

**Effect of Diet, Insulin and Exercise on the Regulation of  
Carbohydrate Metabolism in Health and Type 1 Diabetes**

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## **Thesis Abstract**

The objective of this thesis was to further the understanding of the effect of diet, insulin and exercise on the regulation of carbohydrate metabolism in health and type 1 diabetes. Three studies were undertaken in both non-diabetic healthy volunteers and patients with type 1 diabetes. The first study determined the influence of high fat diet on biochemical and molecular regulators of whole body and muscle metabolism in healthy volunteers. The second study examined the influence of insulin on whole body and muscle metabolism in patients with type 1 diabetes during moderate exercise. The final study compared the influence of insulin and a high carbohydrate diet on liver glycogen concentrations and substrate oxidation during exercise between patients with type 1 diabetes and healthy volunteers.

The main results were, a) 6 days of high fat/low carbohydrate dietary intake did not induce whole-body insulin resistance but caused a shift in intramuscular glucose metabolism from oxidation to glycogen storage when compared to a normal balanced diet. Insulin-stimulated carbohydrate oxidation and muscle PDCa activity were blunted after the high fat diet and this was associated with an up regulation of muscle PDK4 mRNA and protein expression, b) Exercise under hyperinsulinaemic conditions in patients with type 1 diabetes did not spare muscle glycogen utilisation at a time of high exogenous glucose utilization and oxidation, and finally c) Changes in liver glycogen concentration and substrate oxidation during exercise occurred at comparable rates in patients with type 1 diabetes and in healthy controls despite the presence of relative hyperinsulinaemia in the former compared to the latter group.

The key conclusions are, 1) in healthy humans short-term high fat feeding does not induce whole body insulin resistance but impairs basal and insulin-stimulated

carbohydrate oxidation, most likely as a result of fat-induced upregulation of muscle PDK4 protein expression. The precise signaling mechanisms involved in the chronic regulation of PDK4 need to be determined. 2) Contrary to previous observation in non-diabetic individuals, it appears that hyperinsulinaemic conditions in patients with type 1 diabetes do not suppress the exercise-induced changes in muscle and liver glycogen stores. The underlying physiological mechanism(s) behind this apparently divergent response remains to be elucidated.

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Finally, I would like to thank the endless support of my fantastic family (Parkavi, Shianu, Nithilan, mum and dad) who put up with my long periods of absence from home for the last 3 years and with my preoccupation to complete the research. I think there will be huge sigh of relief at home the moment I submit my thesis.



## **Declaration**

The work presented in this thesis is a result of experimental work carried out by myself with invaluable support from research and academic staff based in the School of Biomedical Sciences, University of Nottingham. Data generated from this work contributed to the abstract and research publications as detailed in the next section. A small proportion of the physiological data in Chapter 3 has been previously presented in Luke Norton's PhD thesis. I carried out all the healthy volunteer and patient in-vivo studies with the help of Dr.K.Tsintzas (Lecturer in Physiology) and L.Norton (PhD student), M.Block (Research nurse), J.Littlewood (Research assistant), L.Simpson (Research nurse) and J.Fox (Research Technician). I carried out most of the blood and muscle analytical work under the guidance of Dr.K.Tsintzas except for the plasma catecholamines (S.Sarmad and S.Cordon), blood  $\beta$ -hydroxybutyrate (S.Cordon), plasma glucagon (S.Cordon), deuterated glucose (L.J.C vanLoon) muscle gene and protein expression (K.Jewell and L.Norton) and muscle acetylcarnitine assays (Dr.K.Tsintzas). Physicists Dr.A.Snaar, B.Solanky and E.Leverton carried out the MR spectroscopy analytical work.

I hereby declare that the present thesis has been prepared by me and this thesis is a record of the work done. No part of this thesis has been submitted to any previous application for a higher degree.

**Dr.K.Chokkalingam**

**2007**

## **Publications and presentations from the work described in this thesis**

### **Full Papers**

1) High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: an important role for skeletal muscle pyruvate dehydrogenase kinase 4. K. Chokkalingam, K. Jewell, L. Norton, J. Littlewood, L. J. C. vanLoon, P. Mansell, I. A. Macdonald, and K. Tsintzas. *Journal of Clinical Endocrinology and Metabolism*. 2007 Jan; 92(1): 284-92

2) Exercise under hyperinsulinaemic conditions increases whole-body glucose disposal without affecting muscle glycogen utilisation in type 1 diabetes. Chokkalingam, K., Tsintzas, K., Norton, L., Jewell, K., Macdonald, I., Mansell, P. *Diabetologia* 2007 Feb; 50(2): 414-21

3) Hyperinsulinaemia during exercise does not suppress hepatic glycogen concentrations in patients with type 1 diabetes: a magnetic resonance spectroscopy study. K. Chokkalingam, K. Tsintzas, J. E. M. Snaar, L. Norton, B. Solanky, E. Leverton, P. Morris, P. Mansell, I. A. Macdonald. *Diabetologia* 2007 Sep; 50(9):1921-9.

## **Oral and Poster Presentations**

- 1) Hyperinsulinaemia during exercise does not suppress hepatic glycogen utilisation in patients with type 1 diabetes. (Poster presentation, Annual professional conference of the European Association for the Study of Diabetes, EASD 2006, Copenhagen). Published in abstract form in Diabetologia (2006) Supplement 49: A774.
- 2) Effect of variable Hyperinsulinaemia on substrate utilization in patients with type 1 diabetes (Oral Communication, Annual Professional Conference of the European Association for the Study of Diabetes, EASD 2005, Athens). Published in abstract form in Diabetologia Supplement 1 2005.48:A72
- 3) Short-term high fat diet impairs carbohydrate oxidation without affecting glucose disposal in healthy men (Poster presentation, Annual Professional Conference of the American Diabetes Association ADA 2005, San Diego). Published in abstract form in Online Late breaking abstract book (No: 72).

## **Abbreviations**

$^{13}\text{C}$	Carbon 13
CHO	Carbohydrate
C <sub>OX</sub>	Carbohydrate Oxidation
CPT	Carnitine Palmitoyl Transferase
DAG	Diacyl Glycerol
ERK	Extracellular Signal Related Kinase
ERR	Estrogen Related Receptor
FOXO	Forkhead Box Factors
F <sub>OX</sub>	Fat Oxidation
GI	Glycaemic Index
G-6-P	Glucose 6 Phosphate
GSK	Glycogen Synthase Kinase
HbA <sub>1c</sub>	Glycated Haemoglobin
IMTG	Intramyocellular Triacylglycerol
IRS	Insulin receptor Substrate
LCAC	Long Chain Acyl-CoA
LCFA	Long Chain Fatty Acid
MAPK	Mitogen Activated Protein Kinase
NEFA	Nonesterified Fatty Acid
NMR	Nuclear Magnetic Resonance Spectroscopy
PCR	Polymerase Chain Reaction
PDC	Pyruvate Dehydrogenase Complex
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PGC	PPAR Gamma Coactivator
PI	Phosphatidylinositol
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PPARS	Peroxisome Proliferators Activated Receptors
Ra	Glucose Appearance
Rd	Glucose Disposal
RQ	Respiratory Quotient
RER	Respiratory Exchange Ratio
SREBP	Sterol Regularory Element Binding Protein
TCA	Citric Acid Cycle
TG	Triacyl Glycerol
VO <sub>2</sub>	Oxygen Uptake Rate
VCO <sub>2</sub>	Carbon Dioxide Release Rate

## **Chapter 1: Introduction**

### **1.1 Influence of diet on substrate metabolism**

The cellular mechanisms that regulate the relative contribution of fat and carbohydrate when the availability of either substrate is increased have not been fully elucidated. The glucose-fatty acid cycle was proposed (Randle, Garland et al. 1963) to explain the reduced carbohydrate oxidation when free fatty acid availability was increased. It was suggested that substrates compete with each other to be oxidized as increased free fatty acid availability might suppress the activation of the pyruvate dehydrogenase complex (PDC), thereby inhibiting glycolytic flux and stimulating fatty acid oxidation (Randle, Garland et al. 1963; Randle, Priestman et al. 1994). This hypothesis has been the subject of intensive investigation to further our understanding of cellular mechanisms of insulin resistance. An overview of the cellular regulation of substrate metabolism, insulin signaling network and the changes in insulin mediated substrate metabolism induced by increased free fatty acid availability is discussed in this section.

#### **1.1.1 Cellular regulation of substrate metabolism**

The vast majority of ATP required for muscle contraction is generated through oxidative phosphorylation and the principal substrates are carbohydrate and fat. Glucose or glycogen is first converted to glucose-6-phosphate and then to pyruvate via glycolysis (Fig 1.1a). The rate of pyruvate entry into the mitochondrial matrix and subsequent conversion to acetyl-CoA is regulated by the pyruvate dehydrogenase complex (PDC) as shown in figure 1.1b (Sugden and Holness 2003). In addition to the activation state of PDC, adequate flux through PDC is particularly important in tissues with high ATP demand such as exercising muscle (Constantin-Teodosiu,

Peirce et al. 2004). It was observed in this study that PDC activation was comparable irrespective of the pre-exercise muscle glycogen content. However, due to reduced pyruvate availability in the glycogen-depleted muscle, flux through PDC was 2 fold lower when compared to the non-depleted muscle.

PDC is a multiple enzyme complex composed of three enzyme components namely pyruvate dehydrogenase (PDH), dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase. PDH exists in active (PDH a) and inactive (PDH b, phosphorylated) forms. The activation and inactivation of PDH is regulated by competing PDH kinase and phosphatase respectively (Huang, Gudi et al. 1998; Huang, Wu et al. 2002).

There are four isoforms of PDH kinase (PDK1, 2, 3 and 4). The mammalian PDH kinases exhibit tissue-specific expression (Bowker-Kinley, Davis et al. 1998). PDK2 and PDK4 are highly expressed in skeletal muscle (Wu, Inskeep et al. 1999; Holness, Kraus et al. 2000; Sugden, Langdown et al. 2000). The PDH kinases are allosterically inhibited by pyruvate, high CoA/Acetyl-CoA and high  $\text{NAD}^+/\text{NADH}$  ratios, whereas they are stimulated by a high ATP/ADP ratio (Kerbey, Randle et al. 1976). In addition to allosteric regulation these kinases also appear to be under transcriptional regulation. Administration of a high fat diet for 28 days was associated with significant increases in PDK4 and PDK2 protein expression in rat muscle (Holness, Kraus et al. 2000). The elevated non esterified fatty acids (NEFA) under these dietary conditions appear to serve as ligands for peroxisome proliferators-activated receptors (PPARS) and modulate metabolism accordingly (Berger and Moller 2002). PPAR  $\alpha$  and PPAR  $\delta$  agonists consistently increased the transcript levels of PDK 4 mRNA in animal studies and *in vitro* systems using human muscle cells (Muoio, MacLean et al. 2002; Muoio,

Way et al. 2002; Abbot, McCormack et al. 2005). In contrast, during a hyperinsulinaemic-euglycaemic clamp, levels of both PDK4 and PDK2 mRNA transcripts decreased in response to insulin in Pima Indians (Majer, Popov et al. 1998). The signalling mechanisms by which insulin regulates PDK expression are not clear but may involve one or more of the signalling pathways activated by insulin. It has been suggested that the hormonal regulation of PDK4 expression occurs at the transcriptional level (Huang, Wu et al. 2002). Downstream insulin signalling targets such as protein kinase B (PKB/Akt) and transcription factors such as forkhead box factors (FOXO) appear to be involved in the regulation PDK4 gene expression (Kwon, Huang et al. 2004). FOXO factors (FOXO 1a and FOXO3a) are downstream targets of Akt, and phosphorylation of FOXO factors by Akt excludes it from the nucleus (Biggs, Meisenhelder et al. 1999; Burgering and Kops 2002). FOXO factors directly interact with three insulin response sequences on the promoter of the PDK4 gene and appear to play a key role in the regulation of this gene (Kwon, Huang et al. 2004).

Pyruvate Dehydrogenase Phosphatase (PDP) is responsible for dephosphorylation and hence reactivation of pyruvate dehydrogenase complex (PDC). There are two isoenzymes - PDP 1 and 2. PDP 1 is predominantly expressed in the muscle whereas PDP 2 is expressed in the liver (Huang, Gudi et al. 1998). Of the 2 isoforms, PDP 1 is sensitive to  $\text{Ca}^{2+}$  and is an important regulator of the activity state of PDC (Lawson, Niu et al. 1993), especially in response to exercise (McCormack, Halestrap et al. 1990). It has also been shown in many studies that rapid activation of PDC by insulin is mediated by activation of PDP (Denton, McCormack et al. 1996). However, It should be noted that in chronic insulin resistant states (ie diabetes), changes in the

activity of PDK rather than PDP are responsible for the the impairment in PDC activity normally observed under those conditions (Randle, Priestman et al. 1994).

