single protein meal increases splanchnic blood flow (Brandt, Castleman et al. 1955; Wahren, Felig et al. 1976), which could potentiate the influence of the liver on whole body glucose effectiveness. I speculate that in the HPLC condition, the IVGTT glucose infusion caused the liver to reduce glucose output and begin net glucose uptake more rapidly than in the mixed condition and before an insulin response was able to trigger glucose uptake by skeletal muscle.

The primary measure of insulin sensitivity and glucose effectiveness used in this study was the minimal model developed by Bergman (Bergman, Ider et al. 1979). The minimal model has been shown to systematically overestimate S_g, with the degree of overestimation increasing with increasing insulin response during the first 20 minutes of the IVGTT. As such, caution should be employed when comparing S_g results between groups with differing insulin response (Cobelli, Bettini et al. 1998). However, in the present study there were no between-treatment differences in insulin response during the first 20 minutes of the IVGTT (Figure 8-2) so the overestimation should remain consistent and comparison between treatments is valid.

Normal levels of plasma glucose, plasma FFAs, intramyocellular lipids and insulin sensitivity are maintained when HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. Dietary carbohydrate restriction does not cause circulating FFA to become elevated *per se*. However, it remains probable that circulating carbohydrate status has an important influence on circulating FFA concentration and therefore insulin sensitivity and IMCL accretion in lean, healthy people.

9 Moderate intensity endurance exercise prevents short term starvation induced intramyocellular lipid accumulation but not insulin resistance

9.1 *Introduction*

Sedentary lifestyle is associated with insulin resistance and its co-morbidities (Mikines, Sonne et al. 1989), and conversely, regular physical activity promotes whole body insulin sensitivity (Duncan, Perri et al. 2003). The latter appears to be due primarily to repeated short-term effects of exercise increasing insulin-mediated glucose disposal rather than a long-term adaptation (Heath, Gavin et al. 1983; Richter, Ploug et al. 1985; Mikines, Sonne et al. 1989; Mikines, Sonne et al. 1989; Borghouts and Keizer 2000). Even in athletes undergoing chronic endurance training, a few days of inactivity drastically reduces insulin sensitivity (Burstein, Polychronakos et al. 1985; Oshida, Yamanouchi et al. 1991). However, in both healthy sedentary people (Mikines, Sonne et al. 1989) and endurance trained people who have undergone a few days of inactivity (Heath, Gavin et al. 1983), a single exercise session restores insulin sensitivity to near trained levels.

Whole body insulin sensitivity is also reduced in situations where plasma free fatty acid (FFA) concentration is elevated such as obesity (Charles, Eschwe`ge et al. 1997), intravenous lipid infusion (Dresner, Laurent et al. 1999; Schenk, Cook et al. 2005), low-carbohydrate, high-fat diet (Sparti and Decombaz 1992; Johnson, Stannard et al. 2006) or starvation (Johnson, Stannard et al. 2006). The combination of inactivity and an acute increase in plasma FFA leads, within a few hours, to accretion of intramyocellular lipid

(IMCL) and a simultaneous and proportional reduction in insulin sensitivity (Boden, Lebed et al. 2001). However, the effect of exercise superimposed upon elevated free fatty acids is unclear.

Two studies have directly examined this situation in humans. Schenk *et al.* (Schenk, Cook et al. 2005; Schenk and Horowitz 2007) reported that prior exercise (90 minutes at 65% of VO_{2Peak}) prevented the decrease in insulin sensitivity that otherwise occurs with an overnight lipid infusion, implying that exercise is able to either compensate for or directly prevent a diet-induced decrease in insulin sensitivity. Conversely, Sparti and Decombaz (Sparti and Decombaz 1992) reported that prior exercise (30 minutes at 80 % of estimated maximum heart rate followed by 30 minutes of resistance exercise then ~10 intervals of two minutes at 90 % of maximum heart rate) did not mitigate the insulin resistance observed following a subsequent 36 hour very-low-carbohydrate, high-fat diet. The dissonance between the effects of exercise in these studies may be due to different means of elevating FFAs, but regardless, a better understanding of how these two competing influences affect insulin sensitivity would be useful. In this context, the effect of superimposing physical activity on starvation in physically fit individuals is to date unknown.

While insulin sensitivity has historically been the primary focus of research into glycemic control, glucose effectiveness (i.e. the ability of blood glucose to promote its own disposal and suppress hepatic glucose output) is also an important component of glucose tolerance (Bergman, Ni et al. 1997), especially in patients with impaired insulin secretion. Like insulin sensitivity, glucose effectiveness is decreased by physical inactivity (Nishida, Higaki et al. 2001; Nishida, Tokuyama et al. 2004) and is increased after a single exercise bout (Sakamoto, Higaki et al. 1999). However, the effect of exercise on glucose

effectiveness in a state of elevated FFA and reduced insulin sensitivity has not been examined.

The aim of this study was to test the hypothesis that exercise during starvation would prevent the changes in insulin sensitivity, glucose effectiveness, plasma FFA and IMCL normally observed during starvation without exercise. To this end, lean, physically fit men were exposed to two ~three day periods of starvation with different levels of total energy expenditure, as well as ~three days of standardised moderate carbohydrate diet.

9.2 Materials and Methods

9.2.1 Subjects

Six healthy, physically fit males (age = 38.8 ± 12.7 years, body mass = 72.9 ± 8.8 kg) volunteered for this study. All subjects reported regularly undertaking exercise for more than 1.5 h daily, at least five days per week. Subjects were informed of the study protocol and risks before providing their written consent. The study was approved by the University of Sydney human research ethics committee and conformed to the Declaration of Helsinki.

9.2.2 Preliminary testing

One week prior to participation in the first intervention, submaximal and maximal oxygen uptake tests were performed on an electronically braked cycle ergometer (Lode ergometer, Groningen, The Netherlands). Subjects' oxygen uptake was measured by indirect calorimetry while they cycled for four minutes at each of four constant workloads (100, 150, 200 and 250 W), and then while they cycled to volitional exhaustion at a workload beginning at 100 W and continuously increasing at a rate of 40 $W.min^{-1}$. External power output and VO_2 attained during the final minute of each submaximal workload were used to formulate regression equations ($y = mx + c$) relating oxygen consumption to external workload. These equations, in conjunction with the maximal oxygen consumption obtained from the ramp protocol, were used to derive each subject's workload for the control exercise bout.

On a separate occasion, subjects presented at the laboratory following a twelve hour overnight fast for measurement of resting metabolic rate (RMR) using respiratory gas

analysis during the last 10 minutes of 30 minutes supine rest. Body density was assessed via hydrodensitometry, and percentage body fat then calculated using a two-compartment model of Brozek *et al.* according to Equation 8-1 (Brozek, Grande et al. 1963):

Equation 9-1 $\%BF = (497.1 / BD) - 451.9$ (Brozek, Grande et al. 1963)

Where %BF is the percentage of body mass from fat and BD is the density of the body in $kg.L^{-1}$. Underwater weight was obtained by having the subject sit upon a harness in a weighing pool. Subjects were instructed to breathe out until they submersed, then to remain still until a weight was obtained. Upon surfacing, subjects breathed into a bag containing a mix of oxygen and carbon dioxide to determine residual lung volume by nitrogen dilution (van der Ploeg, Gunn et al. 2000). After equilibrating the gas in the bag with the gas in the lungs, the percentage of oxygen and carbon dioxide in the bag were measured and the remaining volume assumed to be nitrogen or other metabolically inactive gases. Residual lung volume was then calculated according to Equation 9-2.

Equation 9-2 $V_{Latps} = [V_{Bag} (\%N_2 - \%Impure)] / [80 - (\%N_2 + 0.2)] - DS$

(van der Ploeg, Gunn et al. 2000)

Where V_{Latps} is the residual lung volume at ambient temperature and pressure of saturated air, V_{Bag} is the volume of gas in the bag before equilibrating with alveolar gas, $\%N_2$ is the percentage of nitrogen and other inert gasses in the equilibrated bag, DS is the deadspace of the mouthpiece (in this case 50 mL), %Impure is the percentage of nitrogen or other inert gasses in the bag before equilibrating with alveolar gas (in this case 0.5 %) and 0.2 is an estimate of the difference in $\%N_2$ between the equilibrated bag and the alveoli.

9.2.3 Experimental protocol

All subjects underwent three supervised dietary and exercise interventions in random order. A minimum of 25 days washout was allowed after the starvation intervention to allow body composition to return to pre-starvation levels. Each intervention period was 63 h duration and comprised: a water-only starvation diet with no physical activity (inactivity + starvation), a water-only starvation diet with controlled physical activity (exercise + starvation), or a mixed control diet with no physical activity (mixed diet). Beginning at midnight the day before initiation of the diet intervention (36 hours prior to initiation of the diet), subjects refrained from exercise and recorded all dietary intake. Prior to subsequent interventions subjects again refrained from exercise and consumed the same diet as before the first intervention. On the first day of intervention (between 67 hours before the intravenous glucose tolerance test), subjects consumed a standardized preconditioning meal containing 1.5 g carbohydrate.(kg body weight)$^{-1}$ and providing 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein. Four hours later (63 hours before the intravenous glucose tolerance test), participants performed a preconditioning exercise bout consisting of 60 minutes cycling at 65% of the power associated with VO_{2Max}. The combination of the recording of diet and exercise the day before the intervention period, plus the preconditioning meal and exercise, were designed to ensure subjects began all interventions in a similar metabolic state. These measures resulted in 4.5 days (108 hours) of diet and exercise control before each testing session. There were 63 and 15 hours between the last exercise session and the intravenous glucose tolerance test in the inactivity and exercise interventions respectively.

Immediately following the preconditioning exercise, subjects ingested either a carbohydrate snack or water only, according to their allocation to mixed diet or a starvation intervention. The snack, a sports drink containing glucose and sucrose, provided 1 g carbohydrate.(kg body weight)$^{-1}$ (100% energy from carbohydrate) in the mixed diet, or water only in starvation. In the mixed diet, subjects ingested an evening meal two hours later. This meal contained 1.5 g carbohydrate.(kg body weight)$^{-1}$ and supplied 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein. Beginning the following morning, and continuing for the remainder of the mixed dietary treatment (48 h), subjects received a diet which provided energy to match a daily expenditure of 1.5×RMR to maintain energy balance. Diets were designed to deliver 50% of energy from carbohydrate, 35% of energy from fat and 15% of energy from protein. In the starvation conditions, subjects continued a water-only diet until completion of the experimental treatment. In the inactivity + starvation condition, subjects performed no unnecessary physical activity. The exercise + starvation condition was identical except that subjects reported to the laboratory and performed a standardised exercise bout (50% of the power associated with VO_{2Max} for 80 minutes) on the cycle ergometer at 5:00 pm on the second and third days (28 hours and 52 hours after initiation of starvation). Diet composition was quantified via Foodworks (Xyris Software®, Melbourne, Australia) using the New Zealand - Standard database. In all dietary interventions, subjects were instructed to maintain activities of daily living and avoid all forms of recreational exercise. Subjects were based in the university environment returning to their homes to sleep. During the intervention, the researchers met with the subjects at least once a day to ensure compliance with the dietary and exercise protocols.

9.2.4 Determination of intramyocellular lipid content

After 65 h of each diet, *vastus lateralis* proton magnetic resonance (^{1}H-MRS) spectra were obtained using a 1.5 Tesla Gyroscan NT whole-body system (Philips Medical Systems; Best). A 50 x 15 x 15 mm voxel was centred within the *vastus lateralis* at the level of the mid-femur. To allow accurate and repeatable localisation of the voxel, a vitamin E capsule was attached to the skin, and the location of the capsule marked between trials. The leg was fixed with the thigh orientated so that the muscle fibres of the *vastus lateralis* were aligned with the magnetic field of the magnet. Muscle spectra were acquired using the PRESS (point resolved spectroscopy) technique (TR = 5000 ms, TE = 32 ms, 32 measurements, 1024 sample points). Fully automated high-order shimming was performed on the volume of interest to ensure maximum field homogeneity. Excitation water suppression was used to suppress the water signal during data acquisition. Unsuppressed water spectra were also acquired for use as the internal standard. NMR spectroscopy was chosen to determine IMCL content to enable repeat measurements on the same region of muscle and to avoid requiring subjects to endure multiple muscle biopsies.

Spectral data were post-processed by magnetic resonance user interface software (jMRUI version 3.0, EU Project). *Vastus lateralis* IMCL was determined by the ratio of the methylene (—$[CH_2]_n$) resonance from IMCL at 1.3 ppm and intracellular water (Szczepaniak, Babcock et al. 1999). Muscle water signal amplitudes were measured from the non-water suppressed spectrum using HLSVD (Hankel Lanczos Squares Singular Values Decomposition). Water suppressed spectra were manually phased, the creatine CH_3 peak was set to 3.02 ppm, and the residual water peak was deleted using HLSVD. The IMCL methylene resonance was then determined by AMARES fitting using a nine

resonance model [IMCL-CH_3: 0.88 ppm, EMCL-CH_3: 1.19 ppm, IMCL-$(CH_2)_n$: 1.3 ppm, EMCL-$(CH_2)_n$: 1.51 ppm, L-acetate: 2.09 ppm, lipid C2 methylene: 2.24 ppm, creatine-CH_3: 3.02 ppm, trimethylacetate: 3.2 ppm, taurine-N-CH_3: 3.37 ppm]. ^{1}H-MRS processing was performed by an experimenter who was blinded to treatment allocation..

9.2.5 Intravenous glucose tolerance test

Following determination of intramyocellular lipid content, glucose tolerance was assessed by frequently sampled intravenous glucose tolerance test (IVGTT) without modification by insulin infusion (Pacini and Bergman 1986). Venous cannulas were inserted into the antecubital vein of each arm. One cannula was used for glucose infusion while the contralateral cannula was used for venous blood collection. Basal blood samples were collected after 15 min of rest, following which the IVGTT was initiated. Subjects rested in a supine position throughout the IVGTT. The subject was infused with 0.3 g.(kg body mass)$^{-1}$ of glucose (50% anhydrous glucose with sodium bicarbonate or hydrochloric acid for pH adjustment; AstraZeneca, Sydney, Australia) via syringe evenly over two min. Glucose infusion was immediately followed by infusion of 10ml of saline. Venous blood was sampled for determination of plasma glucose and insulin concentrations at 3, 4, 5, 6, 7, 8, 10, 14, 19, 22, 25, 30, 40, 60, 70, 80, 100, 140 and 180 min after the initiation of glucose infusion (Pacini and Bergman 1986). All samples were drawn within 15 s either side of the designated time. IVGTTs were undertaken after a twelve hour overnight fast (or 67 hour fast in the starvation intervention). Additional blood samples were collected at 15, 30, 60 and 120 min for determination of plasma FFA concentrations.

A glucose tolerance test was used to determine insulin sensitivity in order to avoid insulin infusion and the attendant risk of severe hypoglycaemia in these highly insulin sensitive

subjects. Intravenous rather than oral glucose tolerance test was chosen in order to avoid potential confounding effects of diet (including starvation;` Corvilain, Abramowicz et al. 1995) on gastric emptying and subsequent glucose tolerance (Horowitz, Edelbroek et al. 1993).

9.2.6 Blood sampling

Prior to the IVGTT, 3ml of venous blood was sampled by syringe, transferred into EDTA, placed on ice and then centrifuged at 2000 g for eight minutes within 30 min of collection. Plasma was decanted off and stored at –85°C for later analysis of FFA concentration. An additional 2ml of venous blood was drawn into a lithium heparin-pelleted syringe. This sample was mixed and rested on ice for ~5 min, after which 1.3 ml was transferred into blood tubes, centrifuged at 2000 g for eight minutes, and the plasma frozen (–80°C) for later determination of plasma insulin concentration. Residual blood remained on ice in the lithium heparin pelleted syringe for later determination of plasma glucose concentration. Blood for glucose, insulin and FFA measurement was sampled according to these methods during the ensuing IVGTT according to the sampling schedule outlined above.

9.2.7 Analytical procedures and calculations

Plasma glucose concentration was measured by autoanalyser (EML 105, Radiometer, Copenhagen, Denmark). The plasma concentration of FFAs was determined using a Wako NEFA C Test kit (WAKO Chemical, Richmond, VA, USA) scaled for use in a micro-plate (Bio-Rad, Hercules, CA, USA). All measurements were made in duplicate and the mean reported. Glucose tolerance was estimated by the slope of the least-squares linear regression of ln(glucose concentration) vs. time between 10 and 40 minutes of the

IVGTT (K_g) as outlined by Galvin *et al.* (Galvin, Ward et al. 1992). A measure of glucose tolerance was also made by calculation of the incremental area under the curve (iAUC) for plasma glucose vs. time above the basal glucose concentration (Sparti and Decombaz 1992). Insulin sensitivity index (S_i) and glucose effectiveness (S_g) were determined via the Minimal Model analysis of the plasma glucose and insulin response to the IVGTT (Pacini and Bergman 1986) using MINMOD Millennium (version 6.02, MinMod, University of Southern California, Los Angeles, CA, USA). For these calculations, plasma insulin concentrations were converted from units of pmol.L^{-1} to microunits per litre (1 μU.L^{-1} = 6 pmol.L^{-1}) and glucose concentrations were converted from units of mmol.L^{-1} to mg.dl^{-1} (1 mmol.L^{-1} = 18 mg.dl^{-1}).

9.2.8 Analysis

Differences in nutritional intake, all basal measures (plasma glucose, insulin, FFA, and IMCL:water) and differences in indices of glucose tolerance, S_i and S_g between conditions were compared by one-way repeated measures ANOVA. Plasma glucose, insulin and FFA concentrations during IVGTT were compared by two-way repeated measures ANOVA for investigation of treatment and time (diet–time) interactions. When statistical significance was found, Tukey's honestly significant difference test was used to determine where the difference occurred. Pearson correlation coefficients (two-tailed) were used to express the relationship between IMCL:water and S_i. Statistical significance was accepted at $P < 0.05$. All values are expressed as mean ± standard deviation

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9.3 Results

9.3.1 Subject characteristics

Subjects were lean, with an average of 13.5 ± 2.1 % body fat. Their resting metabolic rate was 306 ± 35 (ml O_2.min^{-1}) and their VO_{2Max} was 75.5 ± 16.0 ml.kg^{-1}.min^{-1}.

9.3.2 Diet and exercise

During the mixed diet, subjects had an average daily energy intake of 12299 kJ (49.5 ± 0.7 % from carbohydrate; 34.3 ± 1.2 % from fat; 15.8 ± 0.9 % from protein). Subjects consumed no energy during the starvation conditions. During the starvation+exercise intervention, participants exercised at an average workload of 174 ± 30 W, and performed 1666 ± 288 kJ of external work. All subjects were able to complete the required exercise, although two subjects had to stop for a brief rest after 70 minutes during the last exercise session in the exercise + starvation condition.

9.3.3 Intramyocellular lipid content

The ratio of *v. lateralis* IMCL:water was significantly higher in the inactivity + starvation condition than in the mixed ($p = 0.0003$) or exercise + starvation conditions ($p = 0.009$, Table 9-1). The ratio of IMCL:water was not significantly different between the mixed and exercise + starvation conditions ($p = 0.10$).

Table 9-1. Basal substrate and insulin concentrations and intravenous glucose tolerance test results after 67 hours of dietary intervention.

	Inactivity + Mixed Diet	Exercise + Starvation	Inactivity + Starvation
Fasting glucose ($mmol.l^{-1}$)	4.5 ± 0.3	3.4 ± 0.5*	3.5 ± 0.3 *
Fasting insulin ($pmol.l^{-1}$)	19.8 ± 8.3	10.7 ± 6.5*	8.5 ± 5.1*
Fasting free fatty acids ($\mu mol.l^{-1}$)	379 ± 120	1188 ± 219*	1180 ± 294*
Vastus lateralis			
IMCL:water ratio ($\times 10^{-3}$)	13.6 ± 6.1	18.1 ± 5.8	25.6 ± 5.9*†
S_i ($l.min^{-1}.mU^{-1}$)	16.5 ± 6.8	6.0 ± 1.7*	5.7 ± 1.5*
$S_g \times 1000$ (min^{-1})	4.4 ± 2.2	8.8 ± 3.0*	6.4 ± 2.3

Values are means ± S.D., n = 6 subjects. Between trials comparisons were performed using repeated measures ANOVA. *Significantly different vs. mixed ($p < 0.01$). †Significantly different vs. Exercise + Starvation ($p < 0.05$) S_i, Minimal Model insulin sensitivity; S_g, Minimal Model glucose effectiveness index.

9.3.4 Basal plasma metabolite and insulin concentrations

After the mixed diet, compared to either starvation condition, basal plasma glucose concentrations were higher (inactivity $p = 0.002$, exercise $p = 0.001$) and insulin (inactivity $p < 0.001$, exercise $p = 0.003$) and free fatty acid concentrations were lower (inactivity $p < 0.001$, exercise $p < 0.001$). There was no difference in basal glucose, insulin or free fatty acid concentrations between the starvation conditions (Table 9-1).

9.3.5 Plasma metabolite and insulin responses to IVGTT

In all three treatments, the plasma glucose concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 14 minutes after initiation. Glucose concentration subsequently declined and was not different between treatments and not different from baseline by the last sample of the IVGTT at 180 minutes (Figure 9-1a). There was a significant treatment-time interaction effect ($p = 0.039$) with mixed diet resulting in lower plasma glucose concentrations compared to exercise + starvation at baseline and from 40-100 minutes and compared to inactivity + starvation at baseline and from 30-140 minutes (Figure 9-1a).

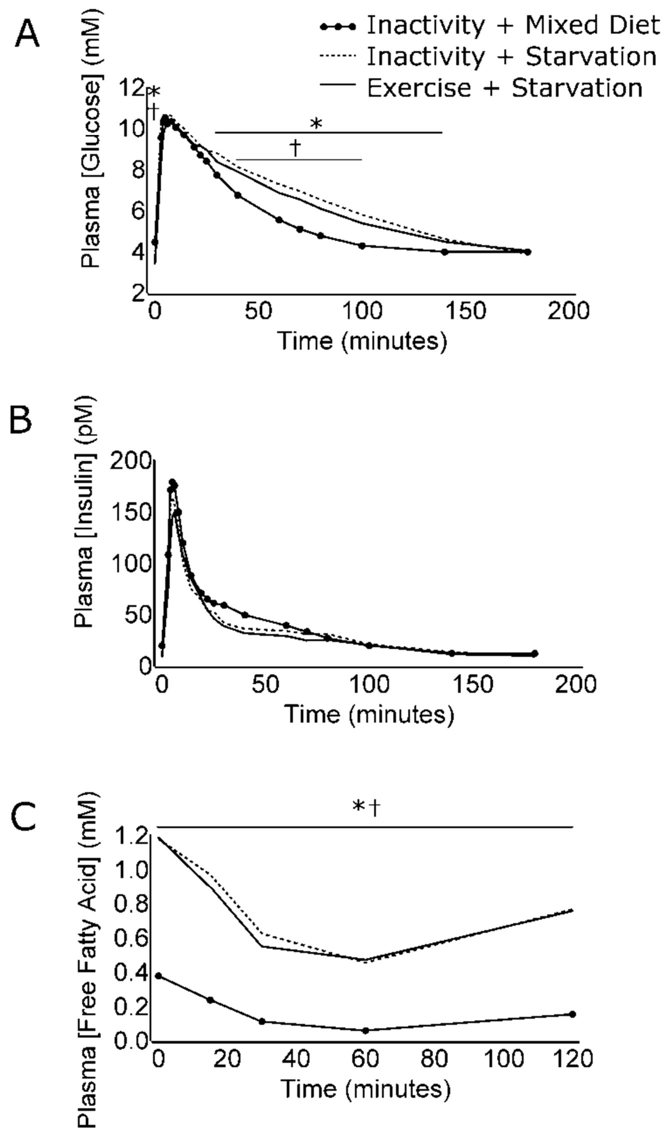

Figure 9-1. Effect of 67 h of inactivity + mixed diet, inactivity + starvation or exercise + starvation on (A) plasma glucose, (B) insulin and (C) free fatty acid concentrations during an intravenous glucose tolerance test. n = 6 subjects. Between trials comparisons were performed using repeated measures ANOVA. * Significantly different inactivity + mixed diet vs. inactivity + starvation ($p < 0.05$). † Significantly different inactivity + mixed diet vs. exercise + starvation ($p < 0.05$). Reprinted from Metabolism Clinical and Experimental, Green, J.G., Johnson, N. A., Sachinwalla, T., Cunningham, C.W., Thompson, M. W., and Stannard, S. R., *Moderate-intensity endurance exercise prevents short-term starvation-induced intramyocellular lipid accumulation but not insulin resistance*, Copyright (2011), doi:10.1016/j.metabol.2011.01.003, with permission from Elsevier.

There were seven data points in which the plasma insulin concentration was below the detectable limit of the assay (6 pM) and a value of 5 pM was assumed for these points. Plasma insulin concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between three and eight minutes after initiation. Insulin concentration subsequently declined and was not different from baseline by 140 minutes (Figure 9-1b). There was no significant treatment-time interaction effect on insulin concentration ($p = 0.18$, Figure 9-1b).

In all treatments, plasma free fatty acid concentration declined after glucose infusion reaching a minimum at 60 minutes, after which time they began to rise again (Figure 9-1c). After the starvation conditions, there were significantly greater ($p < 0.001$) plasma free fatty acid concentrations throughout the IVGTT compared to the mixed diet. There was also a significant treatment-time interaction ($p < 0.001$) during the IVGTT, with plasma free fatty acid concentrations declining more rapidly after starvation than after the mixed diet (Figure 9-1c). There was no difference in free fatty acid concentration between the starvation conditions.

9.3.6 Minimal model analysis

Values for S_i and S_g after each of the interventions are presented in Table 9-1. Compared to after mixed diet, S_i determined by the minimal model was significantly lower after starvation with inactivity ($p = 0.005$) and starvation with exercise ($p = 0.007$). There was no difference in S_i between the starvation conditions. S_g was significantly greater after the exercise + starvation condition than after the mixed diet ($p = 0.022$) but there was no significant difference between starvation conditions ($p = 0.15$) or between inactivity + starvation and mixed diet ($p = 0.38$).

9.4 Discussion

The primary finding of this study is that daily endurance exercise does not mitigate the insulin de-sensitising effect of three days of starvation despite preventing the accumulation of *vastus lateralis* IMCL.

The exercise performed in the starvation condition was considerably less than the habitual training load of the endurance trained subjects used in this study. Nevertheless, subjects found it difficult to complete the last exercise session during starvation, and two subjects required a brief rest to be able to complete the exercise. Previous research where exercise was imposed on starvation has demonstrated a 32 % reduction ($p = 0.025$) in time-to-failure in an incremental exercise test after two days and a 45% reduction ($p < 0.0001$) after four days (Henschel, Taylor et al. 1954). These large decreases in physical capacity demonstrate the challenge of performing physical work during starvation, and suggest that the dose employed in the present study approached the limit of what is achievable in this state.

To increase insulin sensitivity above habitual levels in the very fit endurance trained subjects used in the current study would require a prodigious amount of exercise, more than would be physically possible during starvation. To avoid this methodological difficulty, an intervention of starvation + inactivity was used to reduce insulin sensitivity, and its effect compared to a habitual diet control (mixed diet + inactivity) and a habitual physical activity control (starvation + exercise). In other endurance trained groups of subjects, cessation of training resulted in a 28 % decline in insulin sensitivity after 38 hours (Oshida, Yamanouchi et al. 1991) and a 35% decline after 60 hours (Burstein, Polychronakos et al. 1985), compared to 12 or 14 hours after the last exercise session.

Even when insulin sensitivity was reduced by 50 % after ten days of inactivity, insulin sensitivity was restored to normal levels by a single exercise session approximating a typical training session for the athlete (Heath, Gavin et al. 1983). Thus, the inactivity interventions, which involved 63 hours between the last exercise session and the IVGTT, would normally have markedly reduced insulin sensitivity compared to the exercise intervention, which incorporated two extra exercise sessions and had only 15 hours between the last exercise session and the IVGTT. As such, the lack of difference in insulin sensitivity between starvation + inactivity and starvation + exercise must be due to the starvation overriding or masking any impact of inactivity.

The results of the current study are consistent with previous research in which FFAs were elevated by low-carbohydrate diet (Sparti and Decombaz 1992), but are at odds with the ability of exercise to maintain insulin sensitivity in the face of similar plasma FFA concentrations maintained by lipid infusion (Schenk, Cook et al. 2005; Schenk and Horowitz 2007). A key difference between these two conditions is a difference in carbohydrate status brought about by the methods of raising FFA concentration; in low-carbohydrate diet and in starvation, the increase in FFA concentration is accompanied by a reduction in circulating glucose availability, but with lipid/heparin infusion it is not. This difference may suggest that some minimum level of carbohydrate status is required to enable exercise to increase whole body insulin sensitivity. Taking a teleological perspective, such an interaction between exercise and carbohydrate status would be prudent as an increase in insulin sensitivity with exercise when carbohydrate status is low could produce dangerous hypoglycaemia. However, the current study was not designed to test for a minimum carbohydrate status to enable exercise to affect insulin sensitivity. This effect could be better investigated by comparing the effect of concurrent exercise on

the reduction in insulin sensitivity usually caused by high fat, low carbohydrate diet and high fat, normal carbohydrate diet.

The energy expended due to additional physical activity in the exercise condition is equivalent to the metabolisable energy of approximately 250 g of fat. Assuming an average 18 kg of active muscle mass recruited by the subjects in this study during cycling (Medbo and Tabata 1993; Janssen, Heymsfield et al. 2000), the difference in total IMCL content between exercise and inactivity conditions is approximately 115 g. Thus, the extra oxidation of lipid to meet the energy requirements of exercise could account for the difference in IMCL stores between starvation + exercise and starvation + inactivity despite both conditions having similarly elevated circulating FFA concentration and similarly reduced insulin sensitivity (van Loon and Goodpaster 2006). Such a mechanism is also consistent with the elevation of IMCL stores despite exercise when FFA are elevated by lipid infusion (Schenk, Cook et al. 2005; Schenk and Horowitz 2007) as greater carbohydrate availability and higher plasma insulin concentration in this situation would be expected to decrease FFA oxidation and increase esterification (Rowlands, Johnson et al. 2009). However, differences in oxidation alone cannot explain the ability of exercise to maintain insulin sensitivity in the face of lipid infusion (Schenk, Cook et al. 2005; Schenk and Horowitz 2007) but not starvation or low-carbohydrate diet (Sparti and Decombaz 1992). A possible explanation for this effect is an alteration in the rate of FFA absorption by muscle cells. Membrane bound fatty acid transport protein content increases in response to various physiological stimuli including insulin (Chabowski, Górski et al.), muscle contraction (Holloway, Luiken et al. 2008), and starvation (Turcotte, Srivastava et al. 1997). If myocellular fatty acid absorption capability is increased by low carbohydrate availability then there could be a greater intramyocellular

FFA load in starvation compared to lipid infusion despite a similar extracellular FFA concentration.

The difference in exogenous fat intake in the current study and that of Schenk *et al.* (Schenk, Cook et al. 2005; Schenk and Horowitz 2007) is not likely to be responsible for the difference in the observed effects of exercise on insulin sensitivity. When carbohydrate status is low, as in the current study, circulating FFA will be elevated either from high exogenous fat intake (Dresner, Laurent et al. 1999; Schenk, Cook et al. 2005; Johnson, Stannard et al. 2006) or from increased adipose tissue lipolysis (Stannard, Thompson et al. 2002; Johnson, Stannard et al. 2006). Thus, skeletal muscle cells will be exposed to the same lipid availability regardless of dietary fat intake.

The exercise + starvation condition in the current study resulted in very high glucose effectiveness (S_g). As insulin sensitivity is reduced in both starvation conditions, it seems paradoxical that S_g should be increased. However, it is likely that S_g is not quantitatively important at the low plasma glucose concentrations during the fast (Table 9-1). Increased S_g has important implications for patients with diabetes as it has the potential to improve glycemic control even when insulin secretion is absent. Furthermore, increased S_g provides a convenient mechanism for very rapid initiation of muscle and liver glucose uptake and suppression of hepatic glucose production in response to an increase in plasma glucose.

Increased S_g during starvation could be mediated by reduced muscle glycogen concentration (Borghouts and Keizer 2000). While liver glycogen is substantially depleted after an overnight fast (Nilsson and Hultman 1973), muscle glycogen stores remain near full capacity after a mixed diet, are somewhat reduced after three days

inactivity + starvation and are further reduced after exercise + starvation (Knapik, Meredith et al. 1988). This order parallels the S_g values observed in this study.

The primary measure of insulin sensitivity and glucose effectiveness used in this study was the IVGTT with minimal model analysis developed by Bergman (Bergman, Ider et al. 1979). The minimal model has been shown to systematically overestimate S_g, with the degree of overestimation increasing with increasing insulin response during the first 20 minutes of the IVGTT. As such, caution should be employed when comparing S_g results between groups with differing insulin response (Cobelli, Bettini et al. 1998). However, in the present study there were no between-treatment differences in insulin response during the first 20 minutes of the IVGTT (Figure 9-1) so the overestimation should remain consistent and comparison between treatments is valid.

In summary, three days of starvation causes increased IMCL and circulating FFA concentration and decreased insulin sensitivity. Exercise during starvation prevents the accumulation of IMCL and increases glucose effectiveness yet does not affect the changes in FFAs and insulin sensitivity that occur with inactivity during starvation. I propose that some minimum level of whole body carbohydrate status is required to enable exercise to affect insulin sensitivity.

10 Continued endurance training cannot prevent decreased insulin sensitivity associated with overfeeding

10.1 Introduction

Changes in whole body's energy storage status (Oppert, Nadeau et al. 1995; Danielsson, Fagerholm et al. 2009) and energy turnover (Mikines, Sonne et al. 1988; Hansen, Nolte et al. 1998) strongly influence the whole body response to a set level of circulating insulin. Specifically, physical activity tends to improve insulin sensitivity (Mikines, Sonne et al. 1988; Hansen, Nolte et al. 1998) while fat gain tends to impair it (Oppert, Nadeau et al. 1995; Danielsson, Fagerholm et al. 2009). The effect of obesity on insulin sensitivity has been well studied in rodent models, and an abundance of potential mechanisms have been identified (e.g. changes in circulating lipids, circulating adiponectin and inflammation; see section 4.5.1). Due to ethical and methodological difficulties, there is relatively little published human research examining changes in insulin sensitivity in response to chronic overfeeding.

Whilst the published evidence indicates a decrease in insulin sensitivity in response to simultaneous overfeeding and reduced physical activity (Table 2.3), the design of these studies makes it impossible to distinguish the separate effects of inactivity and overfeeding on insulin action. When subjects were asked to maintain "normal" physical activity levels during chronic energy surplus, Erdman *et al.* (2008) observed a decrease in insulin sensitivity, though no information was supplied as to what level of physical

activity was considered "normal" or whether subjects complied with activity prescription during overfeeding. Only Ohannesian and colleagues (Ohannesian, Marco et al. 1999) have specifically recruited physically active individuals and measured insulin sensitivity after fat gain without reducing physical activity. They found no change in insulin sensitivity, suggesting that continued physical activity may have prevented a fat induced change. Unfortunately, physical activity levels in the Ohannesian *et al.* study were not well controlled during the intervention so the impact of mass gain alone could not be conclusively assessed (Ohannesian, Marco et al. 1999).

Mass gain with overfeeding is clearly the result of energy intake exceeding energy expenditure for a significant time period. Despite this, some individuals appear to be more resistant than others to mass gain in response to a given increase in energy intake (Sims, Goldman et al. 1968; Bouchard, Tremblay et al. 1990; Diaz, Prentice et al. 1992; Levine, Eberhardt et al. 1999), presumably as a result of changes in physical activity energy expenditure that parallel changes in energy intake (Levine, Eberhardt et al. 1999). There is little evidence suggesting what factors might predict an individual's change in body composition in response to imposed overfeeding. However, epidemiological studies have reported that both insulin sensitivity (Swinburn, Nyomba et al. 1991; Wedick, Mayer-Davis et al. 2001; Howard, Adams-Campbell et al. 2004; Pannacciulli, Ortega et al. 2007; Boule, Chaput et al. 2008; Chaput, Tremblay et al. 2008; Wedick, Snijder et al. 2009) and fasting leptin concentration (Ravussin, Pratley et al. 1997; Lissner, Karlsson et al. 1999; Monteleone, Fabrazzo et al. 2002; Wedick, Snijder et al. 2009) are predictive of subsequent free living mass gain in populations prone to mass gain. Conversely, exercise during weight loss helps to maintain lean body mass while accelerating the loss of fat mass (Frey-Hewitt, Vranizan et al. 1990; Rice, Janssen et al. 1999; Tsai, Sandretto et al. 2003; Layman, Evans et al. 2005). If weight gain is analogous to weight loss, then

exercise during weight gain would be expected to maximize lean tissue gain while minimizing fat gain. Any of these factors could conceivably predict the change in body composition in response to overfeeding, but none have been examined experimentally.

This study tested the hypotheses that (a) continued high levels of physical activity would prevent changes in insulin sensitivity in the face of prolonged excess energy intake, and (b) initial insulin sensitivity, fasting leptin concentration or relative energy expenditure would predict the magnitude of body mass and fat gain in response to a given increase in energy intake. To this end we recruited athletes engaged in endurance training and measured changes in insulin sensitivity and body composition before and after four weeks of overfeeding while normal training was maintained.

10.2 Materials and Methods

Subjects

Nine healthy endurance trained individuals (two women and seven men, age 26.8 ± 7.0 years, height 1.78 ± 0.10 m, BMI 22.6 ± 2.9 $kg.m^{-2}$, VO_{2Max} 65.6 ± 8.5 $ml.kg^{-1}.min^{-1}$) volunteered to participate in this study. All subjects regularly undertook endurance exercise training for more than one hour per day at least four days per week. Subjects were informed of the study protocol and risks before providing their written consent. The study was approved by the local institutional review board and conformed to the Declaration of Helsinki.

Protocol

Subjects completed a preliminary characterisation session prior to the beginning of the study. At least three days after characterisation, subjects underwent a one week baseline period which was immediately followed by a four week overfeeding period (**Figure 10-1**). Before and after the overfeeding period subjects underwent an intravenous glucose tolerance test (IVGTT) and dual x-ray absorptiometry (DXA) to determine insulin sensitivity and body composition respectively.

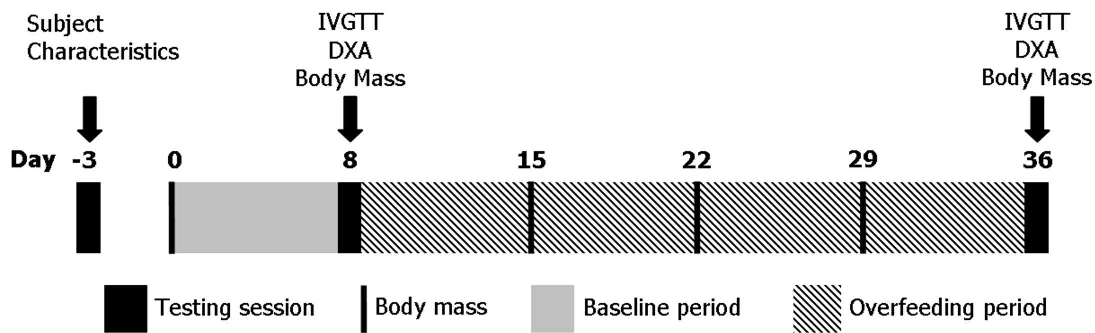

Figure 10-1. Schematic of overfeeding study protocol. The study protocol consisted of a pre-testing session at least three days before the beginning of the baseline period. On day 0 subjects began baseline recording and reported to the lab for weighing. The baseline period lasted seven days. On day eight subjects reported for the lab for IVGTT and DXA measurements. On day nine subjects began the overfeeding protocol and reported to the lab for weighing every seven days. On day 36 subjects again underwent IVGTT and DXA measurements

Subject characterisation

At least three days prior to baseline testing, subjects reported to the laboratory after a 4-hour fast. Subjects sat reclining for 30 minutes while heart rate was recorded using a Polar monitor. Expired gas was collected in a Douglas bag during the last ten minutes for determination of resting metabolic rate by indirect calorimetry. Subjects then stood still for a further ten minutes and expired gas was collected during the final four minutes. Subjects then cycled on an electronically braked cycle ergometer at four submaximal workloads for six minutes while heart rate was recorded and breath-by-breath indirect calorimetry was performed using a metabolic cart. The submaximal workloads were used to determine the relationship between heart rate and energy expenditure for heart rate interpolation. After a brief rest subjects performed an incremental cycle test (increasing 40 $W.min^{-1}$) to exhaustion with breath-by-breath indirect calorimetry to determine

VO_{2Max}. Following this test and a recovery period, subjects were instructed on dietary recording techniques and were provided with food and physical activity diaries, kitchen scales and heart rate monitor.

Establishing baseline

Beginning day 0 subjects consumed their normal diet and performed their normal physical activity. Daily dietary intake was recorded by food diary, physical activity by exercise diary and energy expenditure by heart rate interpolation (Ceesay, Prentice et al. 1989; Ekelund, Yngve et al. 2002). Subjects were instructed to eat as normally as possible, but to avoid eating food that would not be able to be replicated during the overfeeding period. To minimise recording burden, subjects were allowed to use any reliably replicable measure of food quantity (e.g. always filling the coffee cup to the same point) and were only required to weigh food when there was no other replicable means to determine quantity. Subjects recorded time of day, duration, perceived intensity and average heart rate for each exercise session. Subjects' heart rate was recorded at one minute intervals for the entire baseline period except when sleeping or bathing. Researchers were in regular contact with subjects to ensure compliance.

Pre-testing

On day 8, subjects reported to the laboratory at 08:00 after a 12-hour water only fast. After determination of body mass, an IVGTT and additional blood sampling for FFA, triglyceride, cholesterol and leptin determination were performed as described in sections 8.2.5 and 8.2.6. Following the IVGTT, subject's body composition was determined by

DXA scanning using a Hologic Discovery scanner (Hologic Inc., Bedford, Massachusetts, USA) controlled by a qualified DXA operator.

Overfeeding intervention

Dietary intake during baseline was estimated using Foodworks (Xyris Software®, Melbourne, Australia) with the New Zealand - Standard database. In consultation with the subject, additional food was chosen to provide 90 kJ.(kg body mass)$^{-1}$.day^{-1} of additional energy with the same proportions of carbohydrate and protein as the subject's baseline diet, and with the remainder of the energy from fat. Subjects consumed the same absolute amount of alcohol during the intervention as during baseline, resulting in a decline of the relative proportion of total energy from alcohol. The proportion of energy from fat was increased slightly to make up for the shortfall from alcohol. The relative proportions of saturated, monounsaturated and polyunsaturated fat consumed during the intervention were the same as during baseline.

Beginning day nine subjects used their food record to replicate exactly their diet during the baseline week, plus consuming their additional 90 kJ.kg^{-1}.day^{-1}. Subjects used their exercise diary and heart rate monitor to replicate the exercise performed during the baseline week. The researchers were in regular contact with subjects during the intervention to ensure compliance, and subjects attended weekly sessions for weighing and to receive their next weekly supply of food.

Post-testing

On day 36 IVGTT and DXA tests were repeated according to the pre-testing procedure. Following the intervention, subjects' body mass was recorded weekly until it returned to within 0.5 % of baseline.

Analytical procedures and calculations

Plasma glucose concentration was determined by the hexokinase method (Slein 1963). The plasma concentration of free fatty acids was determined using an ACS-ACOD NEFA C Test kit (WAKO Chemical, Richmond, VA, USA) and triacylglycerol (TAG) by lipase/glycerol kinase method (Fossati and Prencipe 1982). Plasma insulin concentrations were determined by radio-immuno assay kit (Millipore, Billerica, MA), total cholesterol by cholesterol esterase/cholesterol oxidase/peroxidise (Allain, Poon et al. 1974), HDL cholesterol by detergent/cholesterol esterase/cholesterol oxidase/peroxidise (HDL Cholesterol Test Kit, Roche Diagnostics, Indianapolis IN, USA), and leptin by double antibody radioimmunoassay (Human Leptin RIA Kit, Linko Research, St. Charles, MO, USA). All measurements were made in duplicate and the mean reported.

Integrated area under curve (AUC) for glucose, insulin and FFA were calculated using the trapezoidal rule. Insulin sensitivity index (S_i) and the acute insulin response to glucose (AIRg) were determined via the minimal model analysis of the plasma glucose and insulin response to the IVGTT (Pacini and Bergman 1986) using MINMOD Millennium (version 6.02, MinMod, University of Southern California, Los Angeles, CA, USA). Relative energy expenditure was calculated by normalising absolute energy expenditure to initial lean body mass. Percent change in body mass was calculated using 100 * (final value – initial value) / (initial value). The fraction of mass gain made up of lean tissue

(lean fraction) was calculated using (change in lean mass) / (change in total body mass). Minimal model glucose effectiveness was not calculated as the difference in insulin response between conditions renders this measure unreliable (Cobelli, Bettini et al. 1998).

Statistical analyses

Pre-post comparisons of fasting variables, S_i and AIRg as well as glucose, insulin and FFA AUCs were made using Student's paired t-test, as was the comparison between reported energy intake and energy expenditure during baseline and body mass before and after baseline. Baseline S_i, baseline fasting plasma leptin concentration, change in S_i and relative energy expenditure were tested for correlation against % change in body mass, change in body fat % and lean fraction. All correlations were graphed and visually inspected for linearity and normality, and the kurtosis and skewness of each variable was calculated. Plasma glucose, insulin and FFA concentrations during the IVGTT were compared by two-way repeated measures ANOVA for investigation of overfeeding, time and overfeeding-time interactions. Where interactions were found Tukey's honestly significant difference test was used to identify when throughout the IVGTT the difference occurred. To test the power of energy expenditure, initial insulin sensitivity, and initial fasting leptin concentration to predict the proportion of lean tissue gained, a post-hoc regression analysis with lean fraction as the dependent variable and relative energy expenditure and initial S_i as the independent variables was performed. Change in S_i was defined as the primary outcome variable while all other statistical tests were defined as secondary outcomes. All 32 secondary outcome p values were adjusted using Benjamini and Hochberg's (Benjamini and Hochberg 1995) linear step-up method for multiple-test false discovery rate correction (Appendix 14.2). To assess the impact of including both male and female subjects, all analyses were repeated with only the male subjects included

(Appendix 14.2). Statistical significance was accepted at $p < 0.05$. All values are expressed as mean ± standard deviation.

10.3 Results

During the baseline period subjects consumed 10790 ± 2408 $kJ.day^{-1}$ as estimated by food diary, expended 12927 ± 3400 $kJ.day^{-1}$ as estimated by heart rate interpolation. The estimated energy expenditure was significantly greater than the estimated energy intake ($p < 0.063$), however the decrease in body mass was not significant (0.14 ± 0.38 kg, $p < 0.33$). Weekly energy intake during the baseline period was made up of 32.0 ± 3.9 % of energy from fat, 48.8 ± 5.5 % from carbohydrate, 16.6 ± 2.4 % from protein, and 2.4 ± 2.4 % from alcohol. During the overfeeding period subjects consumed an additional 6480 ± 1332 $kJ.day^{-1}$ or an additional 181 ± 37 MJ over the entire overfeeding period. Weekly energy intake during the overfeeding period was made up of 33.1 ± 3.8 % of energy from fat, 48.6 ± 5.4 % from carbohydrate, 16.6 ± 2.4 % from protein, and 1.5 ± 1.5 % from alcohol. Subjects chose to consume most of their additional calories in the form of energy dense convenience foods or drinks (e.g. protein bars, cottage cheese, soymilk, soda, custard). Overfeeding was generally well tolerated. Subjects reported subjective feelings of fullness or bloating, reduced motivation, increased strength, increased thirst, and increased stool volume. During the overfeeding intervention, two male subjects each missed two of their scheduled training sessions due to personal commitments, but all other subjects were able to follow their training diary for the entire intervention period. For all subjects, training during the last week of the intervention was identical to training during the baseline week.

Table 10-1. Body composition and plasma metabolite concentrations measured after an overnight fast before and after overfeeding.

	Pre	Post	% change	adjusted P <
Body mass (kg)	72.0 ± 14.8	75.4 ± 16.1	4.7 ± 1.9	0.007
Lean tissue mass (kg)	61.0 ± 13.5	63.5 ± 14.7	3.9 ± 1.6	0.010
Fat mass (kg)	11.0 ± 3.4	12.0 ± 3.6	9.4 ± 7.0	0.013
Body Fat %	15.4 ± 4.7	16.0 ± 4.7	4.4 ± 5.5	0.055
Plasma glucose (mmol.l^{-1})	4.7 ± 0.3	4.7 ± 0.2	1.2 ± 5.5	0.66
Plasma insulin (pmol.l^{-1})	66.2 ± 14.5	69.7 ± 20.5	5.4 ± 24	0.55
Plasma FFA (µmol.l^{-1})	436 ± 164	316 ± 158	-19 ± 49	0.16
Plasma TAG (mmol.l^{-1})	0.76 ± 0.19	0.94 ± 0.36	40 ± 39	0.034
Plasma Cholesterol (mmol.l^{-1})	3.80 ± 0.61	4.32 ± 0.80	14 ± 13	0.039
Plasma HDL (mmol.l^{-1})	0.65 ± 0.10	0.70 ± 0.17	4.9 ± 11	0.26
Plasma Leptin (µg.l^{-1})	2.76 ± 2.19	4.90 ± 4.49	70 ± 52	0.053

Values are means (± S.D.), n = 9 subjects. P values adjusted for multiple comparisons using false discovery rate correction.

The fasting measurements taken before and after overfeeding are presented in **Table 10-1**. There were significant increases in total body mass (**Figure 10-2**) as well as lean and fat mass while body fat % tended to increase. The increase in body mass resulted in an increase in BMI of 1.1 ± 0.5 kg.m^{-2}. There were significant increases in fasting leptin, TAG, and total cholesterol but not in other fasting blood measurements. Subjects returned to within 0.5 % of their baseline weight in 5.6 ± 2.3 weeks after the end of overfeeding (range three to ten weeks).

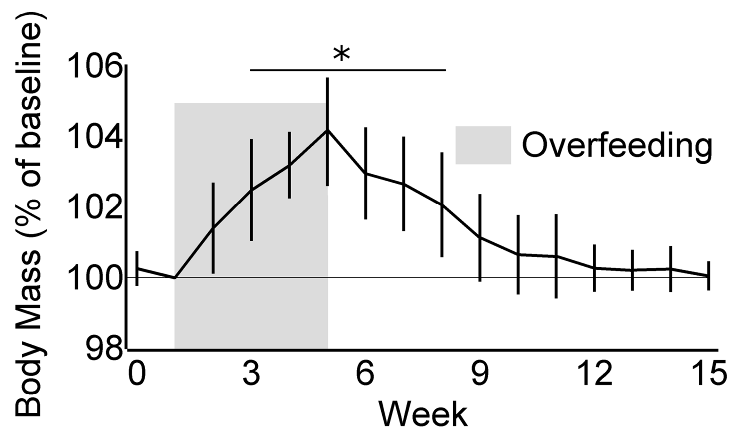

Figure 10-2. Change in body mass during overfeeding and washout. n = 9 subjects. Body mass is presented as a percentage of baseline mass. All subjects gained weight rapidly during the overfeeding period (weeks 1-5). Subjects returned to within 0.5 % of their baseline weight within 3-10 weeks after the end of overfeeding. * significantly different from baseline mass (beginning of week 1).

The results of the IVGTT before and after overfeeding are presented in **Table 10-2**. Overfeeding caused significant increases in the AIRg and insulin AUC, and decreases in FFA AUC in response to the IVGTT. However, there were no significant changes in glucose tolerance or minimal model insulin sensitivity. During the IVGTT there was a significant treatment-time interaction effect on plasma insulin ($p < 0.003$) but not glucose ($p < 0.19$) or FFA concentration ($p < 0.39$) (**Figure 10-3**). Insulin concentrations during the first six minutes of the IVGTT were significantly higher after overfeeding than before ($p < 0.05$, **Figure 10-3**).

Table 10-2. Intravenous glucose tolerance test (IVGTT) results before and after four weeks of overfeeding.