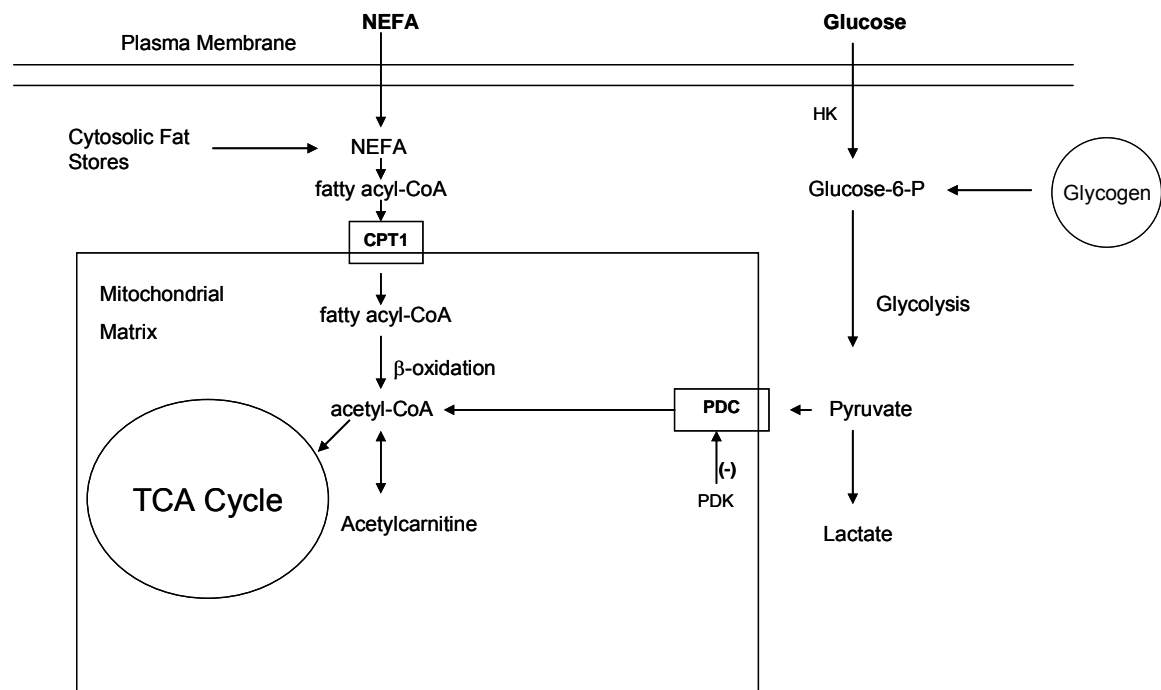


Fig. 1.1a. Oxidative pathways of glucose, glycogen and fatty acids. PDC: Pyruvate dehydrogenase complex; PDK: Pyruvate dehydrogenase kinase; NEFA: Nonesterified fatty acid, TCA: Tricarboxylic Citric acid cycle and CPT: carnitine palmitoyl acyl transferase.



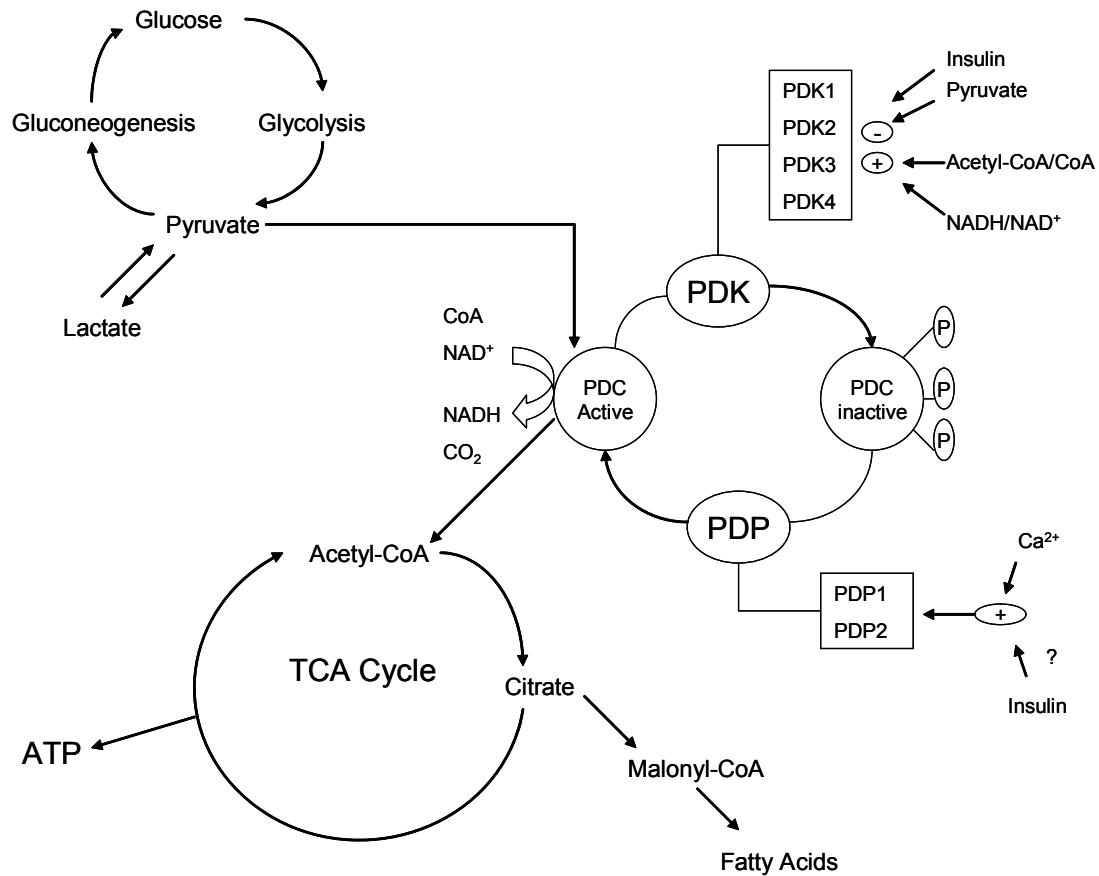


Fig.1.1b. The Pyruvate Dehydrogenase Complex. PDC: Pyruvate dehydrogenase complex; PDK: Pyruvate dehydrogenase kinase; NEFA: Nonesterified fatty acid; TCA: Tricarboxylic Citric acid cycle; PDP: Pyruvate dehydrogenase phosphatase and ATP: Adenosine Triphosphate

### **1.1.2 Insulin Signalling Network**

Insulin signalling impacts many cellular processes, including the metabolism of glucose, protein and lipids. Insulin signalling is triggered by the binding of insulin to its receptor in the plasma membrane of the cell. Once insulin binds to its transmembrane receptor, a complex sequence of proximal, intermediate and distal intracellular phosphorylated events transduces the binding signal to mediate insulin-induced expression of genes involved in glucose and lipid metabolism and a schematic representation is given in figure 1.1c (Biddinger and Kahn 2006). PI 3-kinase is a key intermediate in the network and activates the serine/threonine kinase Akt (also known as protein kinase B, PKB) and the atypical forms of PKC including PKC $\lambda$  and PKC $\zeta$  (Carpenter and Cantley 1996; Shepherd, Withers et al. 1998; Vanhaesebroeck and Alessi 2000). Akt has numerous and diverse intracellular targets (Nakae, Kitamura et al. 2001; Rommel, Bodine et al. 2001). Akt phosphorylates glycogen synthase kinase 3 (GSK3) and deactivates this protein. Deactivation of GSK3 leads to activation of glycogen synthase and results in stimulation of glycogen synthesis. Akt also phosphorylates a nuclear transcription factor FOXO (Forkhead box factors 1 and 3) and leads to its exclusion from the nucleus thereby preventing gene transcription of various enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Both Akt and atypical PKC's appear to promote insulin stimulated glucose uptake via increased translocation of GLUT 4 to the plasma membrane (Kohn, Summers et al. 1996; Bandyopadhyay, Standaert et al. 1997; Bandyopadhyay, Standaert et al. 1999).

Many of insulin's effects on the expression of genes in the glucose metabolic pathway are therefore regulated by FOXO factors, whereas effects on lipogenesis mainly

appear to be mediated via a nuclear transcription factor called sterol regulatory element-binding protein (SREBP)-1c (Horton, Goldstein et al. 2002). SREBPs are a family of three nuclear transcription factors and SREBP-1c is the dominant isoform in liver, adipose tissue and muscle. SREBP-1c is capable of activating the entire program of monounsaturated fatty acid synthesis. Induction of SREBP-1c transcript appears dependent on PI3-Kinase, through activation of PKC $\lambda$  (Azzout-Marniche, Becard et al. 2000; Matsumoto, Ogawa et al. 2002; Matsumoto, Ogawa et al. 2003). There is also evidence to show that SREBPs are also involved in the regulation of glucose metabolism since it interacts with the promoter of hexokinase II (HKII) and that the effect of insulin on HKII gene expression in muscle is mediated by SREBPs (Gosmain, Lefai et al. 2004; Gosmain, Dif et al. 2005).

Another major pathway for insulin signaling is the mitogen-activated protein (MAP) kinase pathway. The activities of MAP kinases such as extra cellular signal related kinases (ERK1 and 2) increase in response to insulin and exercise (Osman, Pendergrass et al. 2000). However, results of *in vitro* studies suggest that the effects of insulin on glucose metabolism are independent of MAP kinase pathway activity (Lazar, Wiese et al. 1995). Therefore, the physiological role of MAP kinase pathway activation by insulin is unclear.

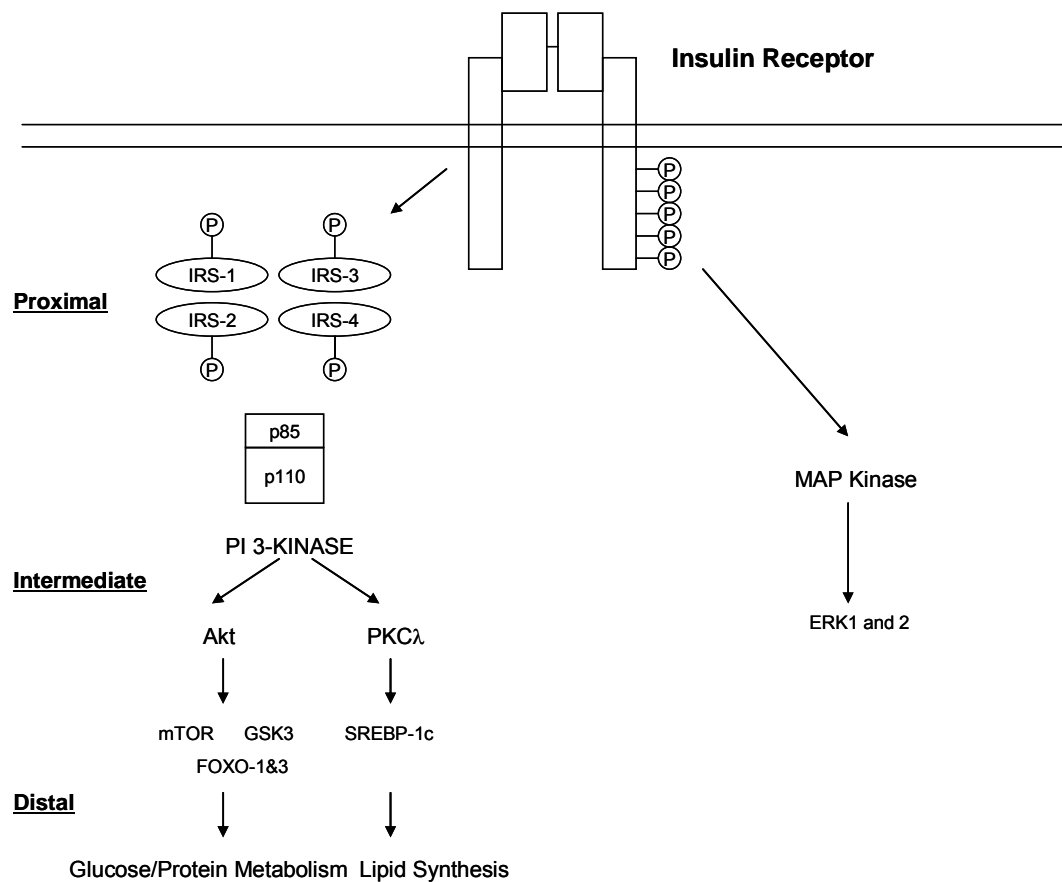


Fig.1.1c. Insulin signalling Network. IRS: Insulin receptor substrate; PI 3-Kinase: Phosphatidylinositol kinase; Akt: Protein kinase B; mTOR: mammalian target of rapamycin; GSK: Glycogen synthase kinase; FOXO: Forkhead transcription factor; PKC: protein kinase C; SREBP: Sterol regulatory element-binding protein and MAP: Mitogen-activated protein kinase, ERK: Extracellular signal related kinase.