	Pre	Post	% change	Adj. P <*
S_i ($l.min^{-1}.mU^{-1}$)	14.5 ± 5.9	9.5 ± 4.1	-34 ± 51	0.08*
Glucose AUC ($mmol.l^{-1}.min$)	194 ± 81	167 ± 35	-13 ± 40	0.20
AIRg	203 ± 82	448 ± 133	93 ± 49	0.002
Insulin AUC ($min.\mu mol.l^{-1}$)	4.4 ± 1.6	7.5 ± 2.2	69 ± 37	0.005
FFA AUC ($min.mmol.l^{-1}$)	30.2 ± 8.0	22.4 ± 8.2	-26 ± 27	0.027

Values are means (± S.D.), n = 9 subjects. *All P values except the primary outcome (S_i) were adjusted for multiple comparisons using false discovery rate correction. S_i, Minimal Model insulin sensitivity index; Glucose AUC, area under glucose vs. time curve during the IVGTT, higher values reflecting worse glucose tolerance; AIRg, acute insulin response to glucose, indicates the rapidity of initial secretion of insulin in response to a glucose challenge; Insulin AUC, area under insulin vs. time curve during the IVGTT, higher values indicating a greater glucose disposal stimulus; FFA AUC, area under free fatty acid vs. time curve during the IVGTT, lower values indicate greater suppression of plasma FFA.

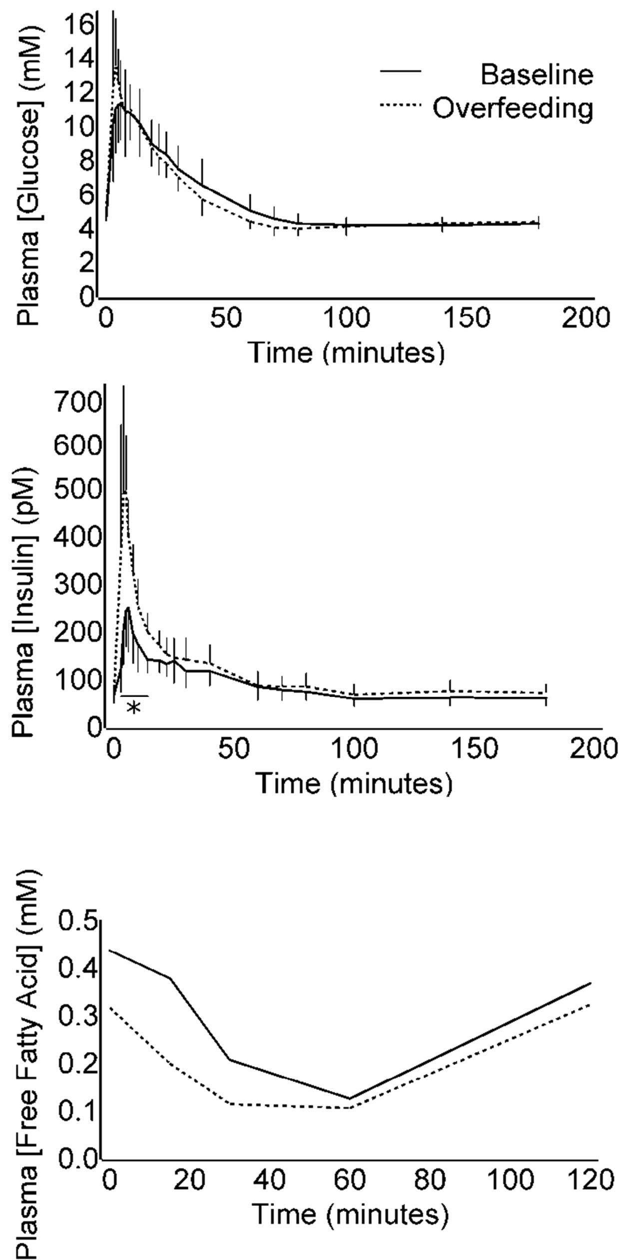

Figure 10-3. Plasma glucose, insulin and free fatty acid (FFA) response to the intravenous glucose tolerance test before and after overfeeding. n = 9 subjects. * individual time points significantly different before vs. after overfeeding. There was also a significant increase in insulin AUC ($p < 0.005$) and decrease in FFA AUC ($p < 0.027$) after overfeeding.

Changes in minimal model insulin sensitivity were significantly correlated with changes in body fat % ($r = -0.77$, $p < 0.032$, **Figure 10-4**), but not with fractional changes in body mass ($r = -0.09$, $p < 0.35$) or lean fraction ($r = 0.66$, $p < 0.080$). Initial insulin sensitivity was also correlated with changes body fat % ($r = 0.90$, $p < 0.009$, **Figure 10-5**) and lean fraction ($r = 0.86$, $p < 0.024$, **Figure 10-5**), but not with fractional changes in body mass ($r = 0.04$, $p < 0.34$). Relative energy expenditure was significantly negatively correlated with lean fraction ($r = -0.79$, $p < 0.031$), but was not fractional change in body mass ($r = 0.001$, $p < 0.93$) or change in body fat % ($r = -0.58$, $p < 0.15$, **Figure 10-6**). Baseline fasting leptin concentration was not significantly correlated with change in body fat % (-0.04, $p < 0.97$), fractional change in body mass (0.03, $p < 0.92$), or lean fraction (-0.24, $p < 0.57$). For all but one of the significant correlations (discussed below), visual inspection confirmed that the relationship appeared linear and the variables appeared normally distributed. Consistent with the visual assessment of normality, all variables had kurtosis scores less than one standard error from zero and all except fasting leptin (1.8 standard errors) and change in body fat % (1.4 standard errors) had skewness scores less than one standard error from zero (Appendix 14.3). The correlation between change in S_i vs. change in body fat % appeared to be heavily influenced by one particularly large value for change in body fat % (**Figure 10-4** lower panel), and when this point was removed the relationship became non-significant, although it remained qualitatively similar ($r = 0.55$, $p = 0.15$). The post-hoc regression analysis ($p = 0.0028$, $R^2 = 0.93$) demonstrated that relative energy expenditure ($p = 0.006$) and initial insulin sensitivity ($p = 0.014$) were independent predictors of lean fraction, but baseline fasting leptin concentration ($p = 0.71$) was not.

When female subjects were excluded from the analysis there was a general reduction in significance due to reduced statistical power. However, the magnitude of correlations and pre-post differences remained similar for all tests performed (Appendix 14.2).

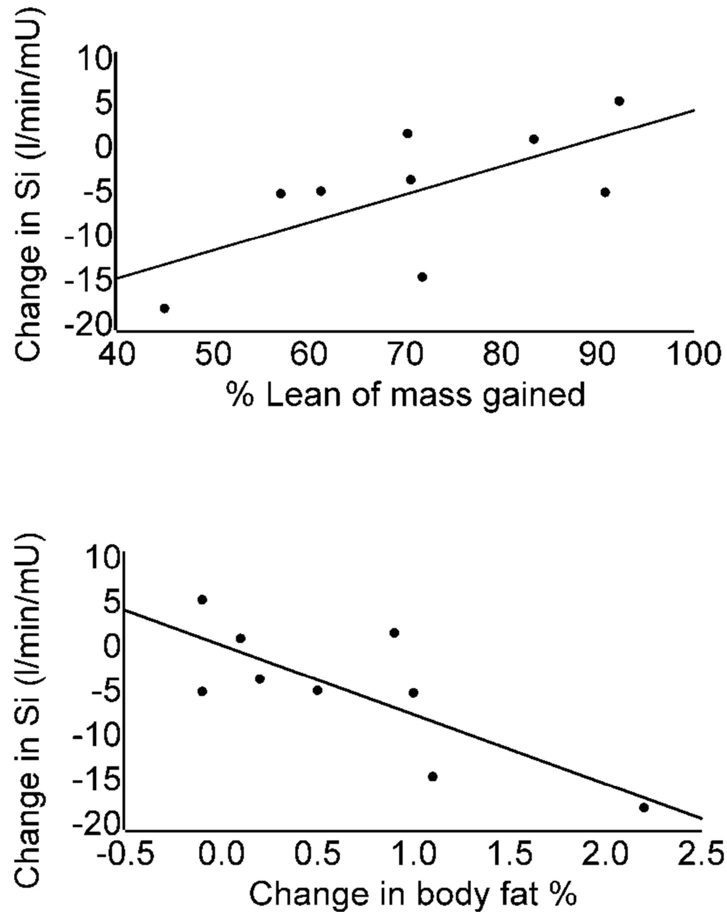

Figure 10-4. Relationship between change in insulin sensitivity (S_i) and change in body fat during overfeeding. n = 9 subjects. The upper panel illustrates the relationship (not significant) between and lean fraction [(change in lean mass)/(change in body mass)*100] and change in S_i ($r = -0.66$, $p < 0.08$). The lower panel illustrates the correlation between change in body fat % (final body fat % - initial body fat %) and change in S_i ($r = -0.77$, $p < 0.032$).

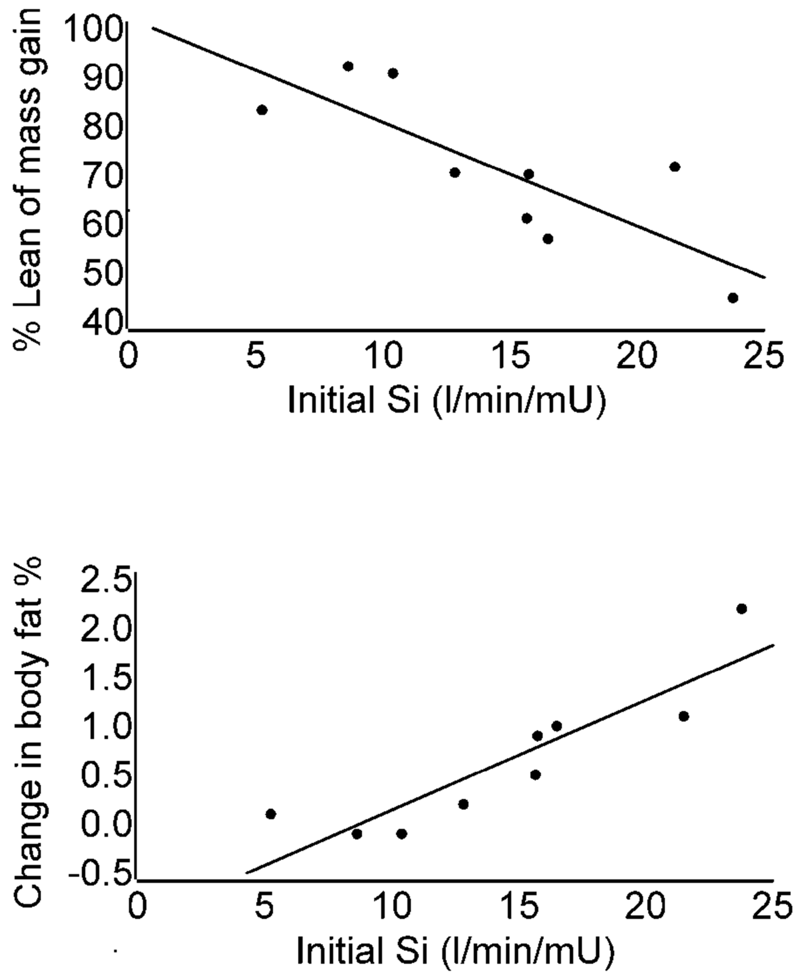

Figure 10-5. Relationship between initial insulin sensitivity (S_i) and change in body fat during overfeeding. n = 9 subjects. The upper panel illustrates the correlation between lean fraction (change in lean mass / change in total mass) and initial S_i ($r = -0.80$, $p < 0.024$). The lower panel illustrates the correlation between change in body fat % (final body fat % - initial body fat %) and initial S_i ($r = 0.90$, $p < 0.009$).

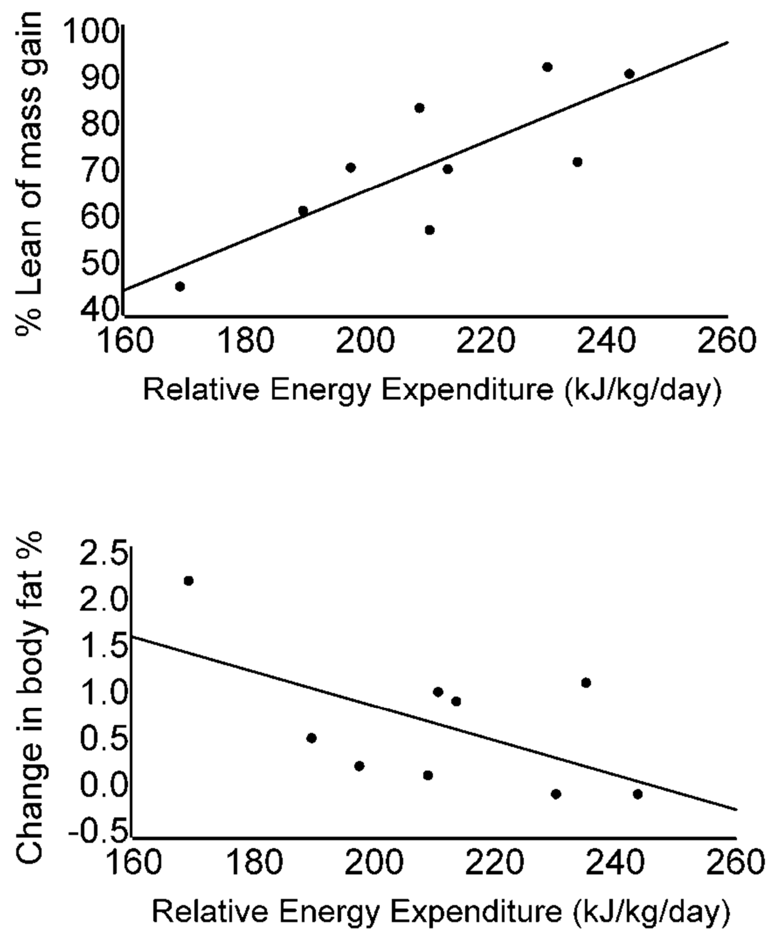

Figure 10-6. Relationship between daily energy expenditure relative to lean tissue mass and change in body fat during overfeeding. n = 9 subjects. The upper panel illustrates the correlation between lean fraction (change in lean mass / change in total mass) and relative energy expenditure ($r = -0.79$, $p < 0.031$). The lower panel illustrates the relationship (not significant) between change in body fat % (final body fat % - initial body fat %) and relative energy expenditure ($r = -0.58$, $p < 0.15$).

10.4 Discussion

This study was primarily designed to test the hypothesis that continued high levels of physical activity would prevent changes in insulin sensitivity in the face of prolonged excess energy intake. Although the decrease in minimal model whole body insulin sensitivity (S_i) did not quite reach significance ($p = 0.08$, **Table 10-2**), changes in S_i were significantly negatively correlated with changes in body fat % ($r = -0.77$, $p < 0.032$) and tended towards a positive correlation with the fraction of mass gained as lean tissue ($r = 0.66$, $p < 0.08$), yet were not related to changes in total body mass ($r = -0.09$, $p < 0.35$). These data strongly suggest that there is real change in S_i with overfeeding that produces fat gain in lean, physically active individuals, but that the current study did not have sufficient statistical power to resolve an S_i main effect. This suggestion is further supported by the increase in the AIRg and the insulin AUC after overfeeding. It is therefore appropriate to reject the hypothesis that continued exercise can prevent the changes in insulin sensitivity usually observed with prolonged overfeeding.

To test the second hypothesis, that initial insulin sensitivity and fasting leptin concentration can predict the magnitude of body mass and fat gain in response to a given increase in energy intake, Pearson correlations were performed between baseline insulin sensitivity and fasting leptin concentration and changes in body mass and body fat during overfeeding. Neither initial insulin sensitivity nor fasting leptin concentration were related to changes in body mass. However, initial insulin sensitivity was strongly positively related to changes in body fat % and negatively related to the fraction of mass gained as lean mass (**Figure 10-5**). That is, subjects with higher baseline insulin sensitivity gained a higher proportion of fat in response to overfeeding than did subjects with lower insulin sensitivity, despite no difference in total body mass gain. To my knowledge, this

study is the first report of insulin sensitivity predicting energy partitioning in response to overfeeding.

As those subjects who gained the most fat mass also had the highest initial insulin sensitivity, it is possible that the correlation between change in body fat and change in insulin sensitivity is not causal. That is, a high baseline insulin sensitivity may have caused both a greater decline in insulin sensitivity and a greater increase in fat mass. As there is abundant physiological evidence demonstrating a mechanistic link between high adiposity and insulin resistance (see section 4.5.1) it seems likely that the decrease in insulin sensitivity was secondary to the increase in fat mass, but the design of the current study cannot test for this mechanism.

The results of the current study are not consistent with the only previous study to measure insulin sensitivity in physically active subjects before and after prolonged overfeeding without also reducing physical activity (Ohannesian, Marco et al. 1999). There are two differences in methodology that could potentially explain the different results from these studies. In the Ohannesian *et al.* (1999) study, subjects were "encouraged to maintain their usual physical activity", but no attempt was made to monitor their physical activity during overfeeding, nor to control their physical activity immediately prior to determination of insulin sensitivity. As such, subjects may have increased their physical activity during overfeeding, particularly in the 24 hours preceding the hyperinsulinaemic-euglycaemic clamp, which may have masked any change in insulin sensitivity. Alternatively, the protocol used by Ohannesian *et al.* (1999) included a 2-week period of energy balance after overfeeding and before the second set of insulin sensitivity measurements, which may have attenuated the effect of overfeeding. However, this possibility seems less likely as, in less active subjects, Erdman *et al.* (2008) observed

decreased insulin sensitivity after similar overfeeding despite four weeks of energy balance before the post-overfeeding measurements.

Within the subjects in the current study, the combination of initial insulin sensitivity and relative energy expenditure was able to predict the proportion of mass gained as lean tissue with a very high degree of accuracy ($R^2 = 0.93$). An explanation for this effect of relative energy expenditure may be provided by the anabolic effect of exercise on skeletal muscle. That is, as exercise stimulates skeletal muscle anabolism and fuel storage (Hamilton and Booth 2000), higher relative energy expenditure (i.e. greater physical activity) could increase skeletal muscle anabolism and result in a higher proportion of energy sequestered in lean tissue. Similarly, the effect of initial insulin sensitivity could be explained by assuming a greater metabolic effect of insulin in response to overfeeding in subjects with higher insulin sensitivity. That is, higher insulin sensitivity may result in greater suppression of adipose tissue lipolysis (Enoksson, Degerman et al. 1998) and therefore a greater proportion of excess energy sequestered in adipose tissue. Likewise, high insulin sensitivity favours postprandial glucose disposal by skeletal muscle at the expense of postprandial lipid disposal (Segal, Albu et al. 1992), which may also direct energy towards adipose tissue during overfeeding. In this sense, higher insulin sensitivity (in combination with overfeeding) could be thought of as analogous to consuming higher glycemic index foods, where increased rates of glucose disposal and oxidation by skeletal muscle result in partitioning of energy towards fat storage (Brand-Miller, Holt et al. 2002). While the current study does not provide direct evidence for these mechanisms, they are physiologically plausible and warrant future investigation.

The relationship between relative energy expenditure and fat gain in the current study is analogous to the results of Sun *et al.* (2002) who found that the proportion of type 1

muscle fibres was inversely correlated with changes in body fat % in response to overfeeding. Similarly, compared to the less active subjects in previous overfeeding studies, the highly physically active subjects in the current study gained a higher proportion of lean tissue. Nearly three quarters (71%) of the mass gained by subjects in the current study was accounted for by lean mass. A search of Web of Science and Google Scholar revealed eight overfeeding studies that report the amount of extra energy provided, changes in body mass, and changes in body composition. Of these studies, seven reported a lower portion of lean mass gain (range 29 to 49 %) than the current study (Norgan and Durnin 1980; Diaz, Prentice et al. 1992; Oppert, Nadeau et al. 1995; Levine, Eberhardt et al. 1999; Ohannesian, Marco et al. 1999; Levitsky, Obarzanek et al. 2005; Forbes, Brown et al. 2007)]. Subjects in the current study also gained mass less "efficiently" than subjects in previous studies. Subjects in the current study gained an average of 18.7 g.(MJ extra energy)$^{-1}$, compared to between 22.9 to 33.1 g.MJ^{-1} in the same seven studies mentioned above. The one study that reported lower mass gain efficiency (16 g.MJ^{-1}) also reported a higher percentage of mass gained as lean tissue (80 %, Meugnier, Bossu et al. 2007). The subjects used by Meugnier *et al.* were lean young men with similar body composition to the subjects in the current study. While their levels of physical activity were not described, their body composition and demographic information suggest that they may have been highly physically active. On balance, the results of the current study and previous studies suggest that highly physically active subjects respond differently to energy excess than do less active subjects, with greater increases in energy expenditure and greater partitioning of mass gain towards lean tissue.

This study employed self-reported diet records to estimate energy intake, a technique that consistently under estimates actual energy intake (Hill and Davies 2001; Trabulsi and Schoeller 2001). To assess the extent of possible under-reporting energy expenditure was

estimated using heart rate interpolation, showing that reported baseline energy intake was less than estimated energy expenditure by 2140 kJ.day^{-1} or 17 % ($p < 0.08$). This degree of under-reporting is in the middle of the range of previously observed under-reporting when food records were compared to doubly-labelled water energy expenditure (Hill and Davies 2001; Trabulsi and Schoeller 2001). During the baseline dietary recording, subjects' body mass decreased by an average of 0.14 ± 0.38 kg, not significantly different from 0 ($p < 0.33$) and equal to the expected average daily standard error of nine subjects' body mass [daily S.E. = (average body mass*daily CV)/√n = (72 kg*0.006)/3 = 0.14 kg] assuming a daily coefficient of variation of body mass in active subjects of 0.6 % (Cheuvront, Carter 3rd et al. 2004). Assuming that the mass change during baseline was entirely accounted for by fat catabolism yielding 37 kJ.g^{-1}, the mass change represents an average energy deficit of 740 kJ.day^{-1}, although the true figure is likely to be somewhat less due to catabolism of less energy dense lean tissue as well as fat. Combined with the expected 310 kJ.day^{-1} increase in basal metabolic rate due to the increase in lean body mass during overfeeding, the effective overfeeding could be at most 1050 kJ.day^{-1} less than the nominal 6480 kJ.day^{-1}. However, even after correcting the nominal energy excess by this amount, the efficiency of mass gain in the current study is only 22 g.MJ^{-1}, still less than reported by seven of the eight previous studies. It therefore seems likely that the low efficiency of mass gain observed in the current study is primarily due to increased energy expenditure during the overfeeding period rather than energy deficit during the baseline period.

The current study was not designed to identify what caused the apparently large increase in energy expenditure during the overfeeding period. Subjects reported that they performed the same training (exercise mode, exercise time and average heart rate) during the overfeeding period as during the baseline period, but the current study did not include

any measurement of energy expenditure or non-programmed physical activity during overfeeding. Heart rate interpolation could not be performed during the overfeeding period as subjects would not tolerate the heart rate monitor for an additional four weeks. However, a less uncomfortable technique to measure energy expenditure, such as doubly labeled water, could have provided useful information. Alternatively, an activity monitor such as an accelerometer or pedometer may have been better tolerated by the subjects and provided insight into any changes in activity patterns during overfeeding.

One possible cause for a greater increase in energy expenditure during overfeeding in the endurance trained subjects used in the current study compared to the relatively sedentary subjects used in previous studies is an interaction between exercise training and the thermic effect of food (TEF). In lean subjects, exercise training (Tagliaferro, Kertzer et al. 1986) and acute exercise (Zahorska-Markiewicz 1980; Segal and Gutin 1983) are both reported to increase TEF, yet this effect is not reported in obese subjects (Zahorska-Markiewicz 1980). Furthermore, the magnitude of reported exercised induced increases in the thermic effect of a single meal (range 20 to 300 kJ) are sufficient to explain the differences in efficiency of weight gain between the current study and previous overfeeding studies.

Despite the low proportion of body fat gained by the subjects in the current study, the metabolic changes observed show directional similarity to the metabolic differences between lean and obese humans reported in cross sectional studies. Changes in fasting plasma metabolite concentrations with overfeeding paralleled the metabolic disturbances present in obese patients with normal glucose tolerance, i.e. elevated plasma triglycerides and total cholesterol but no change in HDL cholesterol or fasting FFA concentrations (Jensen, Haymond et al. 1989; Ko, Chan et al. 1998). Dynamic responses to a glucose

challenge after overfeeding were also similar to those seen in obesity, with increased insulin response (Ludvik, Clodi et al. 1994; Macor, Ruggeri et al. 1997) and greater suppression of FFA (Jensen, Haymond et al. 1989; Handan, Turgan et al. 1995) during the IVGTT. The similarity between the metabolic changes observed with overfeeding in healthy, lean, highly physically active subjects and those present in obesity is further evidence that exercise does not prevent the metabolic changes associated with overfeeding.

Both the current study and the previous chapter of this thesis demonstrate that physical activity cannot prevent reductions in insulin sensitivity associated with dietary challenge. However, unlike the starvation study, the current study did not include a sedentary control so it is not possible to test for an interaction effect of exercise and overfeeding. As such, it is possible physical activity may be able to partially compensate for changes in insulin sensitivity with overfeeding. Nevertheless, the results of both studies suggest that a dietary perturbation without a change physical activity with have a qualitatively similar effect regardless of habitual physical activity level.

The findings of the current study are consistent with a negative feedback system where insulin resistance inhibits mass gain while insulin sensitivity promotes energy storage (**Figure 10-7**), as first suggested by Eckel (1992). This system could explain the reported epidemiological relationship between initial insulin sensitivity and free living mass gain (Swinburn, Nyomba et al. 1991; Wedick, Mayer-Davis et al. 2001; Howard, Adams-Campbell et al. 2004; Pannacciulli, Ortega et al. 2007; Boule, Chaput et al. 2008; Chaput, Tremblay et al. 2008; Wedick, Snijder et al. 2009) as increases in lean tissue mass in less insulin sensitive individuals would increase basal metabolic rate and may decrease energy excess, whereas increases in adipose tissue mass in more insulin sensitive individuals

may not. Such a system may also have aided survival in hunter-gatherer societies. That is, when an individual is very lean, high insulin sensitivity promotes efficient energy storage as adipose tissue. However, when an individual has greater adiposity, low insulin sensitivity promotes functional tissue deposition at the expense of efficient energy storage.

The current study was designed to test whether maintaining a high level of physical activity could prevent the decrease in insulin sensitivity with overfeeding, not to identify a feedback system between insulin sensitivity and body fat % or the mechanisms responsible for such feedback. Furthermore, the current study uses correlation analyses with small subject numbers (n = 9). While there is no evidence of violation of the linearity, normality or homoscedasticity assumptions of the correlations supporting the existence of the proposed feedback system, it is not possible to conclusively validate these assumptions with such small subject numbers. To confirm the existence of the proposed system it would be instructive to include multiple subject groups stratified by initial insulin sensitivity and relative energy expenditure, to include both energy excess and energy deficit interventions, and to increase the total number of subjects. The use of tracer-clamp techniques would allow for the determination of the fate of ingested substrates and the relative insulin sensitivities of adipose, liver and skeletal muscle tissue. Potential mechanisms may best be investigated using the *in vitro* response to insulin of adipose and skeletal muscle cells obtained from subjects with a variety of insulin sensitivities and levels of physical activity.

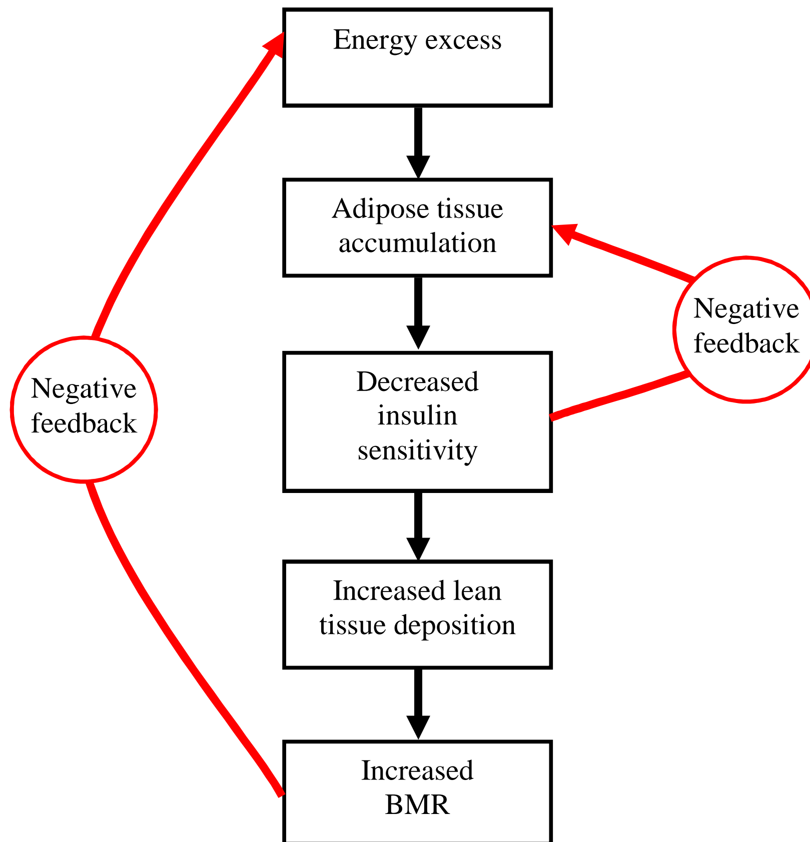

Figure 10-7. Potential negative feedback system involving fat gain and insulin sensitivity. In response to an energy excess high insulin sensitivity results in partitioning of energy towards adipose tissue. Accumulation of adipose tissue decreases insulin sensitivity, directing future energy excess towards lean tissue. Increased accumulation of lean tissue increases energy expenditure and may reduce energy excess.

This study demonstrates that even in healthy, highly physically active athletes, overfeeding resulting in increased fat mass causes decreased insulin sensitivity and alterations in the metabolic profile that are similar to those seen during obesity. Furthermore, these results suggest that the magnitude of fat gain in response to overfeeding is a function of initial insulin sensitivity and level of physical activity. I concluded that exercise cannot prevent the adverse metabolic consequences of obesity.

This conclusion has no bearing on the independent metabolic effects of exercise or the ability of exercise to prevent obesity *per se*.

11 Comparison of indices of insulin sensitivity

As noted in the review of techniques to determine insulin sensitivity (Section 7.1.4), the fasting indices of insulin sensitivity, HOMA and QUICKI, have not been validated in conditions of starvation or overfeeding. The data presented in this thesis provide a ready opportunity for these indices to be validated against the FSIVGTT in these unusual conditions. To this end, Pearson correlations were calculated between Si obtained via FSIVGTT and QUICKI, HOMA IR, and log(HOMA) indices for the pooled data from all studies. Correlations between each of the three indices and Si were weak (r=-0.39, 0.28, and 0.36 for QUICKI, HOMA IR, and log(HOMA) respectively, n=42). Graphical representations of the relationships are presented in Figure 11-1.

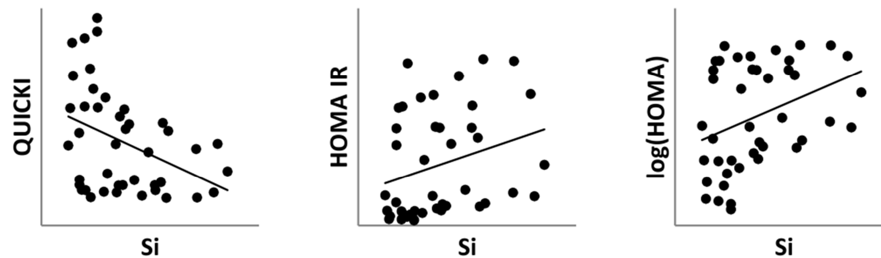

Figure 11-1 Relationships between fasting indices of insulin sensitivity and insulin sensitivity determined from FSIVGTT (Si). Pearson correlations were r=-0.39, 0.28, and 0.36 for QUICKI, HOMA IR, and log(HOMA) respectively, n=42.

The observed correlations between the fasting indices and Si are considerably weaker than the reported correlations between Si and QUICKI for lean sedentary subjects before (r=0.79) and after (r=0.56) undergoing an exercise program (Duncan, Hutson et al. 2001) and for obese (r=0.75) and diabetic (r=0.67) subjects (Katz, Nambi et al. 2000), and obese children and adolescents (r>0.8, Conwell, Trost et al. 2004) but similar to healthy non-obese subjects where physical activity is not specified (r=0.36; Katz, Nambi et al. 2000)

and pre-pubescent children undergoing growth-hormone therapy (r=0.2, Cutfield, Jefferies et al. 2003). Similarly, the correlations in the current data are considerably weaker than reported correlations between Si and HOMA in obese children and adolescents (r<-0.8, Conwell, Trost et al. 2004), but similar to pre-pubescent children undergoing growth-hormone therapy (r=-0.4, Cutfield, Jefferies et al. 2003).

There is a very weak relationship between insulin sensitivity values determined by FSIVGTT and fasting indices in the current study, in children undergoing growth-hormone therapy, and healthy people where physical activity was not controlled. While the current data do not indicate why there was such a weak relationship, it seems likely the assumptions required to estimate insulin sensitivity from a single fasting blood sample are violated in starvation and prolonged overfeeding, particularly the assumption that there is the same rate of appearance of glucose into the bloodstream in all cases.

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These results suggest that fasting indices of insulin sensitivity such as HOMA and QUICKI are not suitable for use in unusual situations such as short-term starvation or overfeeding, despite being a useful tool in situations where their assumptions are likely to be fulfilled.

12 Conclusions and future directions

The research presented in this thesis explores the way a healthy insulin-glucose system adapts to challenging situations, especially those which might be encountered when dietary macronutrient supply is not reliable. Such situations, including periods of caloric surplus, short-term energy restriction with concurrent physical activity, and select macronutrient restriction might have been encountered in hunter-gatherer societies due to seasonality of food and climatic events (Speth and Spielmann 1983). The data presented in this thesis fill gaps in our knowledge of acute adaptations of the insulin-glucose system in healthy people, which in turn provides a framework for the effective design of future experiments. A better understanding of acute insulin resistance in healthy people provides a baseline for comparison with chronic, pathological insulin resistance.

To achieve the overall research objective, the specific goals of each experiment were to: (1) compare the metabolic effects of a high protein, low carbohydrate diet with those of other forms of carbohydrate deprivation; (2) determine whether maintaining a high level of physical activity during short term starvation can prevent the usual decrease in insulin sensitivity; (3) determine whether maintaining a high level of physical activity during chronic overfeeding can prevent the usual decrease in insulin sensitivity; and (4) examine the effect of insulin sensitivity and leptin concentration on the changes in body composition that occur when overfeeding is imposed.

Carbohydrate deprivation in the form of short term high-protein, low-carbohydrate diet did not cause a reduction in insulin sensitivity, in contrast to short term starvation or high-fat, low-carbohydrate diet (Johnson, Stannard et al. 2006). The high protein diet allowed

maintenance of normal blood glucose concentration in the face of a low exogenous carbohydrate supply, probably because of a massive up-regulation of gluconeogenesis in the face of abundant gluconeogenic substrate. Normal blood glucose obviates the need for increased adipose lipolysis to supply fuel for normal metabolism, so circulating FFA concentration is not changed and peripheral tissues do not experience an alteration in their interstitial milieu.

That a high dietary protein intake is able to maintain normal metabolic carbohydrate availability and normal insulin sensitivity has important implications for human survival when carbohydrate is scarce. In hunter-gatherer societies in arctic and colder temperate climates, the diet during the winter months consisted primarily of animal flesh (Speth and Spielmann 1983). In a more contemporary example, the stranded sailors of Shackleton's 1914-1917 antarctic expedition survived for months on Elephant Island with a diet primarily of seal meat (Shackleton 1998). The ability to maintain normal blood glucose concentrations while consuming such a diet maintains cognitive function (Cryer 2007) and cold tolerance (Haight and Keatinge 1973), and obviates the need for functional tissue catabolism to maintain endogenous glucose supply (Garlick, Fern et al. 1983).

The current evidence is consistent with reduced metabolic carbohydrate availability being the trigger for reduced insulin sensitivity during starvation (for a summary see **Table 12-1**). However, it is also possible that high dietary fat intake could be responsible for the reduction in insulin sensitivity that occurs with a low-carbohydrate, high-fat diet (Oakes, Bell et al. 1997; Meugnier, Bossu et al. 2007), while dietary energy restriction could be responsible for the reduction in insulin sensitivity that occurs with starvation. These possibilities could be tested by determining the effect on insulin sensitivity of a low-

carbohydrate diet with normal protein and fat content compared to an isocaloric diet with mixed macronutrient content.

Although maintaining a high level of physical activity during short-term starvation prevented the accumulation of *vastus lateralis* IMCL, it did not alter the elevation of circulating FFA concentration or the reduction of insulin sensitivity normally observed with starvation. That is, despite physical activity, starvation blood glucose concentration is held at sedentary starvation levels by maintaining low insulin sensitivity. This result is consistent with exercise being unable to prevent the decline of insulin sensitivity in the face of high-fat, low-carbohydrate diet (Sparti and Decombaz 1992), but contrasts with the ability of exercise to prevent the reduction in insulin sensitivity that usually occurs with lipid infusion (Schenk, Cook et al. 2005; Schenk and Horowitz 2007).

Table 12-1: Summary of human research describing the effect of dietary interventions and exercise on whole body insulin sensitivity

Diet	Carbohydrate	Protein	Fat	Energy	Effect of intervention on insulin sensitivity	Effect of exercise during intervention on insulin sensitivity	Selected references
Normal balanced diet	↔	↔	↔	↔	No change	Increase	(Maehlum, Felig et al. 1978; Bogardus, Thuillez et al. 1983; Devlin, Hirshman et al. 1987; Mikines, Sonne et al. 1989; Richter, Mikines et al. 1989)
Low carb high fat	-	↔	↑	↔	Decrease	?	(Fery, Bourdoux et al. 1982; Klein and Wolfe 1992; Johnson, Stannard et al. 2006)
Low carb high protein	-	↑	↔	↔	No change	?	(Chapter 8)
Starvation	-	-	-	-	Decrease	No change	(Chapters 8 and 9; (Sparti and Decombaz 1992; Johnson, Stannard et al. 2006)
Low carb	-	↔	↔	↓	?	?	
High carb	↑	↔	↔	↑	Decrease	?	(Schwarz, Neese et al. 1995; Faeh, Minehira et al. 2005)
High fat	↔	↔	↑	↑	Decrease	?	(Oakes, Bell et al. 1997; Oakes, Cooney et al. 1997; Schenk, Cook et al. 2005; Meugnier, Bossu et al. 2007; Schenk and Horowitz 2007)
Low fat	↔	↔	↓	↓	No change	?	(Mostad, Qvigstad et al. 2004)[a]
Long-term low energy	↓	↓	↓	↓	Increase	Increase	(Goodpaster, Katsiaras et al. 2003; Larson-Meyer, Heilbronn et al. 2006)[b]
Long term high energy	↑	↑	↑	↑	Decrease	?	(Chapter 10;(Erdmann, Kallabis et al. 2008)

Subjects are lean and healthy except where noted. Each of the symbols represents the effect of a dietary

manipulation on the intake of a macronutrient or dietary energy: ↔no change from usual intake; ↑greater than normal intake; ↓intake less than normal but greater than zero; -no intake. [a]Subjects are overweight type-2 diabeteic patients. [b]Subjects are obese but otherwise healthy.

A key difference between the lipid infusion (Schenk, Cook et al. 2005; Schenk and Horowitz 2007) and low-carbohydrate dietary protocols (Sparti and Decombaz 1992) is the dietary carbohydrate intake (**Table 12-1**). It is therefore appropriate to ask what effect exercise will have on insulin sensitivity under conditions of high-fat diet with either low-carbohydrate intake or normal-carbohydrate intake. Given the assumption that metabolic carbohydrate availability modulates the ability of physical activity to enhance insulin sensitivity, exercise will attenuate the reduction in insulin-mediated glucose uptake in when carbohydrate intake is normal, but not when carbohydrate intake is eliminated. The existing data (Sparti and Decombaz 1992; Schenk, Cook et al. 2005; Schenk and Horowitz 2007) appear to support this prediction and, if it is confirmed by future research, it will provide further insight into survival during carbohydrate scarcity. In any case, that endurance exercise can be performed during short-term starvation without a further decrease in blood glucose concentration is an important evolutionary adaptation to intermittent food supply. If physical activity *was* to reduce blood glucose below usual fasting levels then hungry people who attempted to hunt or travel to obtain food would risk hypoglycaemia, poor success in food procurement, and possibly death (Cryer 2007). Similarly, hungry people exposed to cold environmental conditions would be unable to sustain blood glucose concentrations while shivering, so would have increased risk of hypothermia (Haight and Keatinge 1973).

Obesity (Thamer, Machann et al. 2003) and short term starvation (Johnson, Stannard et al. 2006) both cause an increase in lipid availability in skeletal muscle cells and a decrease in whole body insulin sensitivity. The results presented in this thesis demonstrate another similarity between the two conditions. Like obesity (Kelley, Goodpaster et al. 1999), short term starvation also causes a reduction in metabolic flexibility. In obesity this inflexibility manifests itself in high basal glucose oxidation but

a reduced ability to increase glucose disposal during exercise or in response to a glucose load (i`.e`. decreased insulin sensitivity, \Kelley, Goodpaster et al. 1999). During short term starvation, decreased insulin sensitivity has been described many times (Chapters 8 and 9; (Defronzo, Soman et al. 1978; Mansell and Macdonald 1990; Horowitz, Coppack et al. 2001; Johnson, Stannard et al. 2006) but this is the first description of a reduced ability to increase glucose disposal during exercise. This finding further supports the importance of fully understanding the adaptations of a healthy metabolism to short term starvation. In particular, the similarity of the metabolic changes caused by obesity and starvation suggest that they are the result of the same physiological signalling pathways, so understanding the mechanisms that reduce insulin sensitivity during starvation can be used to inform research to identify effective treatments for the pathological insulin resistance of obesity.

Exercise during starvation caused a large increase in glucose effectiveness compared to inactivity during starvation. If this effect also operates in insulin resistant obese or type-2 diabetic patients then it has important implications for glycaemic control in these populations. Indeed, increased glucose effectiveness is a likely mechanism explaining the beneficial effects of exercise on glycaemic control in type-2 diabetic patients (Schneider, Amorosa et al. 1984). Furthermore, high glucose effectiveness would be beneficial for lean-healthy people consuming their first meal after a period of starvation. In this situation, high glucose effectiveness would promote rapid glucose storage and help prevent hyperglycaemia and wasteful glycosuria before an insulin response could occur.

Overeating for 4 weeks caused a non-significant ($p = 0.08$) decrease in insulin sensitivity in subjects with a very high level of physical activity. Individual decreases in insulin sensitivity were highly correlated with increases in fat mass, and changes in other

metabolic parameters all showed directional similarity to differences between lean and obese humans. The qualitative agreement of these data suggest that, although this study did not have sufficient statistical power for the decline in insulin sensitivity to reach the 95% threshold, maintaining a high level of physical activity is not able to prevent the decrease in insulin sensitivity that occurs with overfeeding. That is, no matter how much exercise is performed by obese physically active individuals, they will still tend to have lower insulin sensitivity than lean individuals performing a similar amount of physical activity. This result confirms previous data showing that exercise without changes in body composition is not as effective at increasing insulin sensitivity as is exercise and fat loss combined (Dengel, Pratley et al. 1996). However, as relative energy expenditure was a strong independent predictor of energy storage partitioning, the results presented in this thesis also suggest that exercise may aid in decreasing adiposity even if energy balance is not changed.

The results of the starvation and overfeeding studies demonstrate that exercise cannot prevent a dietary induced alteration in insulin sensitivity, in contrast with some previous research (Ohannesian, Marco et al. 1999; Schenk, Cook et al. 2005; Schenk and Horowitz 2007). These result, in combination with the observation that exercise can alter insulin sensitivity under fed conditions (Richter, Mikines et al. 1989) but not during starvation (Chapter 9), are indicative of an interaction effect of exercise and diet on insulin sensitivity. To confirm the importance of such an interaction in modern society, it would be of interest to repeat the overfeeding experiment using subjects with a wide range of habitual physical activity levels.

Initial insulin sensitivity and plasma leptin concentration were not related to changes in total body mass in response to overfeeding. However, initial insulin sensitivity and

relative energy expenditure were strong independent predictors of energy storage partitioning between lean and fat tissue. These relationships correspond with the metabolic actions of insulin to inhibit adipose tissue lipolysis and of exercise to stimulate skeletal muscle hypertrophy.

If, as suggested above, the overfeeding experiment were to be repeated using subjects with a wide range of habitual physical activity levels, it would also provide an opportunity to confirm the correlations between initial insulin sensitivity, relative energy expenditure and energy partitioning. If the relationships were confirmed then an attempt to identify the mechanisms would be called for. An initial investigation would probably be best performed *in vitro* on adipose and skeletal muscle biopsy samples taken from subjects with varying degrees of insulin sensitivity and habitual physical activity and incubated with labelled macronutrients in media simulating postprandial conditions.

If confirmed in less physically active populations, the relationship between insulin sensitivity and energy partitioning may explain the epidemiological association between high insulin sensitivity and long term body mass gain (Swinburn, Nyomba et al. 1991; Pannacciulli, Ortega et al. 2007) by the operation of a negative feedback system between insulin sensitivity and adiposity (**Figure 10-7**). That is, in lean individuals high insulin sensitivity would promote adipose tissue gain and efficient energy storage. Conversely, in individuals with higher adiposity, low insulin sensitivity would promote lean tissue gain, which would increase basal metabolic rate and decrease energy excess (Eckel 1992). This feedback system would also be expected to have been beneficial to our paleolithic ancestors with unsecured food supply, as it would promote efficient energy storage upon initial feeding after a period of starvation, but also promote improvements in physical capability via the deposition of functional tissue when food was abundant.

The results presented in this thesis provide insight into the acute adaptations of the insulin glucose system in lean healthy people. While this knowledge is important in and of itself, it also demonstrates the need for further research. The data presented here inform the design of future studies in lean healthy people and describe physiological relationships that could be important in the treatment of obese insulin resistant patients. Future study should focus on extending our knowledge of the healthy operation of the insulin-glucose system, and on exploring the implications of this knowledge for the pathological insulin resistance of obesity and type-2 diabetes.

13 Glossary

3T3-L1 adipocytes:	A strain of cultured adipocytes commonly used in biochemical research.
AKT:	A serine/threonine-specific protein kinase. Also known as PKB.
ATP:	Adenosine tri-phosphate. The primary “energy currency” of cells.
ATPS:	Ambient temperature and pressure of saturated air.
C2C12 myotubes:	A strain of cultured muscle cells commonly used in biochemical research. Originally obtained from mouse muscle.
C3G:	Guanyl nucleotide exchange protein.
CAP:	C-Cbl-associated protein. A protein involved in lipid-raft insulin signalling.
Cbl:	Crk associated substrate NS-1 B-lineage proto-oncogene. A protein involved in lipid-raft insulin signalling.
Chylomicron:	An apolipoprotein formed in the gut. Chylomicrons transport ingested lipids from the gut via the bloodstream to the liver and some peripheral tissues.
Crk:	C reactive protein.
DAG:	Diacyl-glycerol.
EDTA:	Ethylenediaminetetraacetic acid, an anticoagulant used to treat sampled blood.
FFA:	Free fatty acid/s. Usually refers to total non-esterified fatty acids including those bound to albumin. See also unbound FFA and NEFA.
FSIVGTT:	Frequently sampled intravenous glucose tolerance test.

G6P: Glucose-6-phosphate.

Glucose effectiveness:

The ability of blood glucose to stimulate its own disposal from the bloodstream.

GSK: Glycogen synthase kinase.

GLUT4: Glucose transporter type 4. The primary insulin sensitive glucose transporter present in skeletal muscle.

HMN1 neurons: A strain of cultured neurons commonly used in biochemical research.

IMCL: Intramyocellular lipids. Also referred to as IMTG.

IMTG: Intramyocellular triglycerides. Also referred to as IMCL.

Insulin sensitivity (generalised):

The ability of insulin to stimulate the disposal of glucose from the bloodstream.

Insulin sensitivity (multi-stage euglycaemic clamp):

The plasma insulin concentration required to stimulate half-maximal rate of glucose disposal from the bloodstream for a given plasma glucose concentration.

Insulin sensitivity (hyperinsulinaemic euglycaemic clamp):

The maximal rate of glucose disposal from the bloodstream at hyper-physiological insulin concentration and physiological glucose concentration. In the context of a multi-stage euglycaemic clamp, this same quantity is referred to as “insulin responsiveness”.

Insulin responsiveness:

The maximal rate of glucose disposal from the bloodstream at hyper-physiological insulin concentration and physiological glucose concentration.

IRS1: Insulin receptor substrate 1.

LCACoA: Long chain acyl-coenzyme A.

M: mol.litre^{-1}.

MAPK: Mitogen activated protein kinase.

Metabolic Carbohydrate Availability:

The total glucose availability to peripheral tissues, regardless of the provenance of the glucose.

MRS: Magnetic resonance spectroscopy.

NEFA: Non-esterified fatty acid. See also unbound FFA.

PDH: Pyruvate dehydrogenase.

PDK: Pyruvate dehydrogenase kinase.

PI3K: Phosphatidylinositol 3-kinase.

PKB: Protein kinase B, a serine/threonine-specific protein kinase. Also known as AKT.

PKC: Protein kinase C, a serine-specific protein kinase.

Post absorptive: The period after all food has been absorbed from the gut, but before endogenous carbohydrate stores become diminished.

Post prandial: The period directly following the ingestion of food when absorption from the gut is occurring.

PtdIns: Phosphotidylinositol.

RER: Respiratory exchange ratio. The ratio of oxygen molecules consumed to carbon dioxide molecules produced by respiration.

SD: Standard deviation.

SE: Standard error.

SSPG: Steady state plasma glucose, usually in reference to the insulin suppression test to measure insulin sensitivity.

TC10: A GTPase protein involved in lipid-raft insulin signalling.

VCO_2: The rate of carbon dioxide production of an individual.

VO_2: The rate of oxygen consumption of an individual.

VO_{2Max}: The maximal rate of oxygen consumption achievable by an individual.

Unbound FFA: Non-esterified fatty acids dissolved in an aqueous medium (not bound to plasma albumin or other protein).

14 Appendices

14.1 Calculation of unbound FFA concentration from total plasma FFA

The great majority of FFA in blood plasma is bound to the protein albumin. This binding allows physiological concentrations of FFA to be transported by the blood stream without acting as soaps to dissolve cell membranes. However, FFAs can only be absorbed by tissues when they are dissociated from albumin and dissolved in aqueous solution. Therefore, it is the concentration of unbound FFA rather than total blood FFA that is needed to estimate the rate of FFA uptake by muscle cells.

As long as the molar ratio of total FFA:albumin remains within a physiological range, the concentration of unbound FFA is determined not by the absolute concentration of total FFA but the total FFA:albumin ratio. By assuming a typical value for serum albumin concentration, and by generalising from palmitate to mixed FFA, the unbound FFA concentration for a given total FFA concentration can be calculated as follows.

Equation 14-1 $[FFA] = (K_d.v) / (n-v)$ (Bojesen and Bojesen 1992)

Where: K_d is the equilibrium dissociation constant

v is the ratio of total FFA to serum albumin

n is the number of albumin binding sites

Literature values for the parameters in **Equation 14-1**.

Parameter	Value
Post absorptive FFA concentration	0.4 mM (Johnson, Stannard et al. 2006)
Starvation FFA concentration	1.2 mM (Johnson, Stannard et al. 2006)
Serum Albumin	40 $g.L^{-1}$ or 0.6 mM (Deepak A. Rao; Le)
K_d	34.42 nM (at 37 C with three albumin binding sites, Bojesen and Bojesen 1992)
n	3 (Bojesen and Bojesen 1992)

Using the listed values gives an unbound FFA concentration of approximately 15 $nmol.l^{-1}$ in the post absorptive state and 32 $nmol.l^{-1}$ during starvation. These concentrations correspond to about half maximal FFA uptake by muscle tissue in the post absorptive state and near maximal uptake during starvation (Bonen, Luiken et al. 1998; Turcotte, Swenberger et al. 2000).