### **1.1.3 Influence of high fat diet on glucose metabolism**

High fat diets were first implicated in the etiology of insulin resistance and impaired insulin mediated glucose metabolism in the 1930's (Himsworth 1935). It had been observed that the glycaemic response to a glucose tolerance test was affected by diet composition (Sweeney 1927) and the highest intolerance was observed in subjects who had consumed a very high fat diet. The first study addressing the effect of the composition/type of fat on glucose metabolism was reported in a patient with type 1 diabetes (Kinsell, Walker et al. 1959) who consumed a diet enriched firstly with safflower oil and subsequently synthetic palmitic acid-oleic acid triglyceride. The latter diet resulted in a decrease in the need for exogenous insulin to maintain glycaemia. Subsequently, classic studies by Randle et al (Garland, Newsholme et al. 1964; Randle, Newsholme et al. 1964; Boden, Jadali et al. 1991) showed that increasing NEFA availability inhibited glucose uptake in the rat hemi diaphragm, although the effect was nullified at high insulin concentrations. These studies led to the introduction of the concept of the glucose-fatty acid cycle and to a large number of subsequent investigations in animals and healthy humans. Crossectional and longitudinal studies have shown an increased association between diets rich in saturated fat intake and the development of insulin resistant states like type 2 diabetes and obesity (Marshall, Hoag et al. 1994; Mayer-Davis, Monaco et al. 1997; Tuomilehto, Lindstrom et al. 2001). In addition, short-term (up to 3 weeks) increase in saturated fat intake resulted in greater area under the curve after a glucose tolerance test (Marshall, Hoag et al. 1994; Uusitupa, Schwab et al. 1994; Mayer-Davis, Monaco et al. 1997; Lovejoy, Windhauser et al. 1998; Tuomilehto, Lindstrom et al. 2001) although the evidence is not altogether conclusive (Schwab, Niskanen et al. 1995; Fasching, Ratheiser et al. 1996; Louheranta, Turpeinen et al. 1998; Vessby, Unsitupa

et al. 2001). It could be hypothesized that this was due to variations in adiposity and physical fitness of study participants, and to differences in techniques used for the measurement of insulin action (Storlien, Baur et al. 1996; Louheranta, Turpeinen et al. 1999; Lichtenstein and Schwab 2000). However, controlling for these factors during short-term (up to 3 weeks) *in vivo* studies using iso-energetic high fat dietary interventions did not induce insulin resistance (Borkman, Campbell et al. 1991; Cutler, Gray et al. 1995; Louheranta, Turpeinen et al. 1999; Bisschop, de Metz et al. 2001; Lovejoy 2002). In summary, the metabolic studies in healthy humans where both the dietary fat level and fatty acid profile was modified did not consistently reduce glucose uptake. However, *in vivo* metabolic studies causing acute elevation of NEFA availability by means of an intralipid and heparin infusions readily induces impairment in insulin mediated glucose uptake (Thiebaud, DeFronzo et al. 1982; Ferrannini, Barrett et al. 1983; Boden, Jadali et al. 1991; Yki-Jarvinen, Puhakainen et al. 1991; Boden, Chen et al. 1994). The physiological significance of the latter studies should be interpreted with caution since NEFA concentrations used in these studies were in the supraphysiological range when compared to the physiological elevations in the short-term diet intervention studies. It is not known whether increasing the long-term availability of NEFA within the physiological range would result in similar changes in glucose metabolism. However, the lack of accurate tools to assess dietary fat intake in free-living humans make it difficult to carry out long-term intervention trials.

It had been suggested that the metabolic changes associated with high fat intake are mediated by changes in fatty acid composition of structural membrane lipids in skeletal muscle, which in turn would relate to insulin action (Borkman, Storlien et al. 1993; Vessby, Tengblad et al. 1994; Storlien, Baur et al. 1996; Storlien, Kriketos et al.

1997). Alternatively, it could be due to changes in both mitochondrial and peroxisomal fat oxidation but the evidence is not altogether conclusive (Jump and Clarke 1999; Papamandjaris, White et al. 2000; Piers, Walker et al. 2002). Finally, raising NEFA concentrations has been associated with interference to the insulin-signalling pathway, though the precise location is unclear. It has been observed that reduced muscle glucose uptake is associated with either decreased phosphatidylinositol (PI) 3-kinase activity (Dresner, Laurent et al. 1999) or increased protein kinase C activity (Itani, Ruderman et al. 2002). Accumulation of intracellular fatty acid metabolites such as diacyl glycerol possibly serves as a signal causing insulin resistance (Itani, Ruderman et al. 2002).

Although short-term, high-fat dietary intervention trials have not consistently demonstrated disturbances in whole body insulin mediated glucose uptake, there are effects on intracellular glucose metabolism both in rats (Kim, Wi et al. 1996) and in humans (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001). For example, very low carbohydrate (high fat) diets for 11 days in humans do not induce any changes in whole body glucose uptake both under basal and hyperinsulinaemic conditions (Bisschop, de Metz et al. 2001). However, the high fat diet induced a marked reduction in insulin stimulated glucose oxidation and increased non-oxidative glucose metabolism. The observed increase in non-oxidative glucose disposal has been attributed to an effective insulin stimulated glycogen synthesis due to an increase in glycogen synthase activity (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001) and/or due to increased nonoxidative glycolysis by net release of alanine and lactate (Yki-Jarvinen, Puhakainen et al. 1991).

Increased NEFA availability impairs the ability of insulin to suppress endogenous glucose production. During 11 days of high fat feeding in healthy humans endogenous glucose production was suppressed and the degree of suppression was inversely related to the fat content of the diet (Bisschop, de Metz et al. 2001) and NEFA concentrations (Lewis, Vranic et al. 1997). Insulin suppresses endogenous glucose production via direct effects on the liver, but also indirectly via a reduction in NEFA concentrations (Lewis, Zinman et al. 1996; Lewis, Vranic et al. 1997). Hence, it is likely that the increased NEFA availability inhibits the indirect effect of insulin, thereby causing hepatic insulin resistance.

#### **1.1.4 Non esterified fatty acid induced changes in intracellular regulation of substrate metabolism**

Randle and colleagues were the first to demonstrate that incubation of muscle with NEFA decreased insulin-stimulated glucose uptake (Randle, Garland et al. 1963). The inhibitory effect of NEFA on intracellular glucose metabolism is mediated by inhibition of PDC activity (Kelley, Mookan et al. 1993) and reduction of glycolytic flux via high citrate induced reduction in phosphofructokinase activity and subsequently hexokinase activity (Boden, Chen et al. 1994). Increasing NEFA availability is associated with increased muscle acetyl-CoA content and inhibits muscle PDC activity (Thiebaud, DeFronzo et al. 1982; Boden, Jadali et al. 1991; Kelley, Mookan et al. 1993). Inhibition of PDC activity would cause the intracellular glucose-6-phosphate (G-6-P) concentrations to rise with a decrease in hexokinase II activity and a consequent decrease in glucose uptake and glycogen synthesis. However, infusing intralipid results in supraphysiological concentrations of free fatty acids whereas there is a more physiological increase in free fatty acid concentrations during high fat diet interventions. In humans, high fat feeding has been shown to



result in decreases in both basal and insulin stimulated PDH activity (Putman, Spriet et al. 1993; Cutler, Gray et al. 1995; Peters, St Amand et al. 1998; St Amand, Spriet et al. 2000; Stellingwerff, Spriet et al. 2006) and has been attributed to increases in PDK activity (Peters, St Amand et al. 1998; Peters, Harris et al. 2001). However, there is no consistent evidence in humans that a reduction in PDH activity is associated with impaired insulin stimulated glucose uptake. Whole body insulin resistance is apparently induced by 3 days of high fat feeding (Bachmann, Dahl et al. 2001; Pehleman, Peters et al. 2005) but not by 11 to 21 days (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001). The details of these studies are described in table 1.1. It is possible that acute changes in dietary fat availability (several hours up to 3 days) might induce insulin resistance because of a greater imbalance between plasma NEFA availability and their muscle oxidation, whereas after several days an increase in NEFA availability can be compensated by a greater intramuscular lipid storage and/or utilisation. On the other hand, this difference may also be due to methodological differences in determining insulin resistance in the above studies (oral glucose tolerance test vs. insulin clamps; clamp duration and ambient insulin concentrations). Additional mechanisms, such as a direct effect of elevated NEFAs on early steps of glucose metabolism including glucose transport and phosphorylation have been proposed (Boden, Jadali et al. 1991). These studies showed discordance between the reduction in glucose oxidation and the delay in the inhibition of insulin stimulated glucose uptake. Recent studies using magnetic resonance spectroscopy did not show the increase in muscle glucose-6-phosphate concentrations during an intralipid infusion which might have been expected if fatty acids inhibited glucose metabolism (Roden, Price et al. 1996; Dresner, Laurent et al. 1999; Roden, Krssak et al. 1999). These observations suggest that mechanisms other than impaired glycolytic flux and

intramyocellular accumulation of G-6-P must account for the defect in insulin mediated glucose disposal following lipid infusion to elevate NEFA concentrations. NEFAs could primarily inhibit glucose transport and phosphorylation via interference with the initial steps of the insulin signalling cascade (Dresner, Laurent et al. 1999; Kruszynska, Worrall et al. 2002; Belfort, Mandarino et al. 2005). NEFA would appear to induce insulin resistance by blocking the activation of phosphatidyl-inositol-3-kinase associated with Insulin Receptor 1 (IRS-1) through an increase in intracellular fatty acyl-CoA and diacylglycerol concentrations. This results in the activation of serine kinase PKC- $\theta$ , causing serine phosphorylation of IRS-1. Serine phosphorylated IRS-1 cannot recruit PI-3 kinase and thus inhibits GLUT4 translocation leading to a reduction in insulin stimulated glucose transport (Yu, Chen et al. 2002).

**Table.1.1 Influence of high fat diet on carbohydrate metabolism**

<b>Author</b>	<b>Trial Duration</b>	<b>Metabolic Impact</b>	<b>Glucose Uptake Technique</b>	<b>Fat Content of Diet</b>
Borkman, 1991	3 weeks	No impact on glucose uptake	Clamp	45%
Cutler, 1995	3 Weeks	Decreased Carbohydrate oxidation, increased non-oxidative glucose disposal, no impact on glucose uptake	Clamp	75%
Bachman, 2001	3 days	Decreased glucose uptake	Clamp	
Bisschop, 2001	11 days	Decreased Carbohydrate oxidation, increased non-oxidative glucose disposal, no impact on glucose uptake	Clamp	44 and 83%
Pehleman, 2005	56 hours	Decreased glucose uptake	Glucose tolerance test	73%

## **1.2 Physiological and Metabolic responses to exercise**

Fat and carbohydrate are the principal substrates that fuel aerobic ATP synthesis in skeletal muscle. Carbohydrates are mainly stored as glycogen in skeletal muscle and liver. Endogenous carbohydrate stores are relatively small (~ 0.4 -0.5 kg) compared to fat (9 -15 kg in lean individuals). Muscle glycogen stores can be enhanced with carbohydrate loading (Bergstrom, Hermansen et al. 1967). In addition, small quantities of circulating glucose are available for oxidation. Fat is mainly stored as triacylglycerol in subcutaneous and visceral adipose tissue. A small amount is stored as intramyocellular triacylglycerol (IMTG), plasma non-esterified fatty acid (NEFA) bound to albumin and plasma triglyceride incorporated in circulating lipoprotein particles.