14.2 Raw and false discovery rate corrected P values for comparisons made in the overfeeding study

	Raw P	Corrected	Raw P male only	Corrected male
AIRg TTest	8.73E-05	0.002	0.003	0.007
Insulin ANOVA	0.0001	0.003	0.000	0.002
Insulin AUC TTest	0.000278	0.005	0.004	0.011
Body mass TTest	0.000365	0.007	0.003	0.009
iSi vs dfBF% Pearson	0.000784	0.009	0.001	0.004
Lean tissue mass TTest	0.000793	0.010	0.004	0.014
Fat mass TTest	0.001729	0.013	0.017	0.034
iSi vs dLM/dBM Pearson	0.00947	0.024	0.034	0.056
FFA AUC TTest	0.010167	0.026	0.007	0.018
REExpend vs dLM/dBM Pearson	0.01300	0.030	0.014	0.029
dS_i vs dfBF% Pearson	0.014369	0.032	0.011	0.024
Plasma TAG TTest	0.015598	0.034	0.012	0.026
Plasma Cholesterol TTest	0.018557	0.039	0.036	0.059
Leptin0 TTest	0.030996	0.053	0.024	0.044
Body Fat % TTest	0.03189	0.055	0.081	0.107
Baseline energy balance TTest	0.03772	0.063	0.018	0.037
dS_i vs dLM/dBM Pearson	0.053054	0.080	0.078	0.103
REExpend vs dfBF% Pearson	0.117659	0.146	0.102	0.130
FFA0 TTest	0.132649	0.162	0.118	0.148
Glucose ANOVA	0.1541	0.185	0.530	0.569
Glucose AUC TTest	0.170308	0.203	0.219	0.250
Plasma HDL TTest	0.224909	0.259	0.767	0.811
Baseline mass TTest	0.288485	0.324	0.744	0.786
iSi vs dfBM Pearson	0.299436	0.337	0.360	0.394
dS_i vs dfBM Pearson	0.3105	0.350	0.383	0.421

FFA ANOVA	0.3515	0.392	0.582	0.623
Plasma insulin TTest	0.5022	0.544	0.365	0.401
Leptin vs. dLM/dBM Pearson	0.5287	0.572	0.932	0.981
Plasma glucose TTest	0.6137	0.659	0.893	0.940
Leptin vs dfBM Pearson	0.8737	0.921	0.270	0.303
REExpend vs dfBM Pearson	0.8802	0.929	0.944	0.994
Leptin vs dfBF% Pearson	0.9186	0.969	0.798	0.843

14.3 Skewness and kurtosis of variables used in correlation analyses

For n = 9, the expected standard error of kurtosis is 1.63 and the expected standard error for skewness is 0.82.

	Kurtosis	Skewness
iSi	-0.54	0.08
dBF%	1.21	1.10
dLM/dBM	-0.61	-0.18
dS_i	-0.06	-0.67
REE	-0.26	-0.35
dfBM	-1.15	-0.45
leptin	0.77	1.48

14.4 Raw data from high protein diet study

Table 14-1 Subject characteristics

ID	Body Mass kg	Age years	RMR $l.min^{-1}$	VO_{2Max} $l.min^{-1}$	Body Fat %
1	68.3	28	0.24	5.84	16
2	73.3	62	0.31	4.49	15
3	67.0	32	0.33	4.80	14
4	71.7	41	0.31	6.75	11
5	90.0	28	0.32	4.82	14
6	71.7	41	0.34	6.23	11
Mean	73.7	38.7	0.31	5.49	13.5
S.D.	8.3	12.9	0.04	0.92	2.1

Table 14-2 Dietary intake during mixed diet

ID	Energy $kJ.day^{-1}$	CHO $g.day^{-1}$	Fat $g.day^{-1}$	Protein $g.day^{-1}$
1	13828	416	149	120
2	12008	370	119	113
3	12567	393	117	119
4	12240	385	116	116
5	11520	359	120	104
6	11631	360	109	111
Mean	12299	380	122	114
S.D.	843	22	14	6

Table 14-3Dietary intake during high protein, low carbohydrate diet

ID	Energy kJ.day^{-1}	CHO g.day^{-1}	Fat g.day^{-1}	Protein g.day^{-1}
1	13819	19	562	122
2	12080	12	469	117
3	12587	18	508	113
4	12288	12	481	119
5	11506	12	449	112
6	11609	19	458	108
Mean	12315	15	488	115
S.D.	842	4	42	5

Table 14-4 Magnetic resonance spectroscopy (IMCL amplitude)/(water amplitude) after each dietary intervention. Dimensionless units.

ID	Mixed diet	Starvation	HPLC diet
1	0.024	0.033	0.012
2	0.009	0.017	0.020
3	0.012	0.024	0.014
4	0.012	0.023	0.011
5	0.016	0.031	0.014
6	0.008	0.025	0.011
Mean	0.014	0.026	0.014
S.D.	0.006	0.006	0.003

Table 14-5 Fasting plasma FFA concentration after each intervention (mM)

ID	Mixed diet	Starvation	HPLC diet
1	0.29	1.20	0.23
2	0.22	1.32	0.36
3	0.57	1.51	0.17
4	0.39	1.27	0.76
5	0.37	1.14	0.23
6	0.43	0.64	0.56
Mean	0.38	1.18	0.39
S.D.	0.12	0.29	0.23

Table 14-6 Fasting plasma glucose concentration after each intervention (mM)

ID	Mixed diet	Starvation	HPLC diet
1	4.13	3.65	3.53
2	4.65	3.43	4.23
3	4.20	3.73	4.83
4	4.23	3.83	4.00
5	4.13	3.80	3.95
6	4.10	3.90	3.90
Mean	4.24	3.72	4.07
S.D.	0.21	0.17	0.43

Table 14-7 Fasting plasma insulin concentration after each intervention (pM)

ID	Mixed diet	Starvation	HPLC diet
1	13.5	7.5	11.5
2	15	5	12
3	23	14.5	17.5
4	14	9.5	8
5	12	7.5	8
6	18.5	9.5	12.5
Mean	16.0	8.9	11.6
S.D.	4.1	3.2	3.5

Table 14-8 Minimal model insulin sensitivity after each intervention ($l.min^{-1}.\mu U^{-1}$)

ID	Mixed diet	Starvation	HPLC diet
1	11.64	5.96	17.50
2	14.75	4.24	16.74
3	24.28	7.14	11.45
4	10.26	7.68	10.85
5	12.12	3.98	8.84
6	25.66	5.21	21.35
Mean	16.45	5.70	14.45
S.D.	6.77	1.51	4.81

Table 14-9 Minimal model glucose effectiveness after each intervention (min^{-1})

ID	Mixed diet	Starvation	HPLC diet
1	0.0022	0.0029	0.0112
2	0.0241	0.0061	0.0101
3	0.0054	0.0045	0.0154
4	0.0073	0.0078	0.0156
5	0.0062	0.0079	0.0143
6	0.0029	0.0091	0.0128
Mean	0.0080	0.0064	0.0132
S.D.	0.0081	0.0023	0.0022

Table 14-10 Kg (insulin response to the IVGTT) after each intervention ($mmol.l^{-1}.min^{-1}$)

ID	Mixed diet	Starvation	HPLC diet
1	0.44	0.76	0.92
2	1.10	0.55	2.08
3	1.20	0.79	1.21
4	1.15	0.89	1.00
5	1.49	1.00	2.03
6	1.31	0.81	1.02
Mean	1.11	0.80	1.38
S.D.	0.36	0.15	0.53

Table 14-11 Glucose AUC after each intervention (mmol.l^{-1}.min^{-1})

ID	Mixed diet	Starvation	HPLC diet
1	368	584	364
2	169	435	228
3	252	535	359
4	202	412	242
5	187	554	336
6	269	441	384
Mean	241	493	319
S.D.	73	73	67

Table 14-12 Insulin AUC after each intervention (pmol.l^{-1}.min^{-1})

ID	Mixed diet	Starvation	HPLC diet
1	5185	4406	5080
2	3734	2899	2733
3	5108	5552	3618
4	4281	4840	3963
5	4881	4398	4110
6	2947	2661	2856
Mean	4356	4126	3726
S.D.	884	1127	871

Table 14-13 Plasma glucose and insulin concentrations throughout the IVGTT

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	HPLC diet	Starvation	Mixed diet	HPLC diet	Starvation
1	0	4.5	3.35	3.8	13	5	16
1	3	8.35	11.15	5.35	41	100	13
1	4	8.25	11.5	7.5	105	137	17
1	5	9.5	11.55	8.15	102	141	75
1	6	10.3	11.4	9.65	114	131	91
1	8	9.85	10.95	10.1	97	108	82
1	10	9.7	10.95	9.95	86	100	71
1	14	9.95	10.35	9.35	67	89	53
1	19	9.85	10	9.45	59	69	50
1	22	9.6	9.85	8.9	65	62	52
1	25	9.75	9.4	8.9	62	55	50
1	30	9.1	9.25	8.2	57	46	50
1	40	8.65	8.65	7.5	61	37	53
1	60	7.15	7.75	6.4	65	35	58
1	70	6.5	7.4	6.25	62	32	57
1	80	5.85	7.05	5.55	53	32	46
1	100	5.1	6.35	4.5	38	26	31
1	140	3.7	5	3.35	16	18	13
1	180	3.75	3.95	3.25	14	10	7
2	0	4.75	3.15	4.2	17	5	11
2	3	18.25	8.95	17.65	403	86	277
2	4	15.45	8.4	12.75	380	94	260
2	5	13.65	8.4	12.75	310	101	265
2	6	12.85	8.55	11.85	248	100	43

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	HPLC diet	Starvation	Mixed diet	HPLC diet	Starvation
2	8	12.25	8.75	10.65	164	91	166
2	10	11.5	8.15	10.65	129	60	122
2	14	10	8.4	10.2	97	55	89
2	19	8.5	8.1	8.8	71	46	68
2	22	7.6	7.9	8.25	58	38	58
2	25	7.1	7.55	7.85	46	33.5	41
2	30	6.1	7.5	6.8	37	29	14
2	40	4.9	7.1	5.9	21	24	16
2	60	4.65	6.45	4.95	11	22	20
2	70	4.7	6.35	4.6	11	19	16
2	80	4.7	5.95	4.5	12	17	14
2	100	4.55	5.5	4.4	13	14	14
2	140	4.6	4.55	4.15	16	9	11
2	180	4.55	3.7	4.25	13	5	13
3	0	4.8	3.5	5	35	18	18
3	3	10	14.45	20.3	83	181	106
3	4	10.6	13.35	17.4	126	194	119
3	5	12.7	12.6	16.1	119	168	104
3	6	10.4	12.15	15.2	112	141	86
3	8	10.6	11.7	14.1	107	118	77
3	10	10.45	11.4	13.15	78	96	60
3	14	10.25	10.1	11.95	71	94	47
3	19	9.8	9.95	10.9	71	73	43
3	22	9.7	9.05	10.6	76	62	60
3	25	9.4	8.95	10.45	91	68	49

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	HPLC diet	Starvation	Mixed diet	HPLC diet	Starvation
3	30	8.6	9.3	10.15	93	61	69
3	40	7.3	8.7	8.85	73	72	67
3	60	5.2	7.8	6.85	53	54	58
3	70	4.5	7.1	5.9	41	37	61
3	80	3.95	6.9	5.3	23	45	26
3	100	3.7	5.7	4.75	19	26	20
3	140	4	4.5	4.65	17	12	14
3	180	3.6	3.95	4.65	11	11	17
4	0	4.1	4	4.25	13	10	8
4	3	7.9	8.75	6.1	94	124	16
4	4	8.35	9.6	8.25	213	230	104
4	5	8.75	9.85	9	281	279	146
4	6	8.75	10.1	9.55	257	277	158
4	8	9.15	10.4	9.8	218	229	132
4	10	8.9	10.3	9.3	173	168	94
4	14	8.7	10	9	100	93	56
4	19	8.25	9.5	8.2	76	64	43
4	22	7.7	9.6	7.7	55	49	39
4	25	7.6	9.25	7.35	46	46	39
4	30	7.25	9.05	7.9	46	32	45
4	40	6.35	7.75	6.8	46	29	41
4	60	5.45	6.85	5.85	31	29	38
4	70	4.95	6.55	5.5	27	25	37
4	80	4.65	6.1	4.5	20	25	31
4	100	4.1	5.25	4.05	14	17	22

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	HPLC diet	Starvation	Mixed diet	HPLC diet	Starvation
4	140	4	4.05	3.6	7	6	11
4	180	4.15	3.6	3.65	11	5	8
5	0	4.25	3.5	4	23	5	13
5	3	4.7	10.25	10.5	13	82	68
5	4	6.35	11.6	12.4	62	205	191
5	5	6.95	11.5	11.9	100	195	254
5	6	7.65	11.55	11.8	166	190	248
5	8	8.85	11.3	12.05	198	155	197
5	10	9.1	11.2	12.3	180	120	149
5	14	9.45	10.95	11.15	132	64	104
5	19	8.65	9.95	10.25	97	87	82
5	22	8.15	9.45	9.4	88	64	72
5	25	7.7	9.4	8.7	77	62	58
5	30	7.05	8.85	7.95	67	49	43
5	40	6.1	8.45	6.75	55	32	35
5	60	4.85	7.7	5.75	44	29	30
5	70	4.7	7.55	5.35	34	41	28
5	80	4.75	7.1	5	38	37	27
5	100	4.25	6.45	4.8	24	29	18
5	140	3.7	5.2	3.9	13	23	10
5	180	3.95	4.3	3.8	14	14	12
6	0	4.45	3.75	4.1	18	8	10
6	3	8.05	8.9	7.2	16	73	18
6	4	13.1	9.6	8.8	142	90	41
6	5	11.65	10.05	9.85	162	100	61

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	HPLC diet	Starvation	Mixed diet	HPLC diet	Starvation
6	6	11.5	10.45	9.8	157	107	71
6	8	11.4	10.6	10.85	115	89	61
6	10	10.8	10.15	10.7	74	66	52
6	14	9.85	10.2	10.5	62	56	43
6	19	9.5	9.35	9.95	50	46	35
6	22	9.4	9.05	9.8	47	43	40
6	25	8.85	8.95	9.45	45	36	33
6	30	8.3	8.75	8.55	53	31	29
6	40	7.15	8.05	8.05	41	23	30
6	60	6	7.1	6.65	32	35	34
6	70	5.3	6.75	6.45	25	32	14
6	80	4.8	6.1	5.8	16	31	23
6	100	4.1	5.55	5.5	11	14	17
6	140	4	4.4	4.05	6	13	13
6	180	4	3.9	3.9	10	11	6

Table 14-14Plasma FFA concentrations throughout the IVGTT

ID	Sample min	Concentration mM		
		Mixed diet	Starvation	HPLC diet
1	0	0.29	1.20	0.23
1	15	0.27	1.07	0.19
1	30	0.14	0.72	0.07
1	60	0.06	0.50	0.03
1	120	0.07	1.16	0.05
2	0	0.22	1.32	0.36
2	15	0.18	1.11	0.32
2	30	0.12	0.61	0.10
2	60	0.11	0.48	0.05
2	120	0.22	0.67	0.27
3	0	0.57	1.51	0.17
3	15	0.43	1.04	0.10
3	30	0.19	0.72	0.09
3	60	0.07	0.53	0.09
3	120	0.25	0.64	0.70
4	0	0.37	1.14	0.23
4	15	0.16	0.79	0.29
4	30	0.06	0.68	0.13
4	60	0.03	0.32	0.08
4	120	0.23	0.71	0.08
5	0	0.43	0.64	0.56
5	15	0.30	0.80	0.39
5	30	0.12	0.38	0.11
5	60	0.08	0.20	0.03
5	120	0.06	0.37	0.17

ID	Sample min	Concentration mM		
		Mixed diet	Starvation	HPLC diet
6	0	0.39	1.27	0.76
6	15	0.11	1.02	0.63
6	30	0.07	0.65	0.27
6	60	0.02	0.70	0.18
6	120	0.12	1.06	0.24

14.5 Raw data from exercise during starvation study

Table 14-15 Subject characteristics

ID	Body Mass kg	Age years	RMR $l.min^{-1}$	VO_{2Max} $l.min^{-1}$	Body Fat %
1	68.3	28	0.24	5.84	16
2	73.3	62	0.31	4.49	15
3	67.0	32	0.33	4.80	14
4	71.7	41	0.31	6.75	11
5	90.0	28	0.32	4.82	14
6	71.7	41	0.34	6.23	11
Mean	73.7	38.7	0.31	5.49	13.5
S.D.	8.3	12.9	0.04	0.92	2.1

Table 14-16 Dietary intake during mixed diet

ID	Energy $kJ.day^{-1}$	CHO $g.day^{-1}$	Fat $g.day^{-1}$	Protein $g.day^{-1}$
1	13828	416	149	120
2	12008	370	119	113
3	12567	393	117	119
4	12240	385	116	116
5	11520	359	120	104
6	11631	360	109	111
Mean	12299	380	122	114
S.D.	843	22	14	6

Table 14-17 IMCL amplitude/water amplitude after each dietary intervention. Dimensionless units.

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	0.024	0.033	0.019
2	0.009	0.017	0.015
3	0.012	0.024	0.018
4	0.012	0.023	0.017
5	0.016	0.031	0.029
6	0.008	0.025	0.012
Mean	0.014	0.026	0.018
S.D.	0.006	0.006	0.006

Table 14-18 Fasting plasma FFA concentration after each intervention (mM)

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	0.29	1.20	1.09
2	0.22	1.32	1.24
3	0.57	1.51	1.39
4	0.39	1.27	1.42
5	0.37	1.14	1.15
6	0.43	0.64	0.83
Mean	0.38	1.18	1.19
S.D.	0.12	0.29	0.22

Table 14-19 Fasting plasma glucose concentration after each intervention (mM)

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	4.13	3.65	3.15
2	4.65	3.43	3.70
3	4.20	3.73	3.95
4	4.23	3.83	4.15
5	4.13	3.80	3.85
6	4.10	3.90	3.68
Mean	4.24	3.72	3.75
S.D.	0.21	0.17	0.34

Table 14-20 Fasting plasma insulin concentration after each intervention (pM)

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	13.5	7.5	10.5
2	15	5	5
3	23	14.5	15.5
4	14	9.5	7
5	12	7.5	12
6	18.5	9.5	12
Mean	16.0	8.9	10.3
S.D.	4.1	3.2	3.8

Table 14-21 Minimal model insulin sensitivity after each intervention ($l.min^{-1}.\mu U^{-1}$)

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	11.64	5.96	6.70
2	14.75	4.24	5.96
3	24.28	7.14	4.36
4	10.26	7.68	7.71
5	12.12	3.98	7.76
6	25.66	5.21	3.71
Mean	16.45	5.70	6.04
S.D.	6.77	1.51	1.70

Table 14-22 Minimal model glucose effectiveness after each intervention (min^{-1})

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	0.0022	0.0029	0.0055
2	0.0241	0.0061	0.0076
3	0.0054	0.0045	0.0082
4	0.0073	0.0078	0.0104
5	0.0062	0.0079	0.0071
6	0.0029	0.0091	0.0139
Mean	0.0080	0.0064	0.0088
S.D.	0.0081	0.0023	0.0030

Table 14-23 Glucose AUC after each intervention (mmol.l^{-1}.min^{-1})

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	368	584	498
2	169	435	447
3	252	535	535
4	202	412	429
5	187	554	411
6	269	441	334
Mean	241	493	442
S.D.	73	73	70

Table 14-24 Insulin AUC after each intervention (pmol.l^{-1}.min^{-1})

ID	Mixed diet	Starvation inactivity	Starvation exercise
1	5185	4406	3041
2	3734	2899	2635
3	5108	5552	6371
4	4281	4840	3980
5	4881	4398	4258
6	2947	2661	1163
Mean	4356	4126	3574
S.D.	884	1127	1757

Table 14-25 Plasma glucose and insulin concentrations throughout the IVGTT

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	Starvation inactivity	Starvation exercise	Mixed diet	Starvation inactivity	Starvation exercise
1	0	4.5	3.35	2.9	13	5	8
1	3	8.35	11.15	7.6	41	100	38
1	4	8.25	11.5	8.5	105	137	85
1	5	9.5	11.55	9.4	102	141	111
1	6	10.3	11.4	9.5	114	131	112
1	8	9.85	10.95	10.35	97	108	106
1	10	9.7	10.95	9.4	86	100	89
1	14	9.95	10.35	9.2	67	89	82
1	19	9.85	10	8.85	59	69	66
1	22	9.6	9.85	8.8	65	62	62
1	25	9.75	9.4	8.65	62	55	53
1	30	9.1	9.25	8.45	57	46	43
1	40	8.65	8.65	7.75	61	37	35
1	60	7.15	7.75	6.35	65	35	28
1	70	6.5	7.4	6.5	62	32	29
1	80	5.85	7.05	6.05	53	32	26
1	100	5.1	6.35	5.4	38	26	23
1	140	3.7	5	4.15	16	18	13
1	180	3.75	3.95	3.4	14	10	13
2	0	4.75	3.15	2.8	17	5	5
2	3	18.25	8.95	7	403	86	100
2	4	15.45	8.4	10.9	380	94	120
2	5	13.65	8.4	10.4	310	101	110
2	6	12.85	8.55	10	248	100	100
2	8	12.25	8.75	9.6	164	91	74

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	Starvation inactivity	Starvation exercise	Mixed diet	Starvation inactivity	Starvation exercise
2	10	11.5	8.15	9.4	129	60	67
2	14	10	8.4	8.7	97	55	54
2	19	8.5	8.1	8.5	71	46	44
2	22	7.6	7.9	8.3	58	38	34
2	25	7.1	7.55	8.1	46	33.5	30
2	30	6.1	7.5	7.9	37	29	25
2	40	4.9	7.1	7.4	21	24	19
2	60	4.65	6.45	6.8	11	22	17
2	70	4.7	6.35	6.6	11	19	13
2	80	4.7	5.95	6	12	17	15
2	100	4.55	5.5	5.4	13	14	14
2	140	4.6	4.55	4.8	16	9	9
2	180	4.55	3.7	4.6	13	5	5
3	0	4.8	3.5	3.6	35	18	23
3	3	10	14.45	13.3	83	181	153
3	4	10.6	13.35	12.9	126	194	167
3	5	12.7	12.6	12.3	119	168	150
3	6	10.4	12.15	12.45	112	141	134
3	8	10.6	11.7	12.1	107	118	111
3	10	10.45	11.4	11.6	78	96	122
3	14	10.25	10.1	10.8	71	94	118
3	19	9.8	9.95	10.35	71	73	83
3	22	9.7	9.05	10.1	76	62	76
3	25	9.4	8.95	9.8	91	68	67
3	30	8.6	9.3	9.35	93	61	66
3	40	7.3	8.7	8.9	73	72	62

ID	Sample	Glucose			Insulin		
	min	mM			pM		
		Mixed diet	Starvation inactivity	Starvation exercise	Mixed diet	Starvation inactivity	Starvation exercise
3	60	5.2	7.8	7.7	53	54	56
3	70	4.5	7.1	7.2	41	37	45
3	80	3.95	6.9	6.75	23	45	48
3	100	3.7	5.7	5.9	19	26	31
3	140	4	4.5	4.9	17	12	12
3	180	3.6	3.95	4.3	11	11	8
4	0	4.1	4	3.6	13	10	11
4	3	7.9	8.75	9.9	94	124	125
4	4	8.35	9.6	9.65	213	230	222
4	5	8.75	9.85	10.7	281	279	286
4	6	8.75	10.1	10.6	257	277	308
4	8	9.15	10.4	10.35	218	229	254
4	10	8.9	10.3	10.55	173	168	184
4	14	8.7	10	10.1	100	93	118
4	19	8.25	9.5	9.45	76	64	92
4	22	7.7	9.6	9.5	55	49	61
4	25	7.6	9.25	9.1	46	46	50
4	30	7.25	9.05	8.4	46	32	36
4	40	6.35	7.75	7.85	46	29	26
4	60	5.45	6.85	6.9	31	29	23
4	70	4.95	6.55	6.5	27	25	19
4	80	4.65	6.1	6.1	20	25	19
4	100	4.1	5.25	5.45	14	17	18
4	140	4	4.05	4.35	7	6	6
4	180	4.15	3.6	4.1	11	5	13
5	0	4.25	3.5	3.55	23	5	11

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	Starvation inactivity	Starvation exercise	Mixed diet	Starvation inactivity	Starvation exercise
5	3	4.7	10.25	5.9	13	82	27
5	4	6.35	11.6	7.5	62	205	96
5	5	6.95	11.5	8.4	100	195	163
5	6	7.65	11.55	9.45	166	190	188
5	8	8.85	11.3	10.25	198	155	171
5	10	9.1	11.2	10.4	180	120	146
5	14	9.45	10.95	10.1	132	64	112
5	19	8.65	9.95	9.3	97	87	86
5	22	8.15	9.45	9.25	88	64	73
5	25	7.7	9.4	8.85	77	62	61
5	30	7.05	8.85	8	67	49	46
5	40	6.1	8.45	7.45	55	32	32
5	60	4.85	7.7	6.4	44	29	34
5	70	4.7	7.55	6.15	34	41	30
5	80	4.75	7.1	5.9	38	37	30
5	100	4.25	6.45	5.2	24	29	25
5	140	3.7	5.2	4.45	13	23	20
5	180	3.95	4.3	3.8	14	14	13
6	0	4.45	3.75	4.2	18	8	6
6	3	8.05	8.9	7	16	73	17
6	4	13.1	9.6	8.8	142	90	41
6	5	11.65	10.05	9.3	162	100	52
6	6	11.5	10.45	9.4	157	107	56
6	8	11.4	10.6	9.4	115	89	47
6	10	10.8	10.15	9.4	74	66	42
6	14	9.85	10.2	9.6	62	56	28

ID	Sample min	Glucose mM			Insulin pM		
		Mixed diet	Starvation inactivity	Starvation exercise	Mixed diet	Starvation inactivity	Starvation exercise
6	19	9.5	9.35	8.6	50	46	20
6	22	9.4	9.05	8.9	47	43	19
6	25	8.85	8.95	9.2	45	36	16
6	30	8.3	8.75	8.1	53	31	16
6	40	7.15	8.05	8	41	23	17
6	60	6	7.1	7	32	35	17
6	70	5.3	6.75	6.3	25	32	13
6	80	4.8	6.1	5.8	16	31	15
6	100	4.1	5.55	5	11	14	11
6	140	4	4.4	4.3	6	13	9
6	180	4	3.9	4.1	10	11	8

Table 14-26 Plasma FFA concentrations throughout the IVGTT

ID	Sample min	Concentration mM		
		Mixed diet	Starvation inactivity	Starvation exercise
1	0	0.29	1.20	1.09
1	15	0.27	1.07	0.76
1	30	0.14	0.72	0.45
1	60	0.06	0.50	0.39
1	120	0.07	1.16	0.82
2	0	0.22	1.32	1.24
2	15	0.18	1.11	1.04
2	30	0.12	0.61	0.58
2	60	0.11	0.48	0.46
2	120	0.22	0.67	0.64
3	0	0.57	1.51	1.39
3	15	0.43	1.04	1.01
3	30	0.19	0.72	0.64
3	60	0.07	0.53	0.52
3	120	0.25	0.64	0.61
4	0	0.37	1.14	1.15
4	15	0.16	0.79	0.82
4	30	0.06	0.68	0.53
4	60	0.03	0.32	0.49
4	120	0.23	0.71	0.99
5	0	0.43	0.64	0.83
5	15	0.30	0.80	0.79
5	30	0.12	0.38	0.46
5	60	0.08	0.20	0.34
5	120	0.06	0.37	0.55

ID	Sample min	Concentration mM		
		Mixed diet	Starvation inactivity	Starvation exercise
6	0	0.39	1.27	1.42
6	15	0.11	1.02	0.98
6	30	0.07	0.65	0.64
6	60	0.02	0.70	0.64
6	120	0.12	1.06	0.96

14.6 Raw data from overfeeding study

Table 14-27 Subject characteristics

ID	Gender	Age	Height	VO_{2Max}	RMR
		years	m	$ml.kg^{-1}.min^{-1}$	$kJ.day^{-1}$
1	M	29	1.69	74.4	5868
2	M	22	1.73	60.8	12201
3	M	24	1.80	72.9	7869
4	F	23	1.78	53.2	6898
5	M	23	2.00	61.4	12229
6	M	29	1.81	72.0	9136
7	F	25	1.65	53.6	5573
8	M	44	1.75	71.2	6477
9	M	22	1.77	71.2	9416
Mean		26.8	1.78	65.6	8407
S.D.		7.0	0.10	8.5	2533

Table 14-28 Diet and energy expenditure

ID	Energy intake kJ.day^{-1}	Overfeeding extra energy kJ.day^{-1}	Diet Fat %	CHO %	Pro %	Alc %	Energy expenditure kJ.day^{-1}
1	7141	5742	34	49	17	1	10682
2	11525	7650	33	45	18	3	15398
3	12026	6138	35	45	15	3	13704
4	10805	6048	38	47	15	0	9889
5	12814	9306	28	53	15	4	18406
6	15013	6192	26	59	15	0	14237
7	8708	4572	34	52	14	0	8473
8	8641	5931	28	49	19	4	9622
9	10436	6714	32	40	21	7	15929
Mean	10790	6477	32.0	48.8	16.6	2.4	12927
S.D.	2408	1336	3.9	5.5	2.4	2.4	3400

Table 14-29 Body composition and body mass before and after overfeeding

ID	Mass kg	BMI $kg.m^{-2}$	DXA BF %	Fat mass kg	Lean mass kg
			Pre		
1	63.8	22	11.7	7.5	56.3
2	85.0	28	15.2	12.9	72.1
3	68.2	21	12.7	8.7	59.5
4	67.2	21	25.5	17.1	50.1
5	103.4	26	14.8	15.3	88.1
6	68.8	21	12.0	8.3	60.5
7	50.8	19	20.8	10.6	40.2
8	65.9	22	13.7	9.0	56.9
9	74.6	24	12.4	9.3	65.3
Mean	72.0	22.6	15.4	11.0	61.0
S.D.	14.8	2.9	4.7	3.4	13.5
			Post		
1	65.0	23	12.2	7.9	57.1
2	90.6	30	16.1	14.6	76.0
3	69.6	21	12.6	8.8	60.8
4	70.8	22	25.7	18.2	52.6
5	109.4	27	14.9	16.3	93.1
6	73.8	23	13.1	9.7	64.1
7	53.2	20	21.8	11.6	41.6
8	69.6	23	15.9	11.1	58.5
9	77.0	25	12.3	9.5	67.5
Mean	75.4	23.7	16.1	12.0	63.5
S.D.	16.1	3.3	4.7	3.6	14.7

Table 14-30 Fasting blood metabolites before and after overfeeding

ID	Insulin	Glucose	Leptin	FFA	Cholesterol	HDL	TAG
	pM	mM	$ng.ml^{-1}$	mM	mM	mM	mM
				Pre			
1	53.8	5.0	1.1	0.47	3.55	0.64	0.56
2	71.5	4.6	1.7	0.27	2.99	0.51	0.43
3	76.0	4.6	1.3	0.39	3.39	0.56	0.69
4	52.1	4.3	7.1	0.66	3.23	0.85	0.48
5	51.2	4.3	2.8	0.70	3.67	0.68	0.54
6	94.3	4.8	1.4	0.33	3.79	0.61	1.00
7	54.5	4.4	5.9	0.24	4.58	0.60	0.69
8	70.1	5.2	2.1	0.35	4.80	0.74	0.75
9	72.8	4.9	1.5	0.53	4.20	0.69	0.89
Mean	66.3	4.7	2.8	0.44	3.80	0.65	0.67
S.D.	14.5	0.3	2.2	0.16	0.61	0.10	0.19
				Post			
1	54.3	5.0	1.5	0.68	4.68	0.60	1.14
2	96.9	4.7	4.2	0.13	3.09	0.61	0.47
3	84.7	4.9	1.2	0.20	3.88	0.60	0.85
4	37.4	4.8	15.0	0.33	4.12	1.13	0.60
5	72.6	4.5	3.7	0.28	4.34	0.74	0.81
6	98.3	4.6	2.8	0.29	4.22	0.64	1.39
7	61.5	4.4	9.2	0.43	4.51	0.70	0.62
8	66.3	4.9	4.4	0.25	6.08	0.70	1.51
9	55.0	4.9	2.0	0.27	3.94	0.59	1.07
Mean	69.7	4.7	4.9	0.32	4.32	0.70	0.94
S.D.	20.5	0.2	4.5	0.16	0.80	0.17	0.36

Table 14-31 Minimal model and AUC data from the IVGTT before and after overfeeding

ID	S_i	S_g	AIRg	Glucose AUC	Insulin AUC	FFA AUC
	$l.min^{-1}.mU^{-1}$	min^{-1}		mM.min	pM.min	μM min
			Pre			
1	15.7	0.061	269	130	3503	-20.5
2	15.7	0.024	345	309	4216	-13.2
3	8.6	0.021	186	224	6400	-4.2
4	12.8	0.020	253	98	4061	-38.4
5	5.2	0.017	147	328	6694	-55.2
6	21.5	0.022	208	215	2524	-12.3
7	16.5	0.008	169	137	2900	-5.3
8	23.8	0.047	252	152	3388	-18.9
9	10.4	0.020	308	151	6079	-30.5
Mean	14.5	0.027	237	194	4418	-22.1
S.D.	5.9	0.016	66	81	1574	16.7
			Post			
1	10.7	0.047	574	125	6599	-44.9
2	17.2	0.029	425	136	5135	-5.5
3	13.9	0.020	287	210	7140	2.3
4	9.1	0.027	560	171	8308	-18.0
5	6.1	0.028	404	197	10413	-18.7
6	6.8	0.020	468	187	7494	-17.6
7	11.2	0.025	278	114	4603	-21.1
8	5.6	0.022	362	168	6252	-10.5
9	5.3	0.047	669	197	11286	-6.3
Mean	9.5	0.029	448	167	7470	-15.6
S.D.	4.1	0.011	133	35	2235	13.4

Table 14-32 Plasma glucose and insulin concentrations during the IVGTT before and after overfeeding

ID	Time	Insulin		Glucose	
	min	pM		mM	
		Pre	Post	Pre	Post
1	0	54	54	5.0	5.0
1	3	127	312	10.5	13.6
1	4	296	572	12.8	12.8
1	5	253	554	12.3	11.8
1	6	307	431	11.5	11.7
1	8	225	455	11.4	11.5
1	10	185	357	10.4	11.0
1	14	145	147	14.3	9.2
1	19	125	148	7.3	7.9
1	22	99	144	7.0	7.1
1	25	102	107	6.2	6.8
1	30	77	102	5.6	6.2
1	40	78	88	4.9	5.0
1	60	62	45	4.3	4.0
1	70	55	59	4.2	4.6
1	80	65	59	4.5	4.1
1	100	40	61	4.6	4.3
1	140	39	68	4.5	4.5
1	180	50	48	4.6	4.6
2	0	72	97	4.6	4.7
2	3	119	355	16.0	14.1
2	4	126	540	13.3	13.5
2	5	111	420	14.2	13.1
2	6	98	460	14.6	12.5
2	8	97	363	13.5	4.8

ID	Time min	Insulin pM		Glucose mM	
		Pre	Post	Pre	Post
2	10	58	244	13.3	11.5
2	14	87	256	11.9	10.1
2	19	151	177	11.2	8.6
2	22	175	142	11.2	7.6
2	25	251	151	10.6	7.0
2	30	168	152	8.7	6.5
2	40	140	106	8.7	4.3
2	60	109	123	5.5	4.1
2	70	80	80	4.9	3.5
2	80	80	106	4.2	4.2
2	100	59	76	3.8	3.3
2	140	70	66	3.4	4.0
2	180	75	81	3.9	4.4
3	0	76	85	4.6	4.9
3	3	120	125	11.3	11.7
3	4	242	273	12.8	15.1
3	5	267	345	12.2	14.0
3	6	213	347	11.6	13.7
3	8	194	271	11.0	12.3
3	10	169	246	11.4	12.1
3	14	157	210	9.8	11.0
3	19	153	195	9.6	10.5
3	22	122	176	9.3	10.0
3	25	147	176	9.1	9.3
3	30	163	201	8.5	8.6
3	40	169	156	7.2	6.3
3	60	101	115	5.4	4.0

ID	Time min	Insulin pM		Glucose mM	
		Pre	Post	Pre	Post
3	70	99	116	4.2	3.3
3	80	81	97	3.9	3.2
3	100	84	85	4.4	3.5
3	140	101	102	4.3	3.9
3	180	85	90	4.6	4.2
4	0	52	37	4.3	4.8
4	3	207	757	9.8	15.6
4	4	266	680	8.6	15.5
4	5	337	432	8.5	14.1
4	6	232	355	8.5	14.0
4	8	178	308	8.4	13.0
4	10	166	233	8.1	12.4
4	14	165	235	7.8	11.0
4	19	179	148	7.0	8.8
4	22	135	153	6.4	8.9
4	25	124	102	7.4	7.8
4	30	91	82	5.5	6.1
4	40	94	138	4.1	4.9
4	60	38	39	3.5	4.1
4	70	42	58	3.7	4.0
4	80	57	33	3.7	3.8
4	100	26	16	4.1	4.1
4	140	53	43	4.1	4.1
4	180	37	42	3.9	4.1
5	0	51	73	4.3	4.5
5	3	146	153	8.0	8.1
5	4	164	357	9.9	12.2

ID	Time min	Insulin pM		Glucose mM	
		Pre	Post	Pre	Post
5	5	171	618	10.7	13.0
5	6	154	423	11.1	8.1
5	8	134	309	11.4	12.6
5	10	131	249	11.0	10.8
5	14	118	223	9.6	9.9
5	19	103	200	9.9	9.7
5	22	114	164	10.0	9.2
5	25	108	164	9.3	8.6
5	30	116	161	9.4	7.7
5	40	114	202	8.8	6.6
5	60	114	97	7.0	4.7
5	70	116	80	6.1	3.9
5	80	115	130	5.7	3.8
5	100	70	83	4.3	4.0
5	140	56	105	4.1	4.3
5	180	54	100	4.0	4.5
6	0	94	98	4.8	4.6
6	3	191	624	15.6	13.1
6	4	288	505	14.2	16.5
6	5	269	565	13.0	12.1
6	6	293	404	12.5	11.8
6	8	191	317	12.2	10.8
6	10	205	290	11.7	10.1
6	14	125	173	10.5	9.8
6	19	144	210	9.4	8.0
6	22	130	225	8.4	7.5
6	25	115	181	8.7	8.5

ID	Time min	Insulin pM		Glucose mM	
		Pre	Post	Pre	Post
6	30	119	213	8.1	7.3
6	40	113	183	6.8	6.3
6	60	105	114	5.1	4.7
6	70	92	116	4.7	4.1
6	80	86	103	4.2	4.1
6	100	67	94	4.0	3.9
6	140	74	100	4.2	4.1
6	180	73	64	4.4	4.1
7	0	55	62	4.4	4.4
7	3	57	282	5.7	8.0
7	4	93	288	6.4	9.3
7	5	172	383	7.7	9.3
7	6	214	261	8.4	9.4
7	8	200	245	8.0	9.6
7	10	169	139	8.4	8.8
7	14	136	144	8.0	7.9
7	19	111	118	7.9	7.4
7	22	105	93	7.6	7.1
7	25	127	140	7.4	6.7
7	30	78	97	7.1	6.1
7	40	84	108	6.0	5.1
7	60	67	66	5.1	4.2
7	70	56	83	4.5	3.9
7	80	53	80	4.0	3.5
7	100	56	59	3.7	4.0
7	140	46	45	3.7	4.0
7	180	41	81	4.0	4.0

ID	Time	Insulin		Glucose	
	min	pM		mM	
		Pre	Post	Pre	Post
8	0	70	66	5.2	4.9
8	3	70	83	8.6	6.5
8	4	152	281	12.2	9.5
8	5	314	439	12.9	10.7
8	6	392	453	13.9	10.8
8	8	221	325	11.6	9.8
8	10	214	231	11.7	10.0
8	14	154	191	10.0	10.0
8	19	125	157	8.7	8.6
8	22	146	143	8.4	8.3
8	25	109	154	7.8	8.1
8	30	98	121	6.9	7.7
8	40	115	114	6.3	7.1
8	60	68	98	5.1	5.3
8	70	72	103	4.8	4.9
8	80	59	77	4.5	4.6
8	100	65	66	4.6	4.9
8	140	58	72	4.6	4.9
8	180	57	77	4.6	4.9
9	0	73	55	4.9	4.9
9	3	83	706	7.1	20.6
9	4	209	937	9.1	17.0
9	5	293	692	9.7	15.5
9	6	352	500	10.0	14.0
9	8	326	318	10.5	12.4
9	10	274	283	11.2	12.3
9	14	185	217	9.6	11.0

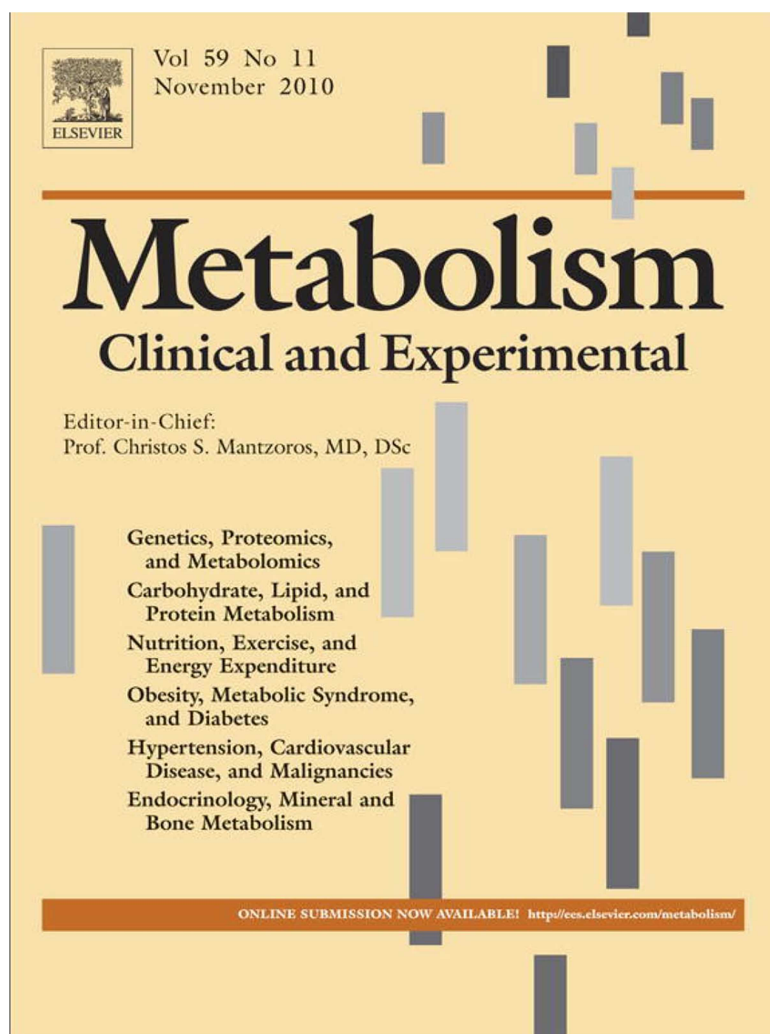
ID	Time min	Insulin pM		Glucose mM	
		Pre	Post	Pre	Post
9	19	170	190	9.8	9.3
9	22	168	136	9.3	8.2
9	25	174	137	8.8	7.8
9	30	158	151	7.7	6.9
9	40	154	109	5.9	5.6
9	60	100	82	4.2	4.4
9	70	95	76	3.9	4.3
9	80	78	85	3.8	4.3
9	100	70	78	4.2	4.8
9	140	63	85	4.6	4.8
9	180	80	68	4.6	4.7

Table 14-33 Plasma FFA concentrations throughout the IVGTT before and after overfeeding

ID	Sample	FFA (mM)	
		Pre	Post
1	0	0.47	0.68
1	15	0.49	0.21
1	30	0.14	0.10
1	60	0.12	0.11
1	120	0.54	0.70
2	0	0.27	0.13
2	15	0.27	0.10
2	30	0.20	0.06
2	60	0.07	0.06
2	120	0.19	0.12
3	0	0.39	0.20
3	15	0.60	0.18
3	30	0.16	0.11
3	60	0.12	0.15
3	120	0.71	0.43
4	0	0.66	0.33
4	15	0.58	0.19
4	30	0.32	0.13
4	60	0.18	0.11
4	120	0.40	0.29
5	0	0.70	0.28
5	15	0.24	0.21
5	30	0.34	0.11
5	60	0.14	0.08
5	120	0.18	0.11
6	0	0.33	0.29
6	15	0.32	0.21

ID	Sample	FFA (mM)	
		Pre	Post
6	30	0.16	0.11
6	60	0.11	0.11
6	120	0.40	0.16
7	0	0.24	0.43
7	15	0.37	0.30
7	30	0.21	0.18
7	60	0.15	0.11
7	120	0.17	0.47
8	0	0.35	0.25
8	15	0.25	0.28
8	30	0.17	0.13
8	60	0.13	0.08
8	120	0.25	0.22
9	0	0.53	0.27
9	15	0.31	0.13
9	30	0.19	0.12
9	60	0.13	0.15
9	120	0.49	0.43

14.7 Peer-reviewed article: Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated



Reprinted from Metabolism Clinical and Experimental, 59, Green, J.G., Johnson, N. A., Sachinwalla, T., Cunningham, C.W., Thompson, M. W., and Stannard, S. R., *Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated*, 1633-1641, Copyright (2010), with permission from Elsevier.

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Metabolism Clinical and Experimental 59 (2010) 1633 – 1641

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Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated

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Received 14 September 2009; accepted 19 March 2010

Abstract

It has been speculated that dietary carbohydrate restriction is solely responsibly for mobilization of endogenous lipid stores, elevation of plasma free fatty acid (FFA) concentration, and an associated reduction in insulin sensitivity seen in starvation and low-carbohydrate diets. In 6 healthy men, dietary carbohydrate was eliminated but gluconeogenic substrate supply was maintained by 3 days of very low-carbohydrate/high-protein (HPLC) diet. Results were compared with 3-day starvation and 3-day mixed-carbohydrate diet. Intramyocellular lipid (IMCL) concentration was measured by ^{1}H magnetic resonance spectroscopy, and insulin sensitivity was determined by intravenous glucose tolerance test. Fasting plasma glucose was significantly reduced ([starvation] 3.5 ± 0.3 vs [HPLC] 4.2 ± 0.4 and [mixed] 4.5 ± 0.3 mmol L^{-1}, $P < .01$), and IMCL to water ratio (25.6 ± 5.9 vs 13.6 ± 6.1 and 13.6 ± 3.3 × 10^{-3}, $P < .01$) and fasting FFA (1179 ± 294 vs 387 ± 232 and 378 ± 120 μmol L^{-1}, $P < .05$) were significantly elevated after starvation but were unchanged after HPLC. Minimal model insulin sensitivity was reduced after starvation (5.7 ± 1.5 vs 14.5 ± 4.8 and 16.5 ± 6.8 L min^{-1} mU^{-1}, $P < .05$). Plasma glucose, plasma FFAs, IMCLs, and insulin sensitivity are maintained when an HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. We conclude that dietary carbohydrate restriction does not cause circulating FFA to become elevated. However, it remains possible that circulating carbohydrate status has an important influence on plasma FFA and therefore insulin sensitivity in healthy people.
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1. Introduction

The pathologic association between excess adiposity and the development of insulin resistance is well established [1,2]. On the other hand, insulin resistance has also been shown to be a rapidly adaptive, short-term physiologic response in lean, physically fit humans. To[AU1] fully understand the pathophysiology linking obesity and insulin resistance, we must understand how "healthy" insulin resistance can develop in the lean, physically fit individual. In the latter group, "healthy" insulin resistance is coincident with changes in fat partitioning induced by high-fat diets and lipid heparin infusion [3], situations that result in elevated blood lipids, whereas in the former group, it can be improved with thiazolidinediones [4] and nicotinic acid [5], agents that reduce blood lipids. The common denominator in reduced insulin sensitivity in both groups appears to be accumulation of lipid or its precursors within skeletal muscle fiber [6,7] resulting from a mismatch between fatty acid uptake from the circulation and oxidation by muscle [8]. Although lean, endurance-trained individuals are very insulin sensitive but reportedly possess higher muscle lipid content, situations

Institutional approval: The study was approved by the local institutional human ethics committee and conformed to the Declaration of Helsinki.

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doi:10.1016/j.metabol.2010.03.013

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where the intramyocellular lipid (IMCL) store is increased also reduce insulin sensitivity within this group [6,9].

In vitro research has also demonstrated a causative relationship between elevated concentrations of lipids in the extracellular environment, increased accumulation of IMCL [10], impairment of insulin signaling [11], and reduced glucose uptake by the muscle fiber [10,12]. However, although insulin resistance may be caused by an excess of lipid at a cellular level, it is not exclusively a function of dietary fat intake.

A reduction in muscle and whole-body insulin sensitivity is also brought about by short-term (3-day) starvation [6,13,14]. Lean, physically fit men experience equivalent reduction in circulating carbohydrate, accumulation of IMCL, and reduction in insulin sensitivity when exposed to 2 different forms of dietary carbohydrate restriction: starvation or very low-carbohydrate/high-fat diet [6]. Thus, despite a vast difference in whole-body energy intake, at a cellular level, starvation and high-fat/low-carbohydrate diet both result in IMCL accumulation and insulin resistance. The common factors between starvation and low-carbohydrate/high-fat diet–induced insulin resistance in healthy individuals are reduced endogenous (circulating) carbohydrate concentration and increased circulating free fatty acid (FFA) concentration.

It has been speculated that, in lean, healthy individuals, dietary carbohydrate restriction independently mediates IMCL accumulation and insulin resistance [15]. However, previous research has not been able to minimize exogenous carbohydrate intake without also reducing circulating carbohydrate concentrations. As such, it is not clear whether the root cause of insulin resistance in lean, healthy individuals is endogenous or exogenous carbohydrate restriction.

High dietary protein intake provides an excess supply of gluconeogenic substrates and, in conjunction with minimal dietary carbohydrate (<5% of energy), causes an up-regulation in hepatic gluconeogenesis [16-20]. By ensuring high dietary protein intake, yet minimizing carbohydrate intake, we can increase the rate of hepatic gluconeogenic substrates and presumably support normal endogenous carbohydrate concentrations despite minimal exogenous supply. By uncoupling exogenous and endogenous carbohydrate supply, it is possible to determine the independent effects of each on IMCL accumulation and insulin sensitivity.

The aim of this study was to compare the effects of moderate-carbohydrate diet vs 2 forms of carbohydrate restriction, very low-carbohydrate/high-protein (HPLC) diet and starvation, on insulin sensitivity and IMCL. We exposed lean, physically fit men to 2- to 3-day dietary treatments that eliminated dietary carbohydrate but supplied differing amounts of gluconeogenic substrates, namely, starvation and HPLC diet, as well as approximately 3 days of standardized moderate-carbohydrate diet. We hypothesized that the carbohydrate restriction diets would induce equivalent reductions in levels of blood glucose with an associated increase in circulating FFA, increase in IMCL accumulation, and reduction in insulin sensitivity when compared with the moderate-carbohydrate diet.

2. Materials and methods

2.1. Participants

Six healthy, physically fit men (age = 38.8 ± 12.7 years, body mass = 72.9 ± 8.8 kg) volunteered for this study. All participants reported regularly undertaking exercise for more than 1.5 hours daily at least 5 days per week. Participants' physical characteristics are given in Table 1. Participants were informed of the study protocol and risks before providing their written consent. The study was approved by the local institutional human ethics committee and conformed to the Declaration of Helsinki.

2.2. Preliminary testing

One week before participation in the dietary intervention, submaximal and maximal oxygen uptake tests were performed on an electronically braked cycle ergometer (Lode ergometer, Groningen, the Netherlands) as previously described [21]. External power output and Vo_2 attained during the final minute of each submaximal workload and the maximal ramp were used to formulate regression equations from which workloads for the control exercise bout were derived. On a separate occasion, participants presented at the laboratory after a 12-hour overnight fast for measurement of resting metabolic rate (RMR) using respiratory gas analysis lying supine after 30-minute rest. Body density was assessed via hydrodensitometry; and percentage body fat was then calculated using a 2-compartment model, as previously described [22]. Underwater body weight measurements were corrected for measured residual lung volume using the methods of van der Ploeg et al [23].