Exercise is a potent stimulator of human metabolism. Sensitive feedback and feed-forward neuroendocrine mechanisms are involved and these key metabolic processes are discussed in this section. The human body responds in an integrated fashion to meet both the substrate and oxygen demands of the working muscle while maintaining whole body homeostasis. NEFA, muscle glycogen, blood glucose and triglycerides (intramuscular and lipoprotein derived) contribute substantially to meet exercise-induced energy needs (Romijn, Coyle et al. 1993; van Loon, Greenhaff et al. 2001). The metabolic responses to exercise are dependent on intensity and duration of exercise and are classified in table 1.2a (Zinman, Ruderman et al. 2004). During exercise of varying intensities (25 -85 %  $\text{VO}_2$  max), muscle glycogen and plasma glucose are the major fuels consumed and the rate of glycogenolysis is most rapid during the first 5-10 minutes of exercise (Hultman 1967; Romijn, Coyle et al. 1993; van Loon, Greenhaff et al. 2001). The ATP needs of contracting muscle are met initially by oxygen-independent ATP production (PCr hydrolysis and anaerobic

glycolysis) due to the delay in oxygen dependent ATP synthesis (Bangsbo, Gollnick et al. 1990). The lag in oxygen dependent ATP resynthesis was initially attributed to inertia in skeletal muscle blood flow and thereby oxygen delivery to contracting muscles (Knight, Schaffartzik et al. 1993; Richardson, Knight et al. 1995). However, subsequent investigations suggested that mitochondrial ATP synthesis is not limited by blood flow but due to metabolic inertia (acetyl group deficit) in mitochondrial ATP production (Grassi, Gladden et al. 1998; Greenhaff, Campbell-O'Sullivan et al. 2002; Roberts, Loxham et al. 2002). As exercise continues and blood flow to muscle increases, blood borne substrates become increasingly important sources of energy. The total body turnover of glucose is increased two, three and five times during mild, moderate and severe exercise respectively. Depending on the exercise intensity and duration, muscle glucose uptake increases substantially (Wahren, Felig et al. 1971) and accounts for 30 to 40 % of the total oxygen consumed by muscle. During low intensity exercise (~ 30% of maximal oxygen uptake) for 4 hours, the rate of glucose utilization peaks at 90 to 180 minutes of exercise and then declines as free fatty acid utilization increases to meet energy needs (Ahlborg, Felig et al. 1974). It was previously thought that NEFAs provided the majority of the fat oxidized by skeletal muscle during low and moderate intensity exercise (Romijn, Coyle et al. 1993; van Loon, Greenhaff et al. 2001). However, it was subsequently shown that the contribution of plasma derived fatty acid oxidation was probably overestimated and conversely triglyceride oxidation was underestimated (van Loon, Greenhaff et al. 2001). There is an equal contribution from plasma NEFA and triglyceride fat sources (intramyocellular and circulating lipids) to energy needs irrespective of exercise intensity after an overnight fast. However, when exercise intensity increased to 75%  $\dot{V}O_2$  max, whole body fat oxidation reduced by 30% and this was accounted for by a

reduction in utilisation of both plasma NEFA and total triglycerides (intramuscular and lipoprotein derived). The type of substrate used during exercise of differing intensities is summarized in figure 1.2a (Ahlborg, Felig et al. 1974; Romijn, Coyle et al. 1993; van Loon, Greenhaff et al. 2001).

**Table 1.2 Classification of exercise intensity, based on physical activity lasting up to 60 min (Zinman, Ruderman et al. 2004).**

Intensity	Relative intensity		
	VO <sub>2max</sub> (%)	% Of Maximal Heart	RPE <sup>#</sup>
		Rate *	
<b>Very light</b>	<20	<35	<10
<b>Light</b>	20-39	35-54	10-11
<b>Moderate</b>	40-59	55-69	12-13
<b>Hard</b>	60-84	70-89	14-16
<b>Very hard</b>	>85	>90	17-19
<b>Maximal</b>	100	100	20

\*Maximal heart rate = 220 – Age. <sup>#</sup> Borg rating of perceived exertion (RPE) 6-20 scale.

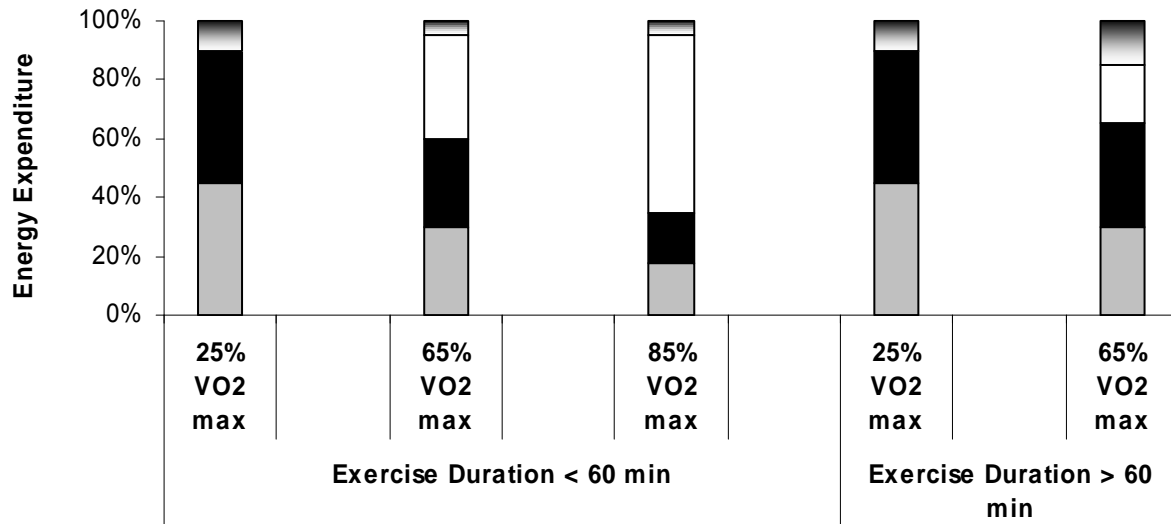


Fig.1.2a. Contribution of different substrates to energy expenditure during exercise. Adapted from Romjin, Coyle et al.1993 and van Loon et al.2001. Open Column-muscle glycogen, closed column-plasma nonesterified fatty acid, black and white column-plasma glucose and grey column-intra muscular/lipoprotein derived triglyceride oxidation.

### **1.2.1 Hormonal responses to exercise**

The hormonal response to exercise is characterized by a fall in plasma insulin concentration (Hunter and Sukkar 1968; Wahren, Felig et al. 1971) and a rise in glucagon, effects that are more pronounced in prolonged or severe exercise (Felig, Wahren et al. 1972; Ahlborg, Felig et al. 1974). Other hormonal changes include elevations of catecholamine, growth hormone and cortisol (Hartley, Mason et al. 1972; Tuttle, Marker et al. 1988). The decrease in insulin is particularly noted in severe exercise despite a modest rise in glucose, suggesting an inhibition in insulin secretion, mediated by the sympathetic nervous system (Wahren, Felig et al. 1971).

The fall in insulin (Wolfe, Nadel et al. 1986; Wasserman, Spalding et al. 1989) and rise in glucagon (Wasserman, Spalding et al. 1989; Hirsch, Marker et al. 1991) are the major determinants of hepatic glucose production during moderate exercise. Insulin secretion is reduced by increased  $\alpha$ -adrenergic inhibition of the pancreatic  $\beta$  cells (Hermansen, Pruett et al. 1970). The fall in insulin is necessary to facilitate hepatic glycogenolysis (Wasserman, Williams et al. 1989) and the rise in glucagon is required for the full increment in both hepatic glycogenolysis and gluconeogenesis (Wasserman, Spalding et al. 1989). Prevention of this physiological response with somatostatin infusion results in attenuation of hepatic glucose output (Wolfe, Nadel et al. 1986; Hirsch, Marker et al. 1991). The fall in insulin is also required to permit increased lipolysis (Wahrenberg, Engfeldt et al. 1987; Wasserman, Lacy et al. 1989).

Adrenaline plays only a minor role in regulating hepatic glucose production as long as the islet hormone responses are intact (Hoelzer, Dalsky et al. 1986). Combined  $\alpha$  and  $\beta$  blockade does not significantly reduce exercise-induced (40%  $\dot{V}O_2$  max) hepatic



glycogenolysis in healthy humans (Simonson, Koivisto et al. 1984; Hoelzer, Dalsky et al. 1986). In contrast, during high intensity exercise rapid and marked elevation in catecholamines is observed (Calles, Cunningham et al. 1983; Marliss, Simantirakis et al. 1991; Marliss, Simantirakis et al. 1992; Purdon, Brousson et al. 1993). This suggests that increased sympatho-adrenal activity may play a more important role in regulating glucose homeostasis during high intensity exercise.

Catecholamines are also responsible for the stimulation of carbohydrate flux by increasing skeletal muscle glycogenolysis and PDC activation (Watt, Howlett et al. 2001). The increased glycogenolysis appears to be regulated by increased glycogen phosphorylase transformation but the mechanism for increased PDC activity was not clear. However, in the same study glucose uptake during exercise was attenuated by adrenaline infusion and this response is likely to be due to increased intracellular glucose-6-phosphate leading to a decrease in glucose phosphorylation. Catecholamines are also responsible for the stimulation of lipolysis and release of free fatty acids during exercise (Issekutz 1978; Wahrenberg, Engfeldt et al. 1987; Wasserman, Lacy et al. 1989).

In humans there appears to be some redundancy in hormonal responses to exercise (Cryer, Davis et al. 2003). When the fall in insulin and rise in glucagon are prevented simultaneously during exercise, plasma glucose concentrations fall to 2.1mmol/l but then rise and plateau at 3.4 mmol/l probably due to other counter regulatory hormones such as adrenaline (Hirsch, Marker et al. 1991; Marker, Hirsch et al. 1991). However, if changes in islet hormones are prevented together with adrenergic blockade, exercise results in hypoglycaemia (Hoelzer, Dalsky et al. 1986; Tuttle, Marker et al. 1988).

### **1.2.2 Glycaemic response to exercise**

The glycaemic response to exercise is dependent on the intensity and duration of exercise. The concentration of blood glucose changes little in short-term mild to moderate exercise (Wahren, Felig et al. 1971). On the other hand, when the intensity of exercise is high, plasma glucose concentrations initially increase but later decrease as exercise continues (Calles, Cunningham et al. 1983; Marliss, Simantirakis et al. 1992) although genuine hypoglycaemia is rare (Ahlborg, Felig et al. 1974). Plasma glucose homeostasis is maintained by an increase in hepatic glucose output that balances the increase in glucose uptake by working muscles (Ahlborg, Felig et al. 1974). A reduction in insulin concentration and/or an increase in glucagon concentration is necessary if plasma glucose homeostasis is to be maintained (Wolfe, Nadel et al. 1986). When changes in insulin and glucagon were prevented, plasma glucose concentration fell. Any deviation from euglycaemia is usually a result of a mismatch between hepatic glucose production and peripheral glucose utilization.

The liver occupies a pivotal position for maintaining glucose homeostasis. It is able to store large amounts of glycogen and release glucose by glycogenolysis. In addition, the liver can convert substrates such as lactate, glycerol and amino acids to glucose by gluconeogenesis. A large body of information on hepatic glucose metabolism has been derived from experimentation with dogs. Subsequently, the availability of stable radioisotopes allowed the quantification of glucose kinetics and oxidation in healthy humans (Wolfe 1984). Recently  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy has become increasingly available and has made real time noninvasive and repetitive *in vivo* measurements of human hepatic glycogen concentrations possible, permitting the calculation of rates of net hepatic glycogen synthesis and glycogenolysis.

Hepatic glucose production is closely linked to the rate of muscle glucose uptake during exercise and in the fasted state is the sole site of glucose production and release into the blood stream (Wahren, Felig et al. 1971). During exercise (40 min), splanchnic glucose output increases 2 to 5 fold depending on exercise intensity. The increased glucose production with increasing exercise intensity is accounted for by the increase in the rates of net hepatic glycogenolysis whereas the rate of gluconeogenesis remained constant (Petersen, Price et al. 2004). However, with prolonged exercise an increasing contribution is derived from gluconeogenesis (Ahlborg, Felig et al. 1974). During high intensity exercise, the increase in hepatic glucose production can exceed the rise in glucose utilization (Calles, Cunningham et al. 1983; Marliss, Simantirakis et al. 1991). In this situation, the marked rise in glucose production is predominantly related to a sharp increase in catecholamines rather than to changes in insulin and glucagon levels (Marliss, Simantirakis et al. 1991; Marliss, Simantirakis et al. 1992; Marliss and Vranic 2002). In addition, the marked increase in catecholamines results in increased muscle glycogenolysis and increased glucose-6-phosphate (Katz, Sahlin et al. 1991). This is likely to feedback negatively on hexokinase and therefore glucose transport. Hence, a combination of increased hepatic glucose output and reduced glucose disposal results in an increase in arterial glucose levels that extends into the post exercise-state.

The glycaemic response to exercise also depends on the pre-exercise diet. Plasma glucose concentrations are higher following a high carbohydrate diet than a high fat diet (Galbo, Holst et al. 1979). This possibly relates to a higher hepatic glycogen store when fed carbohydrates compared with a high fat diet. A high carbohydrate diet can increase muscle glycogen stores and thereby improve endurance (Sherman, Costill et

al. 1981). Carbohydrate intake before exercise has been shown to increase the relative contribution of carbohydrate oxidation to total energy expenditure and a concomitant reduction in fat oxidation during subsequent exercise (Montain, Hopper et al. 1991; Horowitz, Mora-Rodriguez et al. 1997). The glycaemic response during exercise is also dependent on the glycaemic index of the pre-exercise meal. Exercise (30 min treadmill exercise at 70% maximal oxygen uptake) after a high glycaemic index meal is associated with an initial decline in blood glucose concentrations followed by a slow increase towards the end of exercise (Wee, Williams et al. 2005). Carbohydrate supplementation during exercise increases endurance performance by increasing plasma glucose availability, reducing endogenous glucose production and increasing plasma glucose uptake and oxidation (Jeukendrup, Raben et al. 1999; Jeukendrup, Wagenmakers et al. 1999). Carbohydrate supplementation during exercise could spare muscle glycogenolysis especially under hyperinsulinaemic conditions but the evidence is inconsistent (Tsintzas and Williams 1998). Muscle glycogen sparing is observed during running and intermittent cycling but not during constant cycling.

Finally, endurance training also influences the glycaemic response to exercise. Trained individuals are better able to maintain a stable plasma glucose concentration during prolonged exercise than untrained individuals (Koivisto, Hendler et al. 1982; Jansson and Kaijser 1987). It has also been shown that at a given moderate to high intensity exercise, trained subjects increase their plasma glucose concentration more than untrained subjects (Kjaer, Farrell et al. 1986). Endurance training also decreases plasma glucose turnover and oxidation, and increases fat oxidation during moderate intensity exercise (Karlsson, Nordesjo et al. 1974; Jansson and Kaijser 1987; Coggan, Kohrt et al. 1990).

### 1.2.3 Muscle metabolism

Anaerobic ATP synthesis (PCr hydrolysis and glycogenolysis) meets the initial energy demands at the onset of exercise and also during high intensity exercise. At rest and during exercise lasting more than 10 min, the vast majority of ATP required for muscle contraction is generated through oxidative phosphorylation (aerobic ATP resynthesis). The principal substrates are carbohydrate and fat and the process is schematically represented in figure 1.1a. Within the muscle cell, glucose or glycogen is first converted to glucose-6-phosphate and then to pyruvate via glycolysis. Pyruvate then enters the mitochondria and is converted to acetyl-CoA by PDC. Fat stores in the form of triacylglycerol (TG) are first hydrolysed to glycerol and non-esterified fatty acids (NEFAs). NEFAs are subsequently activated to fatty acyl-CoA and transported across the mitochondrial membrane by a carnitine dependent transport system. Inside the mitochondria, fatty acyl-CoA is cleaved in a stepwise fashion to acetyl-CoA ( $\beta$ -oxidation). From this point on, fat and carbohydrate metabolism follow the same biochemical pathways. Acetyl-CoA enters the citric acid cycle (TCA-cycle) to proceed through a series of biochemical reactions coupled to the electron transport system. The electrochemical energy generated is used to couple Adenosine Diphosphate (ADP) and inorganic phosphorous (Pi) to form ATP. 1 mol of glucose generates 36 mol of ATP whereas 1 mol of palmitate generates 130 mol of ATP.

Muscle glycogen content decreases substantially during increasing exercise intensity and the time to exhaustion is proportional to pre-exercise glycogen content (Bergstrom, Hermansen et al. 1967; Bergstrom and Hultman 1967; Hermansen, Hultman et al. 1967; Price, Rothman et al. 1994; van Loon, Greenhaff et al. 2001). Muscle glycogen availability is implicated with the onset of fatigue and hence

determines exercise capacity during prolonged moderate to high intensity exercise (Hultman and Greenhaff 1991; Febbraio and Dancy 1999; Hargreaves 2004). This possibly relates to the inability of glycogen depleted muscle to maintain the required rate of oxidative ATP resynthesis (Sahlin, Katz et al. 1990).

Carbohydrate oxidation increases with increasing exercise intensity with a reciprocal decline in fat oxidation (Romijn, Coyle et al. 1993; van Loon, Greenhaff et al. 2001). Similarly, there are reciprocal changes in carbohydrate and fat oxidation during increased NEFA availability (Boden, Jadali et al. 1991). Multiple mechanisms regulate the relative contribution of fat and carbohydrate oxidation under varying physiological conditions. Initially, Randle's glucose-fatty acid cycle (Randle, Garland et al. 1963) was proposed in order to explain the reduction in carbohydrate oxidation and increase in fat oxidation during increased NEFA availability. Under those conditions, a fat-induced suppression of PDC activation could lead to a decrease in glycolytic flux and glucose transport. However, the glucose-fatty acid cycle does not appear to operate during high intensity exercise (Dyck, Putman et al. 1993; Romijn, Coyle et al. 1995). Secondly, high glycolytic rates as observed during high intensity exercise could limit fat oxidation (Odland, Howlett et al. 1998) by increased accumulation of acetyl-CoA (Constantin-Teodosiu, Carlin et al. 1991) leading to increases in the cytosolic production of malonyl-CoA. Malonyl-CoA is the product of Acetyl-CoA carboxylase reaction and is found in liver, heart, adipose tissue (McGarry, Mills et al. 1983). Malonyl-CoA is the first intermediate in the synthesis of long chain fatty acids (LCFA). Malonyl-CoA allosterically binds to Carnitine Palmitoyl Transferase (CPT-1), inhibiting the enzyme and the transfer of LCFA into the mitochondria for fat oxidation. However, the evidence is inconsistent, since subsequent exercise studies have not demonstrated a rise in Malonyl-CoA (Dean,

Daugaard et al. 2000). Finally, free muscle carnitine availability could explain the decrease in fat oxidation that occurs during high intensity exercise (van Loon, Greenhaff et al. 2001). Carnitine is a co-factor for the transport of LCFA across the inner mitochondrial membrane and the detailed role of carnitine in the regulation of muscle substrate use is reviewed in detail elsewhere (Stephens, Constantin-Teodosiu et al. 2007). Carnitine acts as a sink for acetyl group storage during high flux through the PDC reaction, i.e. when the rate of acetyl group production exceeds its rate of its utilisation by the tricarboxylic acid cycle (Sahlin 1990; Constantin-Teodosiu, Carlin et al. 1991). Hence, under those conditions limited free carnitine availability could compromise the transport of LCFA across the inner mitochondrial membrane and lead to the decline in fat oxidation observed during high intensity exercise.

### **1.3 Influence of type 1 diabetes on substrate metabolism during exercise**

Glucoregulation during various types of exercise is quite variable and can be difficult to predict in patients with type 1 diabetes. The normal physiological response to exercise cannot occur in diabetic individuals since regulation of insulin release has been lost. This results in a mismatch in hepatic glucose production and muscle glucose utilization. In addition, type 1 diabetes is characterised by increased lipolysis and this is more apparent during exercise (Wahren, Hagenfeldt et al. 1975; Jensen, Caruso et al. 1989; Wahrenberg H 1989). During moderate exercise (45%  $\dot{V}O_2$  max), carbohydrate oxidation was significantly lower in patients with type 1 diabetes when compared to controls (Raguso, Coggan et al. 1995). On the other hand, fat oxidation was higher in patients with type 1 diabetes when compared to controls, the main source apparently being intramuscular triglycerides. In contrast, during intense

exercise (75%  $\dot{V}O_2$  max) substrate oxidation was similar between controls and patients with type 1 diabetes. Hence, during moderate exercise there is a shift from carbohydrate to fat metabolism in patients with type 1 diabetes but these differences appear to be resolved during high intensity exercise (Fig.1.3a). In addition to intensity and duration of exercise, the physiological and metabolic response to exercise are dependent on factors unique to patients with diabetes such as preceding glycaemic control, circulating insulin concentrations, insulin injection site and counter regulatory failure. The following sections discuss these factors.



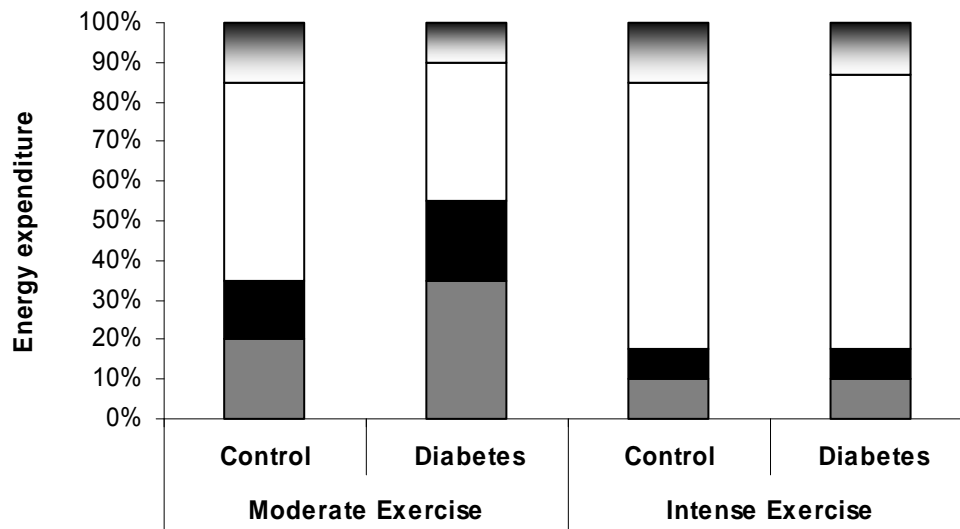


Fig.1.3a. Contribution of different substrates to energy expenditure during 30 min of moderate (45%  $\text{VO}_2$  max) and intense (75%  $\text{VO}_2$  max) exercise in controls and patients with type 1 diabetes. Circulating insulin concentrations during exercise were significantly higher (~3 fold) in the diabetes group when compared to control. Adapted from Raguso et al.1995. Open column-muscle glycogen, closed column-plasma non-esterified fatty acid, black and white column-plasma glucose and grey column-intra muscular/lipoprotein derived triglyceride oxidation.

### **1.3.1 Hyperinsulinaemia**

It is clearly impossible to replicate the fall in insulin concentration that occurs during exercise in non-diabetic individuals, by using exogenous subcutaneous insulin injections in patients with type 1 diabetes. Most exercise in patients with type 1 diabetes will therefore be undertaken at higher than physiological insulin concentrations. In addition, the absorption of subcutaneously administered insulin can be exaggerated by exercise (Zinman, Murray et al. 1977; Koivisto and Felig 1978) and by inadvertent intramuscular injection (Frid, Ostman et al. 1990). Hence, patients with type 1 diabetes who do not make appropriate adjustments in insulin dosage risk becoming hypoglycaemic during exercise and this risk increases with duration and intensity of exercise (Schiffrin and Parikh 1985; Rabasa-Lhoret, Bourque et al. 2001).

Hyperinsulinaemic conditions during exercise in healthy volunteers can both increase peripheral glucose uptake (DeFronzo, Ferrannini et al. 1981; Wasserman, Geer et al. 1991) and suppress splanchnic glucose output (Zinman, Murray et al. 1977) with a consequent risk of exercise-induced hypoglycaemia if the exercise period is sufficiently long (Wasserman and Zinman 1994). The direct and indirect suppressive effect of peripheral hyperinsulinaemia on hepatic glucose output has been extensively investigated in dogs (Sindelar, Balcom et al. 1996; Lewis, Vranic et al. 1997; Sindelar, Chu et al. 1997; Cherrington, Edgerton et al. 1998; Sindelar, Chu et al. 1998). An increase in portal insulin concentration rapidly inhibits net hepatic glucose output caused by the suppression of hepatic glycogenolysis (direct effect). An increase in peripheral insulin concentrations caused a similar reduction in hepatic glucose output due to the suppression of gluconeogenic precursor uptake by the liver (indirect effect). However, there are limited studies in humans.

### **1.3.2 Underinsulinisation (Chronic and Acute)**

The metabolic effects of exercise are dependent on the prevailing degree of metabolic control (Berger, Berchtold et al. 1977). In moderately controlled patients (glycated haemoglobin between 8 and 9.5%), exercise induced a reduction in blood glucose but increased blood levels of NEFA and lactate. In ketotic patients where insulin levels are particularly low, exercise induced an additional increase in blood glucose concentration, lactate, ketone body and branched chain amino acid formation. A greater fraction of the glucose released by the liver during exercise is gluconeogenic in origin under conditions of insulin deficiency (Wahren, Hagenfeldt et al. 1975). The rise in blood glucose stems from an impairment in the exercise-induced increase in glucose utilization accompanied by normal hepatic glucose production (Vranic and Wrenshall 1969).