2.3. Experimental protocol

All participants underwent 3 supervised dietary interventions in random order, each separated by at least 7 days. Each diet period was of 67 hours' duration and comprised a water-only starvation diet, an HPLC diet, or a mixed control diet. Dietary intake and physical activity before initiation of each diet were strictly controlled as previously described [6].

Table 1
Participants' physical characteristics

Body fat (%)	13.5 ± 2.1
RMR (mL O_2 min ^{1})	306 ± 35
Vo_{2max} (mL kg ^{1} min ^{1})	75.5 ± 16.0

Values are mean ± standard deviation. Vo_{2max} indicates maximal oxygen consumption.

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Upon initiation of the diet, participants ingested one of the following: a carbohydrate snack, a protein snack, or nothing, according to their allocation to the mixed diet, HPLC diet, or starvation, respectively. This meal provided 1 g carbohydrate per kilogram body weight (100% energy from carbohydrate) in the mixed diet, an isocaloric meal supplying 1% of energy from carbohydrate and 99% from protein in the HPLC diet, or water only in the starvation diet. Except for the starvation diet, participants ingested an evening meal 2 hours later. In the mixed diet, this meal contained 1.5 g carbohydrate per kilogram body weight and supplied 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein. In the HPLC diet, this was an isocaloric meal supplying 2% of energy from carbohydrate, 35% of energy from fat, and 63% of energy from protein. Beginning the following morning and continuing for the remainder of the dietary treatment (48 hours), participants received diets in both the mixed and HPLC treatments that provided energy to match a daily expenditure of 1.5× RMR to maintain energy balance. Diets were designed to deliver 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein in the mixed diet and negligible carbohydrate, 35% of energy from fat, and 65% of energy from protein in the HPLC diet. After exercise in the starvation treatment, participants continued a water-only diet until completion of the experimental treatment. Diet composition was quantified via Foodworks (Xyris Software, Melbourne, Australia) using the New Zealand–Standard database. In all dietary interventions, participants were instructed to maintain activities of daily living and avoid all forms of recreational exercise.

2.4. Determination of IMCL content

After 65 hours of each diet, vastus lateralis proton magnetic resonances (^{1}H magnetic resonance spectroscopy) were obtained as previously described [6].

Spectral data were postprocessed by magnetic resonance user interface software (jMRUI version 3.0, EU Project). Vastus lateralis IMCL content was determined by the ratio of the methylene (—$[CH_2]_n$) resonance from IMCL at 1.3 ppm and intracellular water [24]. A 10-resonance model was used to determine IMCL concentration as we have detailed previously [6]. Muscle water signal amplitudes were measured from the non–water-suppressed spectrum using Hankel Lanczos squares singular values decomposition. The ^{1}H magnetic resonance spectroscopy processing was performed by an experimenter who was blinded to treatment allocation.

2.5. Intravenous glucose tolerance test

After determination of IMCL content, participants reported to the laboratory where glucose tolerance was assessed by frequently sampled intravenous glucose tolerance test (IVGTT) without modification by insulin infusion as previously described [6,25]. Intravenous rather than oral glucose tolerance test was chosen to avoid potential confounding effects of diet (including starvation) [26] on gastric emptying and subsequent glucose tolerance [27]. The IVGTTs were undertaken after a 12-hour overnight fast (or 67-hour fast in the starvation intervention). Additional blood samples were collected at 15, 30, 60, and 120 minutes for determination of plasma FFA concentrations.

2.6. Blood sampling

Before the IVGTT, 3 mL of venous blood was sampled by syringe, transferred into EDTA, placed on ice, and then centrifuged at 2000*g* for 8 minutes within 30 minutes of collection. Plasma was decanted off and stored at −85°C for later analysis of FFA concentration. An additional 2 mL of venous blood was drawn into a lithium heparin–pelleted syringe. This sample was mixed and rested on ice for approximately 5 minutes, after which 1.3 mL was transferred into blood tubes and centrifuged at 2000*g* for 8 minutes, and the plasma was frozen (−85°C) for later determination of plasma insulin concentration. Residual blood remained on ice in the lithium heparin–pelleted syringe for later determination of plasma glucose concentration. Blood for glucose, insulin, and FFA measurement was sampled according to these methods during the ensuing IVGTT (1 mL for EDTA) according to the sampling schedule outlined above.

2.7. Analytical procedures and calculations

Plasma glucose concentration was measured by autoanalyzer (EML 105; Radiometer, Copenhagen, Denmark). The plasma concentration of FFAs was determined using a Wako NEFA C Test kit (WAKO Chemical, Richmond, VA) scaled for use in a microplate (Bio-Rad, Hercules, CA). All measurements were made in duplicate, and the mean is reported. Glucose tolerance was determined by the rate of decline in plasma glucose concentration between 10 and 40 minutes of IVGTT (K_g) as outlined by Galvin et al [28] where K_g is the slope of the least squares linear regression of ln(glucose concentration) vs time between 10 and 40 minutes of the IVGTT. A measure of glucose tolerance was also made by calculation of the incremental area under the curve (iAUC) for plasma glucose above the basal glucose concentration for glucose vs time [29]. Insulin sensitivity index (S_i) and glucose effectiveness (S_g) were determined via the minimal model analysis of the plasma glucose and insulin response to the IVGTT [25] using MINMOD Millenium (version 6.02, MinMod; University of Southern California, Los Angeles, CA). For these calculations, plasma insulin concentrations were converted from units of picomoles per liter to microunits per liter (1 μU L^{-1} = 6 pmol L^{-1}), and glucose concentrations were converted from units of millimoles per liter to milligrams per deciliter (1 mmol L^{-1} = 18 mg dL^{-1}).

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Table 2
Forty-eight–hour dietary analysis of group daily macronutrient intake

	As a percentage energy intake			Relative to body mass (kg)		
	Mixed	HPLC	Starvation	Mixed	HPLC	Starvation
Carbohydrate (%)	49.5 ± 0.7	2.0 ± 0.4*	0 ± 0*,†	5.2 ± 0.7	0.2 ± 0.1*	0 ± 0*,†
Fat (%)	34.3 ± 1.2	34.6 ± 1.4	0 ± 0*,†	1.6 ± 0.2	1.6 ± 0.2	0 ± 0*,†
Protein (%)	15.8 ± 0.9	63.4 ± 1.1*	0 ± 0*,†	1.7 ± 0.3	6.7 ± 1.0*	0 ± 0*,†
Energy (kJ d $^{-1}$)	12299 ± 843	12314 ± 842	0 ± 0*,†	168.9 ± 23.7	169.1 ± 23.5	0 ± 0*,†

Group daily macronutrient intake as percentage energy intake and relative to body mass in kilograms. Values are means ± SD; n = 7 participants.
* Significantly different vs mixed diet ($P < .001$).
† Significantly different vs HPLC diet ($P < .001$).

Indices of estimated whole-body insulin sensitivity were also obtained via (*a*) the ratio of the iAUC for plasma glucose to insulin (SIAUC) [30], (*b*) the method proposed by Galvin et al [28]:

$$SI_{Galvin} = K_g/(\text{iAUC for insulin between 0 and 40 minutes of IVGTT})$$

and (*c*) the index of insulin sensitivity (ISI) described by Matsuda and DeFronzo [31], assuming its application to the IVGTT:

$$ISI = 10000/\left([\text{glucose}]_r \times [\text{insulin}]_r\right) \times \left([\text{glucose}]_{IVGTT} \times [\text{insulin}]_{IVGTT}\right)$$

where $[\text{glucose}]_r$ is the resting glucose concentration in milligrams per deciliter, $[\text{insulin}]_r$ is the resting insulin concentration in milliunits per liter, $[\text{glucose}]_{IVGTT}$ is the mean concentration of glucose during IVGTT in milligrams per deciliter, and $[\text{insulin}]_{IVGTT}$ is the mean concentration of insulin during the IVGTT in milliunits per liter.

2.8. Analysis

Differences in nutritional intake, all basal measures (plasma glucose, insulin, FFA, and IMCL to water ratio), and differences in indices of glucose tolerance, insulin sensitivity, and S_g between conditions were compared by 1-way repeated-measures analysis of variance. Plasma glucose, insulin, and FFA concentrations during IVGTT were compared by 2-way repeated-measures analysis of variance for investigation of treatment and time (diet-time) interactions. Pearson correlation coefficients (2-tailed) were used to express the relationship between IMCL to water ratio and S_i. Statistical significance was accepted at $P < .05$. All values are expressed as mean ± standard deviation.

3. Results

3.1. Dietary intake

Carbohydrate intake was significantly lower in the HPLC diet than in the mixed diet ($P < .001$), the diets providing 15 ± 4 and 389 ± 22 g d^{-1}, respectively. Dietary protein intake was significantly higher in the HPLC vs the mixed diet ($P < .001$, Table 2), yet daily fat and energy intakes were not different between the HPLC and mixed diets. Despite our efforts to abolish its intake, dietary carbohydrate consumption in the HPLC was significantly greater than during starvation ($P < .001$, Table 2), though by only 0.2 (±0.1) g kg^{-1} d^{-1}.

3.2. IMCL content

The ratio of vastus lateralis IMCL to water ratio was significantly higher in the starvation condition (25.6 ± 5.9 × 10^{-3}) than in the mixed (13.6 ± 6.1 × 10^{-3}, $P < .01$) or HPLC (13.6 ± 3.3 × 10^{-3}, $P < .01$) conditions. The ratio of IMCL to water was not different between the mixed and HPLC conditions ($P > .99$, Fig. 1). Intramyocellular lipid content demonstrated a significant within-subject correlation between the mixed and starvation conditions

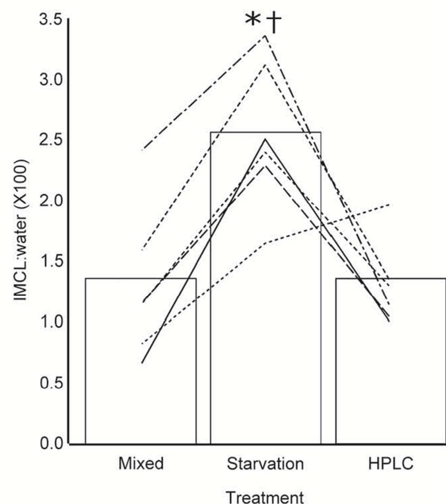

Fig. 1. Effect of 67 hours of mixed diet, HPLC diet, or starvation on IMCL to water ratio. n = 6 subjects. *Significantly different from mixed diet ($P < .01$). †Significantly different from HPLC diet ($P < .01$). Dashed lines indicate individual subject data.

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(r = 0.82, P < .05), but there were no significant correlations between the HPLC condition and either the mixed or starvation condition.

3.3. Basal plasma metabolite and insulin concentrations

Basal plasma glucose concentrations were lower after starvation than after either the mixed (P < .01) or HPLC (P < .05) diet. There was no difference in basal glucose concentration between the mixed and HPLC diets (P = .63, Table 3). The basal plasma insulin concentration was significantly higher after the mixed diet than after either starvation (P < .01) or the HPLC diet (P < .05), but there was no significant difference in basal plasma insulin between starvation and the HPLC diet (Table 3). The basal plasma FFA concentration was significantly higher after starvation than after either the mixed or HPLC diet (P < .01 for both), and there was no difference between basal plasma FFA between the mixed and HPLC diets (Table 3).

3.4. Plasma metabolite and insulin responses to IVGTT

In all 3 treatments, the plasma glucose concentration increased rapidly after the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 14 minutes after initiation. Glucose concentration subsequently declined and was not different between treatments and not different from baseline by the last sample of the IVGTT at 180 minutes (Fig. 2A). There was a significant treatment-time interaction effect (P < .05), with starvation resulting in greater plasma glucose concentrations compared with mixed from 30 to 140 minutes and compared with HPLC from 60 to 140 minutes (Fig. 2A). There was also a significant within-subject main effect of treatment, with the overall glucose concentration throughout the IVGTT being significantly lower after the mixed diet (7.6 ± 2.9 mmol L^{-1}) than after either the HPLC diet (8.1 ± 3.4 mmol L^{-1}, P < .05) or starvation (8.2 ± 2.5 mmol L^{-1}, P < .01).

There were 7 data points in which the plasma insulin concentration was below the detectable limit of the assay (6 pmol L^{-1}), and a value of 5 pmol L^{-1} was assumed for these points. Plasma insulin concentration increased rapidly after the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 8 minutes after

Table 3
Basal plasma substrate and insulin concentrations after 67 hours of dietary intervention

	Mixed	HPLC	Starvation
Plasma glucose (mmol L^{-1})	4.5 ± 0.3	4.2 ± 0.4	3.5 ± 0.3*,‡
Plasma insulin (pmol L^{-1})	19.8 ± 8.3	12.7 ± 3.8†	8.5 ± 5.1*
Plasma FFAs (μmol L^{-1})	378 ± 120	387 ± 232	1179 ± 294*,§

Values are means ± SD; n = 6 participants.
* Significantly different vs mixed diet (P < .01).
† Significantly different vs mixed diet (P < .05).
‡ Significantly different vs HPLC diet (P < .01).
§ Significantly different vs HPLC diet (P < .05).

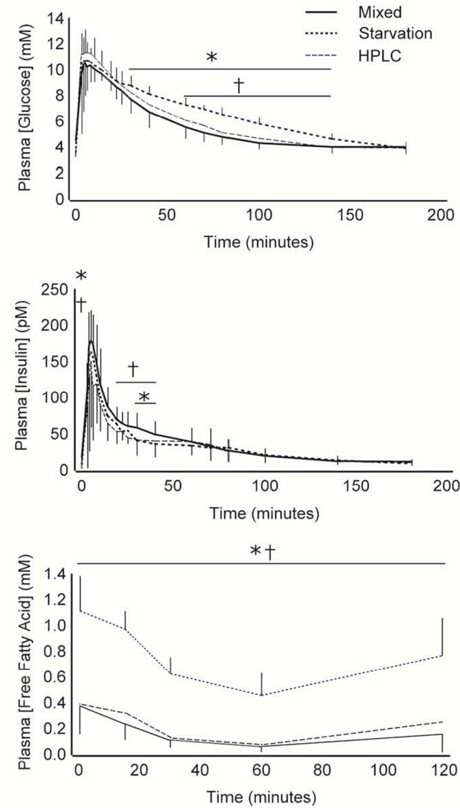

Fig. 2. Effect of 67 hours of mixed diet, HPLC diet, or starvation on plasma glucose, insulin, and FFA concentrations during an IVGTT. n = 6 subjects. *Significantly different, starvation vs mixed diet (P < .05). †Significantly different, starvation vs HPLC diet (P < .05). Error bars indicate 1 standard deviation.

initiation. Insulin concentration subsequently declined and was not different from baseline by 140 minutes and not different between treatments by 40 minutes after initiation of the IVGTT (Fig. 2B). There was a significant treatment-time interaction effect (P < .001), with mixed diet resulting in greater plasma insulin concentrations compared with starvation at baseline and from 30 to 40 minutes and compared with HPLC diet at baseline and from 19 to 40 minutes. Starvation also resulted in significantly greater plasma insulin concentration than HPLC diet at 3 and 19 minutes (Fig. 2B).

In all treatments, plasma FFA concentrations declined after glucose infusion, reaching a minimum at 60 minutes,

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after which time they began to rise again (Fig. 2C). After starvation, there were significantly greater ($P < .001$) plasma FFA concentrations throughout the IVGTT compared with the mixed or HPLC diets. There was also a significant treatment-time interaction ($P < .01$) during the IVGTT, with plasma FFA concentrations declining more rapidly after starvation than after the mixed or HPLC diet (Fig. 2C).

3.5. Minimal model analysis

Insulin sensitivity index determined by the minimal model was significantly lower after starvation compared with after mixed diet ($P < .01$) or HPLC diet ($P < .05$). There was no difference in S_i between the mixed and HPLC diets (Table 4). Glucose effectiveness was significantly greater after the HPLC diet than after either the mixed diet or starvation ($P < .01$ for both, Table 4).

3.6. Glucose tolerance

Glucose tolerance as estimated by K_g was significantly lower after starvation than after the HPLC diet ($P < .05$). The K_g after the mixed diet was not significantly different from the other treatments. Glucose AUC was significantly higher after starvation than the other treatments ($P < .01$ for both), and there was no difference in AUC between the mixed and HPLC diets (Table 4).

3.7. Other estimates of insulin sensitivity

The SIAUC was significantly impaired after starvation ($P < .01$) and HPLC diet ($P < .05$) compared with after the mixed diet and was significantly more impaired after starvation than after HPLC diet ($P < .05$). The SI_{Galvin} was significantly greater after the HPLC diet than after the other interventions ($P < .01$ for both, Table 4). There were no significant differences in ISI between treatments.

Table 4
Effect of 67 hours of mixed diet, HPLC diet, or starvation on indices of glucose tolerance and insulin sensitivity during an IVGTT

	Mixed	HPLC	Starvation
Minimal model			
S_i (L min ^{1} mU ^{1})	16.5 ± 6.8	14.5 ± 4.8	5.7 ± 1.5*,§
S_g × 1000 (min ^{1})	4.4 ± 2.2	13.2 ± 2.2*	6.4 ± 2.3‡
Glucose tolerance			
K_g × 10 ^{2} (mmol L ^{1} min ^{1})	1.1 ± 0.4	1.4 ± 0.5	0.8 ± 0.2§
Glucose iAUC (mmol L ^{1} min)	268 ± 91	339 ± 72	447 ± 50*,‡
Insulin sensitivity indices			
SIAUC × 10 ^{1} (mmol ^{1} μU ^{1})	3.4 ± 1.2	5.3 ± 1.6†	7.6 ± 1.8*,‡
SI_{Galvin} (uU ^{1} min ^{2})	2.9 ± 1.4	4.2 ± 0.7*	2.0 ± 0.4‡
ISI	17.4 ± 3.0	24.4 ± 8.6	26.9 ± 10.0

Values are means ± SD; n = 6 participants. S_i indicates minimal model insulin sensitivity index; S_g, minimal model glucose effectiveness index; K_g, rate of decline of ln(plasma glucose) between 10 and 40 minutes of IVGTT [28], with higher values indicating better glucose tolerance; iAUC, incremental area under curve, with higher values reflecting worse glucose tolerance; SIAUC, insulin sensitivity index = iAUC for glucose/insulin, with higher values reflecting lower insulin sensitivity; SI_{Galvin}, insulin sensitivity determined via the method proposed by Galvin et al (1992) [28], with higher values indicating higher insulin sensitivity; ISI, insulin sensitivity index [31], with higher values indicating lower insulin sensitivity.

* Significantly different vs mixed diet ($P < .01$).
† Significantly different vs mixed diet ($P < .05$).
‡ Significantly different vs HPLC diet ($P < .01$).
§ Significantly different vs HPLC diet ($P < .05$).

4. Discussion

The primary finding of this study is that, in lean, healthy men, carbohydrate restriction in the form of 3 days of HPLC diet does not cause any change in circulating FFA concentration, whole-body insulin sensitivity, or vastus lateralis intramuscular lipid concentration compared with 3 days of mixed, carbohydrate-containing diet. These results contrast with similar protocols that use other forms of dietary carbohydrate restriction (starvation or very high-fat/low-carbohydrate diet) and have produced marked reductions in insulin sensitivity and increases in IMCL concentrations [3,6,32-34].

The almost identical fasting plasma glucose and FFA concentrations after the HPLC and mixed diets, and the marked differences after starvation (Table 3) suggest that there was a continuing supply of glucose entering the blood throughout the HPLC condition. This could be accounted for either by a failure to sufficiently reduce dietary carbohydrate intake in the HPLC diet or by an up-regulation of gluconeogenesis in the face of minimal carbohydrate intake and abundant supply of gluconeogenic substrates. The latter is more likely, as basal insulin concentrations were significantly lower in HPLC than in the mixed condition. Furthermore, we have previously used diets containing approximately 2% of energy from carbohydrate to reduce fasting plasma glucose, increase fasting FFA, and induce insulin resistance and IMCL accumulation almost identically to starvation [6].

To the authors' knowledge, no study has measured the rate of gluconeogenesis or gluconeogenic enzyme activity in humans who have consumed a high-protein/very low-carbohydrate (<5% of energy from carbohydrate) diet. However, where carbohydrate is not eliminated, increased protein intake up-regulates gluconeogenesis. Humans who routinely consume greater amounts of protein have increased overnight-fasted hepatic glucose output and an increased proportion of hepatic glucose output from gluconeogenesis (~65% vs ~45%) compared with controls matched for age, sex, body mass index, and energy intake [35,36]. Infusion of a physiologic dose of gluconeogenic amino acids after an overnight fast doubles the rate of gluconeogenesis [37,38]. If somatostatin is also infused to prevent changes in insulin and glucagon concentrations, amino acid infusion results in increased gluconeogenesis and hyperglycemia [37]. Similarly, when carbohydrate intake was eliminated by 3 to 4 weeks of starvation in obese humans, infusion of alanine caused massive up-regulation of gluconeogenesis and hyperglycemia [16,17].

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In the present study, the large amounts of protein consumed would be expected, at least initially, to increase both gluconeogenesis and hepatic glucose output. However, the reduced fasting insulin concentration in HPLC compared with mixed diet suggests that as liver glycogen stores were depleted, glycogenolysis and total hepatic glucose output declined despite increased gluconeogenesis. This is consistent with data showing that gluconeogenesis accounts for only 47% of hepatic glucose output after 14 hours of fasting but 93% after 42 hours of starvation [39]. Thus, in the HPLC condition, we succeeded in eliminating dietary carbohydrate yet maintaining an endogenous carbohydrate supply, albeit at a reduced rate.

It has been proposed that one physiologic stimulus for IMCL accumulation and an associated insulin resistance in the skeletal muscle of lean, healthy men is an absence of dietary carbohydrate [6]. According to this proposal, dietary carbohydrate restriction, reduced blood glucose, and subsequent suppression of insulin and increased circulating FFAs encourage elevations in IMCL, which in turn protects blood glucose concentrations by blunting glucose disposal by skeletal muscle [6]. In the current study, dietary carbohydrate restriction and replacement with protein did not cause a reduction in blood glucose; so the alterations in circulating FFA and IMCL were also absent. The present results suggest therefore that circulating carbohydrate status rather than exogenous carbohydrate intake is an important factor influencing circulating FFA concentration and therefore IMCL accumulation and changes in insulin sensitivity.

It is important to note that carbohydrate restriction is not the only possible cause of elevated FFA. Circulating FFA concentration also increases in response to high dietary fat intake [40,41] even when carbohydrate intake is very high [42]. This effect is likely to be responsible for some of the elevation in circulating FFA observed during a very low-carbohydrate/high-fat diet [40,41]. However, this increase in circulating FFAs from an exogenous source is clearly the result of different physiologic processes to the increase in circulating FFAs from an endogenous source, as seen in starvation conditions [6,32,33] and under investigation in the current study.

The possibility exists that the physiologic stimulus for elevated circulating FFA in starvation conditions is dietary protein or energy restriction per se rather than circulating carbohydrate status. However, in an evolutionary context, the most parsimonious explanation remains that endogenous FFA production is increased in response to reduced circulating glucose. To conclusively demonstrate that circulating carbohydrate status is the stimulus for elevated circulating FFAs in low-energy conditions, it would be necessary to show elevated circulating FFAs in response to a normal-fat, normal-protein, and low-carbohydrate diet and normal circulating FFAs in response to an energy-matched low-fat, normal-protein, and moderate-carbohydrate diet.

The well-established link between an increase in circulating FFA concentration, accumulation of IMCL and its intermediates, and insulin resistance within a homogenous population [43] is further supported by our data that show a good correlation between IMCL to water ratio and S_i (Pearson coefficient $r = -0.68$, $P < .01$). In the present study, it is also important to consider the potential for other effects of a high protein intake on insulin sensitivity. Where dietary carbohydrate intake is not eliminated, high-protein diet [36] and intravenous infusion of amino acids [38] both decrease insulin sensitivity. Conversely, high protein intake triggers an increase in myocellular insulin-like growth factor 1 concentration [44,45], which in turn could increase muscular insulin sensitivity [46] or mask any decrease that would otherwise have occurred. However, as the relationship between IMCL and insulin sensitivity is maintained in the present study, it is unlikely that other effects of dietary protein are quantitatively important.

Another striking feature of the results of current study was the very high glucose effectiveness indicated by high S_g values in the HPLC condition. The S_g is an estimate of the ability of circulating glucose to trigger its own removal from the blood. The high glucose effectiveness in the HPLC condition is also reflected in the high values for SI_{Galvin}, as this estimate does not include measures of insulin concentration and so will be inflated by high glucose effectiveness (Table 4). To conclusively determine the tissues responsible for the high S_g values would require tracer techniques that were not used in the current study. Notwithstanding, it seems likely that the liver was the main site of altered non–insulin-stimulated glucose disposal during the IVGTT in the HPLC condition. As discussed above, hepatic glucose output is up-regulated by high-protein feeding. In rats, when protein is administered without simultaneous carbohydrate feeding, the increased rate of hepatic glucose output depletes liver glycogen despite increased gluconeogenesis [47,48]. It is teleologically prudent that the liver should replenish its glycogen stores before skeletal muscle, as glycogen in skeletal muscle is difficult to recycle for use by glucose obligate tissues [49]; and in any case, it seems likely that skeletal muscle glycogen stores were not depleted in the HPLC condition, as plasma glucose concentration did not change. Furthermore, a single protein meal increases splanchnic blood flow [50,51], which could potentiate the influence of the liver on whole-body glucose effectiveness. We speculate that, in the HPLC condition, the IVGTT glucose infusion caused the liver to halt glucose output and begin net glucose uptake more rapidly than in the mixed condition and before an insulin response was able to trigger glucose uptake by skeletal muscle.

The primary measure of insulin sensitivity and glucose effectiveness used in this study was the minimal model developed by Bergman et al [52]. The minimal model has good correlation with the hyperinsulinemic-euglycemic clamp [53] and low coefficient of variation [54]. Furthermore, the IVGTT will not be affected by alterations in glucose absorption by the gut, as might be expected after 72 hours of starvation or HPLC diet. The minimal model has

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been shown to systematically overestimate S_g, with the degree of overestimation increasing with increasing insulin response during the first 20 minutes of the IVGTT. As such, caution should be used when comparing S_g results between groups with differing insulin response [55]. However, in the present study, there were no between-treatment differences in insulin response during the first 20 minutes of the IVGTT (Fig. 2); so the overestimation should remain consistent, and comparison between treatments is valid.