A preferential utilization of NEFA over carbohydrate is observed in patients with type 1 diabetes during moderate exercise in insulin deficient states (Wahren, Hagenfeldt et al. 1975; Standl, Lotz et al. 1980; Wahren, Sato et al. 1984) and this possibly relates to increased NEFA availability under these conditions. A shift from fat to carbohydrate utilization can be achieved by increasing the availability of insulin during moderate exercise (Zinman, Murray et al. 1977; Raguso, Coggan et al. 1995).

### **1.3.3 Hypoglycaemia**

Hypoglycaemia (Blood glucose < 3.5 mmol/l) can occur not only during exercise (Berger, Berchtold et al. 1977; Sonnenberg, Kemmer et al. 1990; Kemmer 1992) but also for up to 24 h afterwards (MacDonald 1987; Hernandez, Moccia et al. 2000). This is attributable to a combination of several factors unique to patients with type 1 diabetes such as relative hyperinsulinaemia during exercise (Zinman, Murray et al. 1977; Koivisto and Felig 1978; Shilo, Sotsky et al. 1990; Rabasa-Lhoret, Bourque et al. 2001); reduced mobilization of endogenous hepatic glycogen stores (Cline, Rothman et al. 1994; Hwang, Perseghin et al. 1995; Kishore, Gabriely et al. 2006) and counter regulatory secretory deficits during exercise (Cryer 1985; Davis, Mann et al. 2000; Galassetti, Tate et al. 2003).

Furthermore, a vicious cycle (Fig.1.3b) of exercise and hypoglycaemia has been proposed (Ertl and Davis 2004), whereby an episode of hypoglycaemia or exercise can feed forward to down regulate neuroendocrine and autonomic nervous system responses to a subsequent episode of either stress, thereby increasing the risk of further hypoglycaemia (Galassetti, Mann et al. 2001; Galassetti, Tate et al. 2003). However, the underlying physiological mechanism remains unclear. Hence, reducing pre exercise insulin levels and adjusting carbohydrate intake prior to, during, and following exercise in conjunction with self-monitoring of blood glucose are essential to avoid the vicious cycle of blunted counter regulatory responses to exercise and hypoglycaemia.

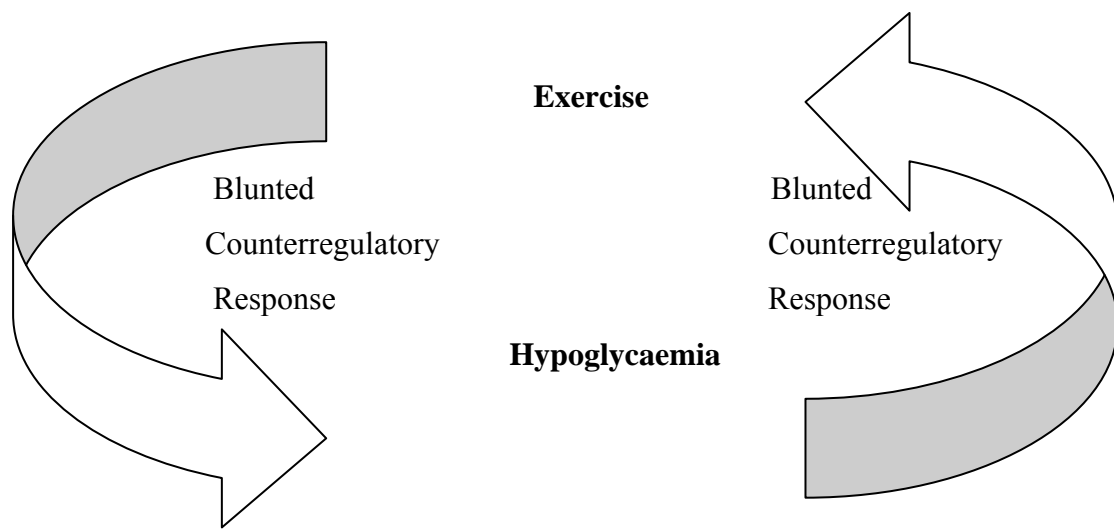


Fig.1.3b. Vicious cycle of exercise and hypoglycaemia

#### **1.3.4 Counterregulatory hormones**

Counterregulatory response is progressively blunted with increasing duration of diabetes, irrespective of the presence or absence of autonomic neuropathy (Fanelli, Pampanelli et al. 1997; Meyer, Grossmann et al. 1998). In well controlled patients with diabetes on intensive insulin therapy, counterregulatory response is also impaired and this has been attributed to deficient glucagon response (Cryer and Gerich 1985; Amiel, Tamborlane et al. 1987). However, in poorly controlled patients with type 1 diabetes but preserved autonomic response, there is an increased catecholamine, glucagon, cortisol and growth hormone response to exercise (Berger, Berchtold et al. 1977; Tamborlane, Sherwin et al. 1979). Patients with autonomic neuropathy (Schneider, Vitug et al. 1991; Bottini, Boschetti et al. 1997) demonstrate blunted catecholamine responses irrespective of the degree of metabolic control.

The role of counter regulatory hormones (glucagon and catecholamines) during exercise is dependent on ambient insulin concentrations (Wasserman and Zinman 1994). The glucagon response is exaggerated when insulin availability is reduced and results in increased glucose output, increased ketogenesis and worsened metabolic control. Increasing insulin availability is associated with reduced glucagon response, thereby leading to decreased hepatic glucose output and increasing the risk of hypoglycaemia. In the absence of advanced autonomic neuropathy, catecholamine responses are exaggerated irrespective of insulin availability during exercise-induced hypoglycaemia. This results in increased lipolysis, impaired glucose utilisation and increased hepatic glucose output and thereby helps to prevent severe hypoglycaemia.

### **1.3.5 Endogenous glycogen stores**

Fasting hepatic glycogen concentrations are consistently lower in patients with type 1 diabetes than in non-diabetic subjects with similar dietary regimens (Petersen, Price et al. 2004; Kishore, Gabriely et al. 2006). Moreover, studies using  $^{13}\text{C}$  magnetic resonance spectroscopy report a defect in liver glycogen synthesis following a mixed meal in poorly-controlled patients with type 1 diabetes (Hwang, Perseghin et al. 1995). Moreover, poorly controlled diabetes is associated with impaired insulin-stimulated muscle glycogen metabolism (Cline, Magnusson et al. 1997). Net rates of muscle glycogen synthesis were reduced in patients with type 1 diabetes when compared to controls. The reduced synthesis was attributed to a reduction in flux through glycogen synthase and a reduction in glucose transport and phosphorylation.

The hepatic glycogen synthetic deficit can be corrected with combined long and short-term improvements in glycaemia (Bischof, Bernroider et al. 2002) but only partially corrected with short-term improvement in glycaemic control (Bischof, Krssak et al. 2001). On the other hand, hyperinsulinaemic and hyperglycaemic conditions also corrected hepatic glycogen synthesis in patients with poorly controlled diabetes (Cline, Rothman et al. 1994). These data suggest that hepatic glycogen synthesis can be normalized by hyperglycaemic and hyperinsulinaemic conditions in patients with type 1 diabetes.

The resting rate of hepatic glucose production is greater in patients with type 1 diabetes than in healthy volunteers and increases in proportion to exercise intensity similar to control subjects (Petersen, Price et al. 2004). The contribution of net hepatic glycogenolysis to glucose production is consistently lower when compared to controls

and the exaggerated rates of glucose production could be entirely accounted by gluconeogenesis.

### **1.3.6 Glycaemic response to exercise**

The glycaemic response to exercise in patients with type 1 diabetes is dependent on both the exercise intensity and ambient insulin concentrations at the time of exercise. However, the major determinant in patients with type 1 diabetes is probably insulin concentration at the time of exercise. The route of insulin administration, delivery device (continuous subcutaneous infusion vs. multiple daily injections), dose and pharmacokinetics determine the ambient insulin concentrations. The resulting variations in serum insulin concentrations and exercise intensity result in variable glycaemic responses to exercise (Zinman, Vranic et al. 1979; Schiffrin and Parikh 1985; Ruegamer, Squires et al. 1990; Sonnenberg, Kemmer et al. 1990; Rabasa-Lhoret, Bourque et al. 2001; Mauvais-Jarvis, Sobngwi et al. 2003). The variable response to exercise is highlighted in figures 1.3 c-f. While mild to moderate intensity exercise may cause both acute and delayed hypoglycaemia, high intensity exercise may result in sustained hyperglycaemia during (Mitchell, Abraham et al. 1988) and after exercise (Purdon, Brousson et al. 1993).

Although not quantified in adults, the incidence of hypoglycaemia during an acute bout of moderate intensity exercise in children and adolescents with type 1 diabetes is reported as 30% (Tansey, Tsalikian et al. 2006). The basal-bolus insulin regimen with both long acting insulin (Levemir or Lantus) as basal insulin and a quick acting preparation (Novorapid or Humalog) for prandial use offers some advantages for those who want to undertake postprandial exercise. Hence, glucose homeostasis can potentially be preserved during postprandial exercise of different intensities and



different durations by appropriate reduction of prandial insulin. There are some pragmatic algorithms available for patients to adjust insulin therapy to counter glycaemic excursions during and after exercise (Table 1.3b). However, the physiological responses to exercise and hence the factors contributing to exercise-induced hypoglycaemia in patients with type 1 diabetes are not fully understood and needs further study.

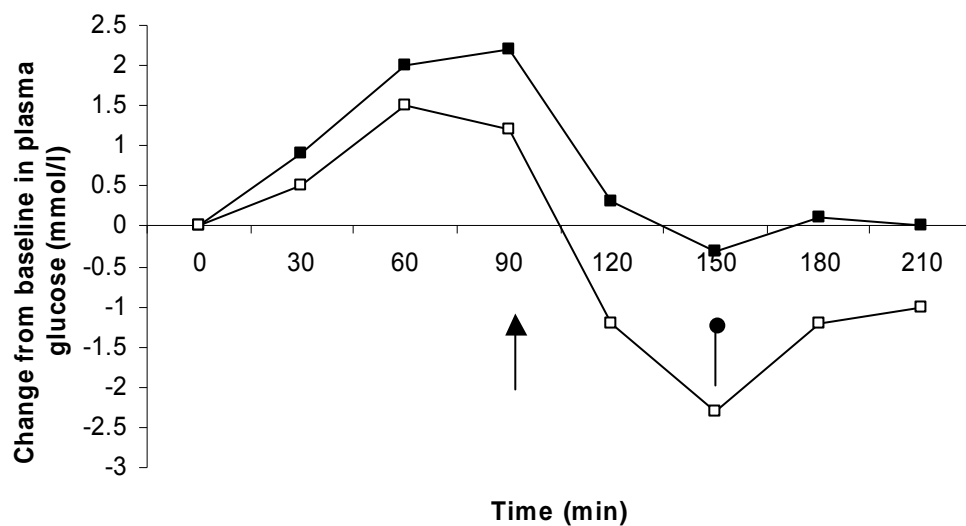


Fig.1.3c. Changes in plasma glucose before, during and after exercise at 25% of  $\text{VO}_2$  max for 60 min (▲-Beginning of exercise and ●-End of Exercise). (■) Pre meal insulin dose was reduced by 50%; baseline plasma glucose was ~ 6 mmol/l. (□) No reduction in premeal insulin dose; baseline plasma glucose was ~ 8.8 mmol/l. (Adapted from Rabasa-Lhoret et al, 2001).

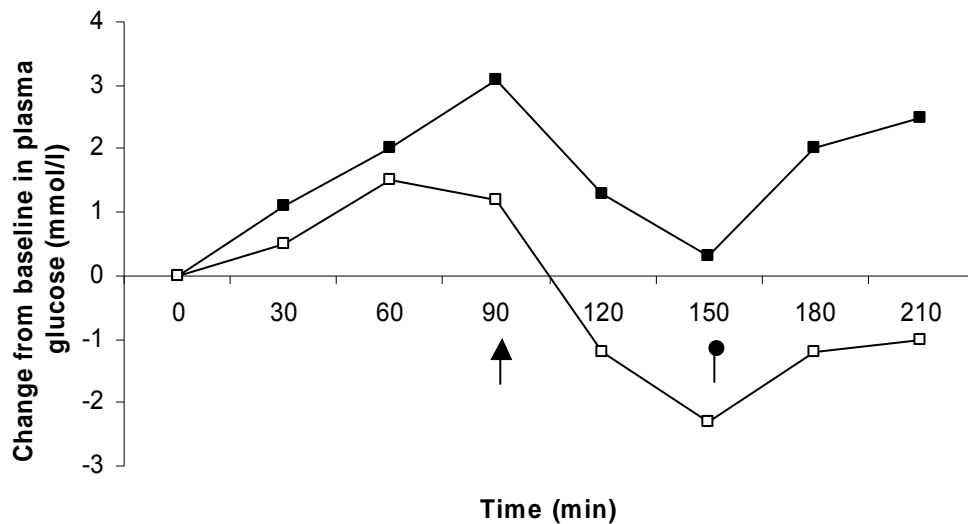


Fig.1.3d. Changes in plasma glucose before, during and after exercise at 50% of  $\text{VO}_2$  max for 60 min (▲-Beginning of exercise and ●-End of Exercise). (□) No reduction in pre meal insulin dose; baseline plasma glucose was  $\sim 10.7$  mmol/l. (■) Premeal insulin dose was reduced by 50%; baseline plasma glucose was  $\sim 9.4$  mmol/l.

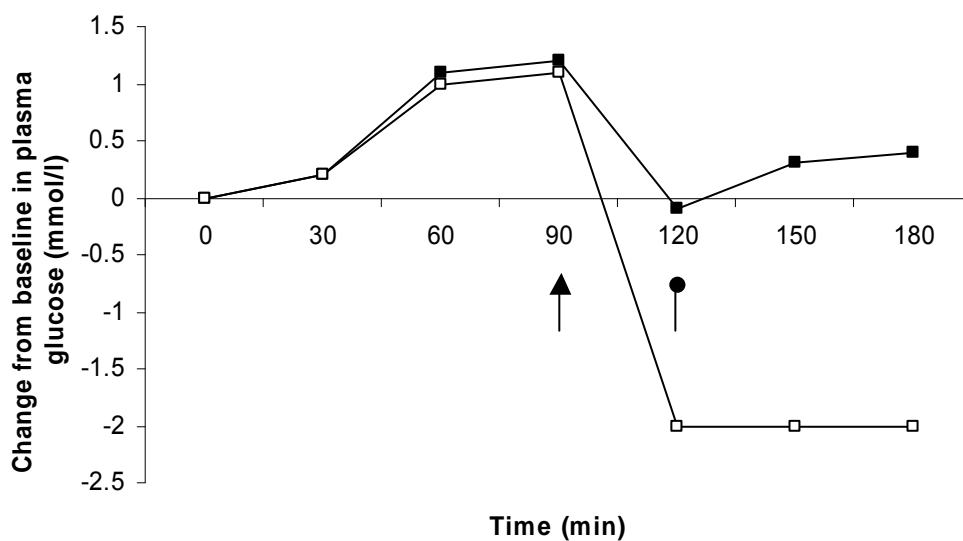


Fig.1.3e. Changes in plasma glucose before, during and after exercise at 50% of  $\text{VO}_2$  max for 30 min (▲-Beginning of exercise and ●-End of Exercise). (□) Pre meal insulin dose was reduced by 50%; baseline plasma glucose was  $\sim 8.7$  mmol/l. (■) Premeal insulin dose was reduced by 75%; baseline plasma glucose was  $\sim 6$  mmol/l.

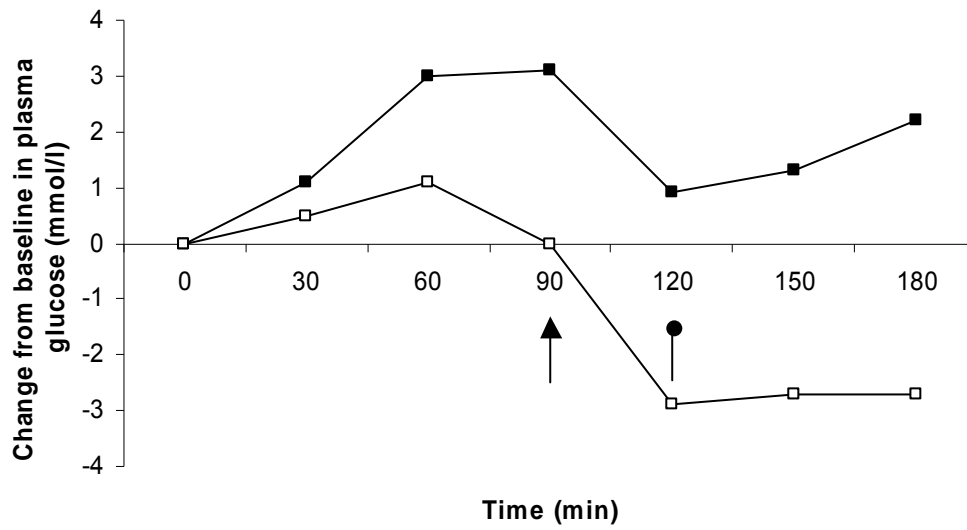


Fig.1.3f. Changes in plasma glucose before, during and after exercise at 75% of  $\text{VO}_2$  max for 30 min (▲-Beginning of exercise and ●-End of Exercise). (■) Pre meal insulin dose was reduced by 25%; baseline plasma glucose was  $\sim 6.8$  mmol/l. (□) No reduction in premeal insulin dose; baseline plasma glucose was  $\sim 8.5$  mmol/l.

**Table 1.3b Guidelines for the reduction of premeal insulin dose in relation to the intensity and duration of postprandial exercise (Adapted from Rabasa-Lhoret et al, 2001)**

<b>Exercise Intensity</b>  <b>(% VO<sub>2</sub> max)</b>	<b>% Dose Reduction</b>	
	<b>30 min of exercise</b>	<b>60 min of exercise</b>
25	25	50
50	50	75
75	75	-

## **1.4 Thesis Structure and Aims**

Diet, exercise and insulin therapy play an important role in the management of type 1 diabetes. The risk of hypoglycaemia is ever-present in insulin-treated diabetes. In addition to the unphysiological nature of insulin therapy, this could be due to a combination of factors such as deficits in glycogen metabolism and hormonal counter regulation. The overall objective of this thesis is to further the understanding of the effect of diet, insulin and exercise on the regulation of carbohydrate metabolism.

Some of the general *in vivo* techniques utilized to study human substrate metabolism are described in chapter 2. Chapter 3 describes a study of short-term dietary manipulation on whole body glucose uptake, muscle intermediary metabolism and cellular regulation of muscle substrate oxidation in healthy volunteers, with particular reference to the mitochondrial pyruvate dehydrogenase (PDH) enzyme complex. It was hypothesised that high fat diet would impair insulin-mediated whole body glucose metabolism via impairment of PDC activity and upregulation of PDK4. In addition it was also hypothesised that high fat diet induced PDK 4 upregulation was mediated through PPAR signalling.

The influence of therapeutic insulin concentrations in patients with type 1 diabetes on whole body, muscle and liver substrate metabolism at rest and during exercise are discussed in chapter 4 and 5. Chapter 4 describes the effects of hyperinsulinaemia approximating to pre-prandial and peak therapeutic insulin concentrations during moderate exercise on whole body and muscle substrate metabolism in patients with type 1 diabetes. It was hypothesized that exercise under peak therapeutic insulin concentrations would increase glucose utilization and oxidation and reduce reliance on intramuscular glycogen as a metabolic fuel. In chapter 5, the *in vivo* changes in

liver glycogen and whole body substrate metabolism both before and after a meal, and during subsequent moderate exercise in patients with type 1 diabetes under therapeutic insulin concentrations were compared to non-diabetic volunteers. It was hypothesized that, when compared to non-diabetic controls, in patients with type 1 diabetes exercise under relative hyperinsulinaemic conditions would suppress hepatic glycogen mobilization and thereby increase the risk of exercise-induced hypoglycaemia.

Finally, chapter 6 provides an overview of the main conclusions from the above studies, clinical implications and further directions for future research.

## **Chapter 2: Methods**

This chapter introduces all the procedures employed in the three studies, i.e. subject recruitment, screening, nutritional interventions, *in vivo* physiological techniques & interventions, analytical methods and statistical analysis.

### **2.1 Recruitment of Study Volunteers**

The Nottingham Research Ethics Committee approved the research study protocols involving patients with diabetes. The University of Nottingham Medical School Research Ethics Committee approved the healthy volunteer studies. All participants received travel and inconvenience expenses for taking part in the research studies described in this thesis.

#### **2.1.1 Recruitment of Healthy volunteers**

Poster advertisements were placed around the University of Nottingham Medical School notice boards seeking volunteers. Interested volunteers received an information sheet that had been approved by The University of Nottingham Medical School Ethics Committee and were given an opportunity to ask questions. Arrangements were made for a medical screening visit once subjects agreed to participate in the study and written informed consent was obtained.

#### **2.1.2 Recruitment of Patients with Type 1 Diabetes**

Potential subjects were identified from the Queen's Medical Centre Diabetes Register and in outpatient clinics. An invitation to participate was posted to each potential subject together with a pre-paid reply envelope. Subjects who did not reply were deemed to have declined the invitation and were not contacted again. Interested subjects were contacted and were given the opportunity to meet in person and discuss the study protocol and the requirements of each study. Arrangements were made for a

medical screening visit once patients agreed to proceed with the studies and written informed consent was obtained.

## **2.2 Screening**

A full medical history was elicited from each healthy volunteer followed by a medical examination. Height and weight (SECA Stadiometer and Weighing scale, Hamburg, Germany) were also measured and used to calculate their body surface area using a nomogram (Weir 1949). A 12 lead electrocardiogram was carried out to screen for underlying cardiac abnormalities. Venous blood was drawn for the measurement of haemoglobin, electrolytes, urea, creatinine, creatine kinase and standard liver function tests. A standard Bruce protocol exercise electrocardiogram test (Cambridge Heart 2000, Reynolds Medical, Herts, UK) was used to screen patients for evidence of ischaemic heart disease. In addition, patients were screened for evidence of retinopathy, nephropathy and poor glycaemic control (Glycated Haemoglobin > 9%). The principal exclusion criterion for healthy volunteers was a history of cardiovascular or cerebrovascular disease. The principal exclusion criteria for patients were the presence of clinically apparent macro vascular complication of diabetes, moderate or severe diabetic retinopathy, diabetes duration greater than 20 years and age less than 18 or greater than 45 years.

## **2.2 Estimation of peak rate of oxygen consumption (Chapters 4 and 5)**

Subjects undertook a continuous incremental cycling test to determine  $\dot{V}O_2$  peak. ECG electrodes were attached to the subject's chest to monitor the heart rate throughout the exercise period (Diascope; Simonson & Weel, Denmark). The subjects pedalled at a speed of 60 revolutions per min (rpm) on an electrically braked cycle



ergometer (Lode Excalibur cycle ergometer; Groningen, Netherlands). The initial workload was 80 Watts and this was increased in steps every 3 minutes by 30-40 watts depending on the subject's physical fitness. A nose clip was worn throughout and the subjects breathed through tubing connected to a gas analyser (Sensormedics Vmax 29; CA, USA) to measure oxygen uptake and carbon dioxide release. The criteria used for attainment of  $\dot{V}O_2$  peak were; heart rate response greater than 90% of predicted maximum heart rate, respiratory exchange ratio ( $RER > 1.10$ ) [British Association of Sport and Exercise Sciences Criteria]. The workload corresponding to 60% of each subject's  $\dot{V}O_2$  peak was calculated from the  $\dot{V}O_2$  - Workload relationship (Pearson Correlation) and used as a constant workload during the main exercise tests.

### **2.3 Basal Intravenous Insulin Replacement Protocol (Chapter 5)**

For the 3 days prior to each visit patients were asked to perform 4-point profiles of capillary glucose concentrations and maintain a food diary. Patients on basal-bolus insulin regimens injected their usual dose of short/rapid-acting insulin prior to their evening meal but omitted their bedtime medium-acting insulin. Patients on twice daily insulin regimens were asked to give a dose of soluble insulin equivalent to the soluble insulin component of their evening biphasic insulin before their evening meal. Patients who were treated with the long acting insulin analogue, Glargine (Lantus<sup>®</sup>) were switched to conventional medium acting Isophane (Insulatard<sup>®</sup>) insulin a week prior to the study visits.

Patients attended the hospital at 9 pm having had a standardised high carbohydrate evening meal (2 g carbohydrate/kg body weight) and slept in the Clinical Nutrition and Investigation Unit at the Queen's Medical Centre, Nottingham. One cannula was inserted in a retrograde fashion under local anaesthetic into a dorsal hand vein and a

second in an ante grade fashion into an antecubital fossa vein of the left upper limb for blood sampling and insulin infusion, respectively. Insulin (5 units Human Actrapid or Novorapid; NovoNordisk, Denmark) diluted in 48 mls 0.9% sodium chloride and 2 mls of patient's venous blood was infused at a rate of  $5 \text{ mU} \cdot \text{m}^{-2} \text{ body surface area} \cdot \text{min}^{-1}$  to maintain glycaemia between 5 and 10 mmol/l. The blood sampling cannula was kept patent by a slow infusion of 0.9 % sodium chloride and blood was taken to measure glucose concentration (HemoCue AB; Sweden) every hour during the night. The indwelling cannula enabled blood to be taken without waking the subject.