5. Conclusion

Normal levels of plasma glucose, plasma FFAs, IMCLs, and insulin sensitivity are maintained when an HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. We conclude that dietary carbohydrate restriction does not per se cause circulating FFA to become elevated. However, it remains probable that circulating carbohydrate status has an important influence on circulating FFA concentrations and therefore insulin sensitivity and healthy IMCL accretion in lean, healthy people.

Acknowledgment

The authors wish to thank David Walton for his assistance with spectroscopy and Dr Patricia Ruell for her assistance with biochemical analysis. This research was supported by the Peter Snell Doctoral Scholarship in Public Health and Exercise Science.

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jackson George Green

Name/Title of Principal Supervisor: Dr Stephen Stannard

Name of Published Paper: Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated

In which Chapter is the Published Work: 13.7

What percentage of the Published Work was contributed by the candidate: 75%

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16/3/2011
Date

GRS Online Version 2– 1 December 2010

14.8 Peer-reviewed article: Moderate-intensity endurance exercise prevents short term starvation-induced intramyocellular lipid accumulation but not insulin resistance

Moderate-intensity endurance exercise prevents short-term starvation-induced intramyocellular lipid accumulation but not insulin resistance

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ARTICLE INFO

Article history:
Received 6 April 2010
Accepted 10 January 2011

ABSTRACT

Exercise has the potential to alleviate the resistance to insulin-mediated glucose uptake precipitated by elevated circulating free fatty acids (FFAs) in conditions such as obesity, lipid infusion, and starvation. In this study, 6 lean healthy men underwent two 3-day periods of starvation with either no exercise or daily endurance exercise (80 min d^{-1} at 50% maximal rate of oxygen consumption) and a 3-day mixed diet without exercise. Insulin sensitivity was determined by intravenous glucose tolerance test, and intramyocellular lipid (IMCL) concentration was measured by ^{1}H magnetic resonance spectroscopy. In both starvation conditions, fasting plasma FFAs were significantly elevated, whereas plasma glucose and whole-body insulin sensitivity were significantly reduced. Vastus lateralis IMCL to water ratio was significantly elevated after starvation without exercise compared with that after starvation with exercise or that after mixed diet. Intramyocellular lipid to water ratio was not different between starvation with exercise and mixed diet. In healthy lean men, exercise during starvation prevents the accumulation of IMCL yet does not affect the starvation-induced changes in FFAs and insulin sensitivity. Unlike during lipid infusion or obesity-induced insulin resistance, exercise cannot overcome the reduction in insulin action caused by starvation. We propose that carbohydrate availability is a key modulator of the combined effects of exercise and circulating FFAs on insulin sensitivity.

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1. Introduction

A sedentary lifestyle is associated with insulin resistance and its comorbidities [1]; and conversely, regular physical activity ensures whole-body insulin sensitivity (Si) [2]. The latter appears to be due primarily to repeated short-term effects of exercise on promoting insulin-mediated glucose disposal rather than some chronic, long-term adaptation [3]. Even in

Author contributions: JGG, NAJ, CWC, and SRS conceived the hypothesis. JGG, NAJ, MWT, and SRS designed the experimental protocol. JGG and CWC developed the protocol for ethical approval. TS, JGG, NAJ, and MWT collected the data. TS, JGG, and NAJ performed blind analysis of NMR spectra. JGG and NAJ analyzed biochemical samples. JGG, NAJ, and SRS interpreted the data. JGG wrote the manuscript with contributions from NAJ, SRS, and CWC.

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doi:10.1016/j.metabol.2011.01.003

Reprinted from Metabolism Clinical and Experimental, Green, J.G., Johnson, N. A., Sachinwalla, T., Cunningham, C.W., Thompson, M. W., and Stannard, S. R., *Moderate-intensity endurance exercise prevents short-term starvation-induced intramyocellular lipid accumulation but not insulin resistance*, Copyright (2011), doi:10.1016/j.metabol.2011.01.003, with permission from Elsevier.

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athletes undergoing chronic endurance training, a few days of inactivity drastically reduces Si [4,5]. However, in both healthy sedentary people [1] and endurance-trained people who have undergone a few days of inactivity [6], a single exercise session restores Si to near trained levels.

Measures of whole-body Si are also reduced in situations where blood plasma free fatty acids (FFAs) are elevated such as obesity [7]; intravenous lipid infusion [8,9]; low-carbohydrate, high-fat diet [10,11]; or starvation [10,12,13]. The combination of inactivity and an acute increase in plasma FFAs leads, within a few hours, to accretion of intramyocellular lipid (IMCL) and a simultaneous and proportional reduction in Si [14]. However, the effect of exercise superimposed upon elevated FFAs is unclear.

Two studies have directly examined this situation in humans. Schenk et al [8,15] reported that prior exercise (90 minutes at 65% of Vo_{2peak}) prevented the decrease in Si that otherwise occurs with an overnight lipid infusion, implying that exercise is able to either compensate for or directly prevent a diet-induced decrease in Si. Conversely, Sparti and Decombaz [11] reported that prior exercise (30 minutes at 80% of estimated maximum heart rate followed by 30 minutes of resistance exercise then ~10 intervals of 2 minutes at 90% of maximum heart rate) did not mitigate the insulin resistance observed following a subsequent 36-hour very low-carbohydrate, high-fat diet. The dissonance between these studies may be due to different means of elevating FFAs; but regardless, a better understanding of how exercise and elevated FFAs interact to affect Si would be useful. In this context, the effect of superimposing physical activity on starvation in physically fit individuals is to date unknown.

Although Si has historically been the primary focus of research into glycemic control, glucose effectiveness (Sg) (ie, the ability of blood glucose to promote its own disposal and suppress hepatic glucose output) is also an important component of glucose tolerance [16], especially in patients with impaired insulin secretion. Like Si, Sg is decreased by physical inactivity [17,18] and is increased after a single exercise bout [19]. However, the effect of exercise on Sg in a state of elevated FFA and reduced Si has not been examined.

The aim of this study was to test the hypothesis that exercise during starvation would prevent the changes in Si, Sg, plasma FFA, and IMCL normally observed during starvation without exercise. To this end, we exposed lean, physically fit men to 2 approximately 3-day periods of starvation with different levels of total energy expenditure, as well as approximately 3 days of standardized moderate-carbohydrate diet.

2. Methods

2.1. Subjects

Six healthy, physically fit men volunteered for this study. Participants' physical characteristics are given in Table 1. All participants reported regularly undertaking exercise for more than 1.5 hours daily, at least 5 days per week. Participants were informed of the study protocol and risks before providing their written consent. The study was approved by the University of Sydney human research ethics committee and conformed to the Declaration of Helsinki.

Table 1 – Participants' physical characteristics

Age (y)	38.8 ± 12.7
Body mass (kg)	72.9 ± 8.8
Body fat (%)	13.5 ± 2.1
RMR (mL O_2 min^{-1})	306 ± 35
VO_{2max} (mL kg^{-1} min^{-1})	75.5 ± 16.0

Values are mean ± standard deviation. VO_{2max} indicates maximal rate of oxygen consumption.

2.2. Preliminary testing

One week before participation in the first intervention, submaximal and maximal oxygen uptake tests were performed on an electronically braked cycle ergometer (Lode ergometer, Groningen, the Netherlands) as previously described [20]. External power output and VO_2 attained during the final minute of each submaximal workload and the maximal ramp were used to formulate regression equations from which workloads for the control exercise bout were derived. On a separate occasion, participants presented at the laboratory following a 12-hour overnight fast for measurement of resting metabolic rate (RMR) using respiratory gas analysis while lying supine after 30-minute rest. Body density was assessed via hydrodensitometry; and percentage body fat was then calculated using a 2-compartment model, as previously described by Brozek et al [21]. Underwater body weight measurements were corrected for measured residual lung volume using the methods of van der Ploeg et al [22].

2.3. Experimental protocol

All participants underwent 3 supervised dietary and exercise interventions in random order. A minimum 7-day washout was allowed after the mixed-diet intervention and 25 days after the starvation interventions. Each intervention period was of 67-hour duration and comprised a water-only starvation diet with no physical activity (inactivity + starvation), a water-only starvation diet with controlled physical activity (exercise + starvation), or a mixed control diet with no physical activity (inactivity + mixed diet). Beginning the day before initiation of the diet intervention (36 hours before initiation), subjects refrained from exercise and recorded all dietary intake. Before subsequent interventions, subjects again refrained from exercise and consumed the same diet as before the first intervention. This preconditioning period plus the intervention period resulted in 4.5 days (108 hours) of diet and exercise control before each testing session. There were 63 hours between the last exercise session and the intravenous glucose tolerance test (IVGTT) in the inactivity conditions and 15 hours between the last exercise session and the IVGTT in the exercise condition.

Upon initiation of the diet, participants ingested either a carbohydrate snack or water only, according to their allocation to mixed diet or a starvation intervention. The snack, a sports drink containing glucose and sucrose, provided 1 g carbohydrate per kilogram of body weight (100% energy from

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carbohydrate) in the mixed diet, or water only in the starvation diets. In the mixed diet, participants ingested an evening meal 2 hours later. This meal contained 1.5 g carbohydrate per kilogram of body weight and supplied 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein. Beginning the following morning and continuing for the remainder of the mixed dietary treatment (48 hours), participants received a diet that provided energy to match a daily expenditure of 1.5× RMR to maintain energy balance. Diets were designed to deliver 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein. In the starvation conditions, participants continued a water-only diet until completion of the experimental treatment. In the inactivity + starvation condition, participants performed no unnecessary physical activity. The exercise + starvation condition was identical except that participants reported to the laboratory and performed a standardized exercise bout (50% of the power associated with maximal rate of oxygen consumption for 80 minutes) on the cycle ergometer at 5:00 PM on the second and third days (28 and 52 hours after initiation of starvation). Diet composition was quantified via Foodworks (Xyris Software, Melbourne, Australia) using the New Zealand–Standard database. In all dietary interventions, participants were instructed to maintain activities of daily living and avoid all forms of recreational exercise. Participants were based in the university environment, returning to their homes to sleep. The researchers maintained a minimum of daily contact with participants to ensure compliance with the dietary and exercise protocols.

2.4. *Determination of IMCL content*

After 65 hours of each diet, vastus lateralis proton magnetic resonance (^{1}H-MRS) spectra were obtained as previously described [10]. Spectral data were postprocessed by magnetic resonance user interface software (jMRUI version 3.0, EU Project). The vastus lateralis IMCL content was determined by the ratio of the methylene (–[CH2]n) resonance from IMCL at 1.3 ppm and intracellular water [23]. A 10-resonance model was used to determine IMCL concentration as we have detailed previously [10]. Muscle water signal amplitudes were measured from the non–water-suppressed spectrum using Hankel-Lanczos squares singular values decomposition. The ^{1}H-MRS processing was performed by an experimenter who was blinded to treatment allocation.

2.5. *Intravenous glucose tolerance test*

Following determination of IMCL content (15 hours after the last exercise session in the exercise + starvation condition), participants reported to the laboratory where glucose tolerance was assessed by frequently sampled IVGTT without modification by insulin infusion as previously described [10,24]. Intravenous rather than oral glucose tolerance test was chosen to avoid potential confounding effects of diet (including starvation [25]) on gastric emptying and subsequent glucose tolerance [26]. The IVGTTs were undertaken after a 12-hour overnight fast (or 67-hour fast in the starvation intervention). Additional blood samples were collected at 15, 30, 60, and 120 minutes for determination of plasma FFA concentrations.

2.6. *Blood sampling*

Before the IVGTT, 3 mL of venous blood was sampled by syringe, transferred into EDTA, placed on ice, and then centrifuged at 2000 *g* for 8 minutes within 30 minutes of collection. Plasma was decanted off and stored at −80°C for later analysis of FFA concentration. An additional 2 mL of venous blood was drawn into a lithium heparin-pelleted syringe. This sample was mixed and rested on ice for approximately 5 minutes, after which 1.3 mL was transferred into blood tubes and centrifuged at 2000 *g* for 8 minutes, and the plasma was frozen (−80°C) for later determination of plasma insulin concentration. Residual blood remained on ice in the lithium heparin-pelleted syringe for later determination of plasma glucose concentration. Blood for glucose, insulin, and FFA measurement was sampled according to these methods during the ensuing IVGTT according to the sampling schedule outlined previously [10,24].

2.7. *Analytical procedures and calculations*

Plasma glucose concentration was measured by autoanalyzer (EML 105; Radiometer, Copenhagen, Denmark). The plasma concentration of FFAs was determined using a Wako NEFA C test kit (WAKO Chemical, Richmond, VA) scaled for use in a microplate (Bio-Rad, Hercules, CA). All measurements were made in duplicate, and the mean was reported. Insulin sensitivity and Sg were determined via the minimal model analysis of the plasma glucose and insulin response to the IVGTT [24] using MINMOD Millennium (version 6.02; MinMod, University of Southern California, Los Angeles, CA).

2.8. *Analysis*

Differences in nutritional intake, all basal measures (plasma glucose, insulin, FFA, and ratio of IMCL to water) and differences in Si and Sg between conditions were compared by 1-way repeated-measures analysis of variance (ANOVA). Plasma glucose, insulin, and FFA concentrations during IVGTT were compared by 2-way repeated-measures ANOVA for investigation of treatment and time (diet-time) interactions. Where statistical significance was found, the Tukey honestly significant difference test was used to determine where the difference occurred. Statistical significance was accepted at $P < .05$. All values are expressed as mean ± standard deviation.

3. Results

3.1. *Diet and exercise*

During the mixed diet, subjects had an average daily energy intake of 12 299 kJ (49.5% ± 0.7% from carbohydrate, 34.3% ± 1.2% from fat, and 15.8% ± 0.9% from protein). Subjects consumed no energy during the starvation conditions. All subjects were able to complete the required exercise, although

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2 subjects had to stop for a brief rest after 70 minutes during the last exercise session in the exercise + starvation condition.

3.2. IMCL content

The ratio of vastus lateralis IMCL to water was significantly higher in the inactivity + starvation condition than in the mixed (P = .0003) or exercise + starvation conditions (P = .009, Table 2). The ratio of IMCL to water was not significantly different between the mixed and exercise + starvation conditions (P = .10). Intramyocellular lipid content demonstrated a significant within-subject correlation between the mixed and inactivity + starvation conditions (r = 0.82, P = .008), but there were no significant correlations between the exercise + starvation condition and either the mixed (r = 0.57, P = .07) or inactivity + starvation (r = 0.6, P = .06) conditions.

3.3. Basal plasma metabolite and insulin concentrations

Compared with either starvation condition, basal plasma glucose (inactivity, P = .002; exercise, P = .001) and insulin (inactivity, P = .0003; exercise, P = .003) concentrations were higher and FFA concentrations were lower (inactivity, P < .0001; exercise, P < .0001) after the mixed diet. There was no difference in basal glucose, insulin, or FFA concentrations between the starvation conditions (Table 2).

3.4. Plasma metabolite and insulin responses to IVGTT

In all 3 treatments, the plasma glucose concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 14 minutes after initiation. Glucose concentration subsequently declined and was not different between treatments and not different from baseline by the last sample of the IVGTT at 180 minutes (Fig. 1A). There was a significant treatment-time interaction effect (P = .039) with mixed diet resulting in higher plasma glucose concentration compared with the starvation interventions at baseline and lower plasma glucose concentration compared with exercise + starvation from 40 to 100 minutes and compared with inactivity + starvation from 30 to 140 minutes (Fig. 1A).

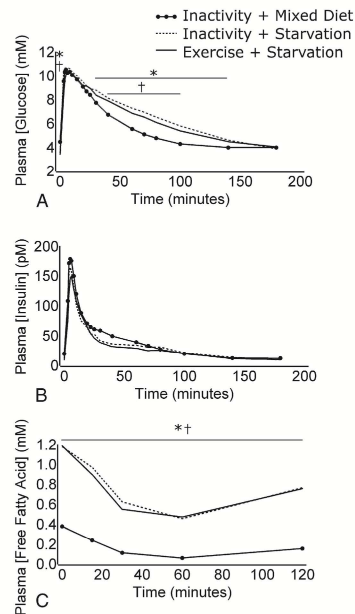

Fig. 1 – Effect of 67 hours of inactivity + mixed diet, inactivity + starvation, or exercise + starvation on (A) plasma glucose, (B) insulin, and (C) FFA concentrations during an IVGTT; n = 6 subjects. Between-trials comparisons were performed using repeated-measures ANOVA. *Significantly different inactivity + mixed diet vs inactivity + starvation (P < .05). † Significantly different inactivity + mixed diet vs exercise + starvation (P < .05).

There were 7 data points in which the plasma insulin concentration was less than the detectable limit of the assay (6 pmol L^{-1}), and a value of 5 pmol L^{-1} was assumed for these points. Plasma insulin concentration increased rapidly following the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 8 minutes after initiation. Insulin concentration subsequently declined and was not different from baseline by 140 minutes (Fig. 1B). There was no significant treatment-time interaction effect on insulin concentration (P = .18, Fig. 1B).

In all treatments, plasma FFA concentration declined after glucose infusion, reaching a minimum at 60 minutes, after which time it began to rise again (Fig. 1C). After the starvation conditions, plasma FFA concentration was significantly greater (P < .0001) throughout the IVGTT compared with the mixed diet. There was also a significant treatment-time interaction (P < .0001) during the IVGTT, with plasma FFA concentration declining more rapidly after starvation

Table 2 – Basal substrate and insulin concentrations and IVGTT results after 67 hours of dietary intervention

	Inactivity + mixed diet	Exercise + starvation	Inactivity + starvation
Plasma glucose (mmol L^{-1})	4.5 ± 0.3	3.4 ± 0.5 *	3.5 ± 0.3 *
Plasma insulin (pmol L^{-1})	19.8 ± 8.3	10.7 ± 6.5 *	8.5 ± 5.1 *
Plasma FFAs (μmol L^{-1})	379 ± 120	1188 ± 219 *	1180 ± 294 *
Vastus lateralis IMCL to water ratio ($\times 10^{-3}$)	13.6 ± 6.1	18.1 ± 5.8	25.6 ± 5.9 *,†
Si (L min^{-1} mU^{-1})	16.5 ± 6.8	6.0 ± 1.7 *	5.7 ± 1.5 *
Sg × 1000 (min^{-1})	4.4 ± 2.2	8.8 ± 3.0 *	6.4 ± 2.3

Values are means ± SD; n = 6 participants. Between-trials comparisons were performed using repeated-measures ANOVA.
* Significantly different vs inactivity + mixed diet (P < .01).
† Significantly different vs exercise + starvation (P < .05).

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than after the mixed diet (Fig. 1C). There was no difference in FFA concentration between the starvation conditions.

3.5. *Minimal model analysis*

Values for Si and Sg after each of the interventions are presented in Table 2. Compared with that after mixed diet, Si determined by the minimal model was significantly lower after starvation with inactivity (P = .005) and starvation with exercise (P = .007). There was no difference in Si between the starvation conditions. Glucose effectiveness was significantly greater after the exercise + starvation condition than after the mixed diet (P = .022), but there was no significant difference between starvation conditions (P = .15) or between inactivity + starvation and mixed diet (P = .38).

4. Discussion

The primary finding of this study is that daily endurance exercise does not mitigate the insulin desensitizing effect of 3 days of starvation despite preventing the accumulation of vastus lateralis IMCL.

The exercise performed in the starvation condition was considerably less than the habitual training load of our endurance-trained subjects. Nevertheless, the subjects found it difficult to complete the last exercise session during starvation; and 2 subjects required a brief rest to be able to complete the exercise. Previous research where exercise was imposed on starvation has demonstrated a 32% reduction (P = .025) in time-to-failure in an incremental exercise test after 2 days and a 45% reduction (P < .0001) after 4 days [27]. These large decreases in physical capacity demonstrate the challenge of performing physical work during starvation and suggest that the dose used in the present study approached the limit of what is achievable in this state.

To increase Si above habitual levels in the very fit endurance-trained subjects used in our study would require a prodigious amount of exercise, more than would be physically possible during starvation. To avoid this methodological difficulty, we used an intervention of starvation + inactivity to reduce Si and compared the results to a habitual diet control (mixed diet + inactivity) and a habitual physical activity control (starvation + exercise). In other endurance-trained groups of subjects, cessation of training resulted in a 28% decline in Si after 38 hours [5] and a 35% decline after 60 hours [4] compared with 12 or 14 hours after the last exercise session. Even when Si was reduced by 50% after 10 days of inactivity, Si was restored to normal levels by a single exercise session approximating a typical training session for the athlete [6]. Thus, our inactivity interventions, which involved 63 hours between the last exercise session and the IVGTT, would normally have markedly reduced Si compared with our exercise intervention, which included 2 extra exercise sessions and only 15 hours between the last exercise session and the IVGTT. As such, the lack of difference in Si between starvation + inactivity and starvation + exercise must be due to the starvation overriding or masking any impact of inactivity.

The results of the current study are consistent with previous research in which FFAs were elevated by low-carbohydrate diet [11], but are at odds with the ability of exercise to maintain Si in the face of plasma FFA concentrations elevated by lipid infusion [8,15]. A key difference between these 2 conditions is a difference in carbohydrate status brought about by the methods of raising FFAs. In low-carbohydrate diet and starvation, the increase in FFAs is accompanied by a reduction in circulating glucose availability; but with lipid/heparin infusion, it is not. This difference may suggest that some minimum level of carbohydrate status is required to enable exercise to increase whole-body Si. Taking a teleological perspective, such an interaction between exercise and carbohydrate status would be prudent, as an increase in Si with exercise when carbohydrate status is low could produce dangerous hypoglycemia.

The energy expended because of additional physical activity in the exercise condition is equivalent to the metabolizable energy of approximately 250 g of fat. Assuming an average 18 kg of active muscle mass recruited by the subjects in our study during cycling [28,29], the difference in total IMCL content between exercise and inactivity conditions is approximately 115 g. Thus, the extra oxidation of lipid to meet the energy requirements of exercise could account for the difference in IMCL stores between starvation + exercise and starvation + inactivity despite both conditions having similarly elevated plasma FFA concentration and similarly reduced Si [30]. This mechanism is also consistent with the elevation of IMCL stores despite exercise when FFAs are elevated by lipid infusion [8,15], as the greater carbohydrate availability and plasma insulin concentration in this situation would be expected to increase carbohydrate oxidation, decrease FFA oxidation, and increase FFA esterification [31]. However, differences in oxidation alone cannot explain the ability of exercise to maintain Si in the face of lipid infusion [8,15] but not starvation or low-carbohydrate diet [11]. A possible explanation for this effect is an alteration in the rate of FFA absorption by muscle cells. Membrane-bound fatty acid transport protein content increases in response to various physiological stimuli including insulin [32], muscle contraction [33], and starvation [34]. If myocellular fatty acid absorption capability is increased by low carbohydrate availability, then there would be a greater intramyocellular FFA load in starvation compared with lipid infusion despite a similar plasma FFA concentration.

The difference in exogenous fat intake in the current study and that of Schenk et al [8,15] is not likely to be responsible for the difference in the observed effects of exercise on Si. When carbohydrate status is low, as in the current study, circulating FFA will be elevated either from high exogenous fat intake [8-10] or from increased adipose tissue lipolysis [10,13]. Thus, skeletal muscle cells will be exposed to the same interstitial milieu regardless of dietary fat intake.

The exercise + starvation condition in the current study resulted in very high Sg. As Si is reduced in both starvation conditions, it seems paradoxical that Sg should be increased. However, it is likely that Sg is not quantitatively important at the low plasma glucose concentrations during the fast (Table 2). Increased Sg has important implications for patients with type 2 diabetes mellitus, as increases in Sg have the potential to improve glycemic control even when insulin secretion is

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absent. Furthermore, increased Sg provides a convenient mechanism for very rapid initiation of muscle and liver glucose uptake and suppression of hepatic glucose production in response to an increase in plasma glucose.

Increased Sg during starvation could be mediated by reduced muscle glycogen concentration [3]. Whereas liver glycogen is substantially depleted after an overnight fast [35], muscle glycogen stores remain near full capacity after a mixed diet, are somewhat reduced after 3 days of inactivity + starvation, and are further reduced after exercise + starvation [36]. This order parallels the Sg values we observed.

The primary measure of Si and Sg used in this study was the IVGTT with minimal model analysis developed by Bergman et al [37]. The minimal model has good correlation with the hyperinsulinemic-euglycemic clamp [38] and low coefficient of variation [39]. Furthermore, the IVGTT will not be affected by alterations in glucose absorption by the gut, as might be expected after a 72-hour fast [25]. The minimal model has been shown to systematically overestimate Sg, with the degree of overestimation increasing with increasing insulin response during the first 20 minutes of the IVGTT. As such, caution should be used when comparing Sg results between groups with differing insulin response [40]. However, in the present study, there were no between-treatment differences in insulin response during the first 20 minutes of the IVGTT (Fig. 1); so the overestimation should remain consistent, and comparison between treatments is valid.

In summary, 3 days of starvation causes increased IMCL and circulating FFAs and decreased Si. The current study shows that exercise during starvation prevents the accumulation of IMCL and increases Sg yet does not affect the changes in Si or plasma FFA concentration. We propose that some minimum level of whole-body carbohydrate status is required to enable exercise to affect Si.

Acknowledgment

The authors wish to thank David Walton for his assistance with spectroscopy and Dr Patricia Ruell for her assistance with biochemical analysis. Funding: This research was supported by the Peter Snell Doctoral Scholarship in Public Health and Exercise Science.

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DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jackson George Green

Name/Title of Principal Supervisor: Dr Stephen Stannard

Name of Published Paper: Moderate-intensity endurance exercise prevents short term starvation-induced intramyocellular lipid accumulation but not insulin resistance

In which Chapter is the Published Work: 13.8

What percentage of the Published Work was contributed by the candidate: 75%

Jackson Green Digitally signed by Jackson Green DN: cn=Jackson Green, o=Massey University, ou=RCMHD, email=J.Green@massey.ac.nz, c=NZ Date: 2011.03.16 12:07:10 +13'00'
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