#### **2.4 Hyperinsulinaemic Clamp (Chapter 3 and 4)**

The insulin clamp was performed according to the previously described method (DeFronzo, Tobin et al. 1979). This technique involves a 10-min prime followed by a continuous intravenous infusion of insulin at a constant rate and a variable infusion of glucose to allow precise control of blood glucose concentrations. An intravenous cannula was placed into a vein in the antecubital fossa of the subject's non-dominant arm using local anaesthetic to minimise any discomfort. The insulin infusion consisted of human soluble insulin (25 units of Human Actrapid; Novo Nordisk, Denmark) mixed with 48 ml of 0.9% saline (Maco Pharma; Cedex, France) and 2ml of the subject's venous blood to prevent the insulin from adhering to the plastic syringe and tubing. Infusion rates corresponding to either 50 or 15  $\text{mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$  were calculated as described in appendix 1, depending on study requirements and infused using a syringe pump (Graseby 3100; Watford, UK). A variable infusion of 20% Dextrose (Baxter Healthcare, Thetford) was given using an infusion pump (IVAC 591, California, USA) via the same cannula. By varying the dextrose infusion rate (CV 3-5%), the blood glucose concentration could be precisely controlled to maintain

euglycaemia (~4.5 mmol/l, chapter 3) or hyperglycaemia (~8 mmol/l, chapter 4) depending on the study requirements. During the clamp, blood glucose concentration was measured (YSI 2300 Stat Plus-D; Yellow Springs Instruments, Ohio, USA) every 5 minutes and the rate of glucose infusion adjusted to maintain the chosen blood glucose concentration. In order to sample arterialised blood the subjects kept the sampling hand in a warming box (55<sup>0</sup> C).

Endogenous glucose production is almost completely suppressed in healthy volunteers when plasma insulin levels exceed 60 mU/l (Rizza, Mandarino et al. 1981). Hence, during steady state conditions, whole body glucose disposal is equivalent to the glucose infusion rate, after an extra cellular space correction for glucose (19% of body weight) and change in blood glucose concentration (DeFronzo et al, 1979). In patients with type 1 diabetes, a correction was made for glycosuria.

## **2.5 Exercise during hyperinsulinaemic clamp (Chapters 4 and 5)**

Minor modifications were made to the method described above in section 2.5. The subject exercised on a cycle ergometer while the insulin and dextrose infusions were running. After the commencement of exercise the glucose infusion rate needed to be increased substantially to maintain glucose concentration at the desired level. In order to sample arterialised blood during exercise the subjects kept the sampling hand in a warming box (55<sup>0</sup> C), which was placed on a table next to the cycle ergometer.

## **2.6 Indirect Calorimetry**

Energy can be changed from one form to the other, i.e. it is conserved, but it cannot be created or destroyed (First law of thermodynamics). During the process of energy transfer, some energy will dissipate as heat (Second law of thermodynamics). These principles apply to biological systems just as they apply to the universe. Indirect

calorimetry measures the respiratory gas exchange during carbohydrate, fat and protein metabolism (Elia and Livesey, 1988). The metabolic rate and type of substrate utilization can be estimated from measurements of whole body oxygen ( $O_2$ ) consumption, carbon dioxide ( $CO_2$ ) production and nitrogen excretion (Weir, 1949; Mansell and Macdonald, 1990; Frayn, 1983). During the oxidation of 1 mol of glucose (180 g) 6 mol of  $O_2$  is consumed and 6 mol of  $CO_2$  is produced. The respiratory exchange ratio (RER) for glucose is thus 1 ( $\dot{V}CO_2/\dot{V}O_2$ ). Whereas during the oxidation of 1 mol of a typical fat (tripalmitate, 861 g) 78 mol of  $O_2$  is consumed and 55 mol of  $CO_2$  is produced. The RER for fat is thus 0.7. The amount of protein oxidized may be estimated from urinary nitrogen excretion. One gram of urinary nitrogen arises from approximately 6.25 g of protein. Alternatively, protein oxidation can also be estimated from urinary urea measurements ( $\sim 90\%$  urinary nitrogen excretion) and changes in the urea pool (Jequier et al, 1987) as described in appendix 2.

If a subject is oxidising c grams of carbohydrate as glucose, f grams of fat per minute and excreting n grams of nitrogen per minute the total oxygen consumption and carbon dioxide production is given by Frayn's equations (Frayn 1983):

$$\dot{V}O_2 \text{ (l/min)} = 0.746 c + 2.03 f + 6.04 n$$

$$\dot{V}CO_2 \text{ (l/min)} = 0.746 c + 1.43 f + 4.89 n.$$

Hence for a given amount of  $\dot{V}O_2$ ,  $\dot{V}CO_2$ , and nitrogen excretion, the substrate oxidation is given by:

$$\text{Carbohydrate Oxidation (g/min)} = 4.55 \dot{V}CO_2 - 3.21 \dot{V}O_2 - 2.87 n$$

$$\text{Fat Oxidation (g/min)} = 1.67\dot{V}O_2 - 1.67 \dot{V}CO_2 - 1.92 n.$$

These equations have been derived assuming that there is an absence of net lipogenesis, or gluconeogenesis, or ketogenesis or any acid-base disturbances.

During a glucose clamp, indirect calorimetry measurements allow for the calculation of whole body carbohydrate oxidation ( $C_{ox}$ ). Non-oxidative glucose disposal during clamp conditions can be calculated as glucose disposal –  $C_{ox}$ .

Resting expired air was collected by means of a ventilated canopy with the subject lying supine and awake on a bed in a quiet room.  $\dot{V}O_2$  and  $\dot{V}CO_2$  measurements were made using a Gas Exchange Monitor (GEM; Nutren Technologies Ltd, Manchester, UK, CV 5-7%). The entire system was calibrated on a monthly basis by carrying out an alcohol burn. The gas analysers were calibrated at the start of each study day with gases of known composition. All measurements were made for 20 to 30min. The measurements from the first 6 min were discarded when calculating the mean  $\dot{V}O_2$  and  $\dot{V}CO_2$  for each subject.

During the exercise studies,  $\dot{V}O_2$  and  $\dot{V}CO_2$  measurements were made by means of a mouthpiece and lightweight tubing connected to an online system (Vmax 29, SensorMedics, Yorba Linda, CA, USA, CV 5-7%). Subjects breathed through the mouthpiece and wore a nose clip during the measurements. A headgear supported the mouthpiece to offload any drag on the teeth. The flow sensor was calibrated before each study by a syringe pump and the online system by gases of known composition. Measurements were made for 4-5 min at intervals of 15 – 20 min and the means of

these were used to calculate the respiratory exchange ratio (RER) and substrate oxidation rates.

## **2.7 Blood Sampling and Analytical Methods (Chapters 3, 4 and 5)**

Arterialised venous blood samples were drawn for the determination of blood metabolite and hormone concentrations. This circumvents the need for, and hazards associated with arterial cannulation. Blood was drawn from a dorsal hand vein cannula (20 GA; BD Venflon, Sweden), placed in a retrograde direction into a vein on the back of the non-dominant hand. The cannulated hand was placed in a warming box set at 55°C (McGuire, Helderma et al. 1976). The cannula was kept patent with a 0.9% sodium chloride infusion (Maco Pharma; Cedex, France). The high temperature causes arteriovenous shunts to open and so venous blood sampled from the hand is “arterialised”. As a result, glucose concentration (Liu, Moberg et al. 1992), plasma catecholamine levels (Liu, Andreasson et al. 1993) and stable isotope concentrations during kinetic studies (Jensen and Heiling 1991) approximate those in true arterial blood.

Blood samples (2-6ml) were collected in SST II Advance (BD Vacutainer) and Lithium heparin plus EGTA (75µl) glutathione tubes (BD Vacutainer) for obtaining serum (left to clot for ~ 30 min) or plasma respectively, centrifuged for 20 min at 3000 rpm and the supernatant was stored at – 80°C for assays of insulin, free fatty acid and catecholamines. An aliquot of blood was collected in EDTA coated tubes containing 500 units of aprotinin, centrifuged and the supernatant was stored in glass tubes at – 80°C for determination of glucagon at a later date. An aliquot of blood was collected in EDTA tubes, centrifuged and the supernatant stored at – 80°C for  $^2\text{H}_2$

glucose measurements. An equal volume of blood and 10% Perchloric acid (PCA) was centrifuged and the supernatant was stored for  $\beta$ -Hydroxybutyrate analysis.

### **2.7.1 Glucose and Lactate**

Whole blood glucose and lactate were measured immediately using a Yellow Springs analyser (YSI 2300 Stat Plus-D; Yellow Springs Instruments, Ohio, USA). The equipment was calibrated every 60 minutes during the experiments. The assays were based on Glucose and L-Lactate oxidase methods for glucose and lactate respectively. The intra assay coefficients of variation (CV) for glucose and lactate were 1.5 % and 2.9 % respectively. The equipment was calibrated every 60 min during the experiments.

### **2.7.2 Catecholamines**

Adrenaline and noradrenalin were measured using high performance liquid chromatography with electrochemical detection. The inter-assay coefficients of variation were 8% for adrenaline and 6% for noradrenalin (Forster and Macdonald 1999).

### **2.7.3 Glucagon**

Glucagon concentration was measured using a double antibody radioimmunoassay produced by Diagnostic Products Corp. Llanberis, Gwynedd, and Wales. Inter-assay CV at 59 pg/ml was 11.9% (n=20) and intra-assay CV at 52 pg/ml was 6.5% (n=20). Full details are outlined in appendix 3.

### **2.7.4 Nonesterified Fatty Acids (NEFA)**

NEFA's were measured using a commercially available kit (WAKO Chemicals; Neuss, Germany). Inter-assay CV was 3.5% and intra-assay CV was 2.4%. Full details are outlined in appendix 4.

### **2.7.5 Insulin**

Serum Insulin was determined using a commercial double antibody radioimmunoassay method produced by Diagnostic Products Corp. Llanberis, Gwynedd, and Wales. Intra assay CV at 39 and 80 mU/l was 5.1% and 3.5% respectively. Inter assay CV at 35 and 95 mU/l was 7.1% and 4.9% respectively. Analytical detail is outlined in appendix 5.

### **2.7.6 $\beta$ - Hydroxybutyrate**

$\beta$  - Hydroxybutyrate was determined enzymatically (Williamson, Mellanby et al. 1962). Intra assay CV was 8.4% and inter assay CV was 11.06%. The procedure is described in appendix 6.

### **2.7.7 Urea**

Plasma and urine urea were determined using a commercially available enzymatic kinetic method (Randox laboratories, Crumlin, UK, CV 3%). The assay procedure is outlined in appendix 7. Measurements of urine urea and plasma urea were used for the calculation of whole body protein oxidation as described in appendix 2.



## **2.8 Muscle Sampling and Analytical Methods**

All muscle samples were obtained from the vastus lateralis muscle using the needle biopsy technique (Bergstrom 1962). The biopsy site was cleaned with iodine solution. Skin, subcutaneous tissue and the fascia lata were infiltrated with 5 to 10 ml of 1% lignocaine. Each sample was taken through a separate skin incision (3-5 mm long). All incisions were made using a surgical blade while the subject was lying on an examination couch. A 5mm Bergstrom needle was employed for muscle sampling and suction was applied to the end of the needle. The size of the muscle samples obtained ranged between 50 and 100 mg wet weight. Firm pressure was applied after sampling to minimize bleeding and steristrips were used to close the incision. Pressure bandages were applied for 24 hours and subjects were encouraged to gently exercise the muscle the following day. For the exercise study, the biopsy site was prepared prior to cycling to minimize the time interval between cessation of cycling and muscle sampling. When more than one biopsy was taken, a distance of at least 3 cm was allowed between sites to minimize sampling errors.

After removing the biopsy needle from the leg, it was immediately immersed in liquid nitrogen. Muscle samples were then removed from the needle and were stored at -80°C until analysis. At a later date, one part of the muscle was used to determine the active form of pyruvate dehydrogenase complex (PDCa) by a previously described method (Constantin-Teodosiu, Cederblad et al. 1991) and another part of the muscle was freeze-dried and washed with 40 % petroleum ether to remove fat. Muscle powdering and extraction of metabolites were carried out as previously described (Harris, Hultman et al. 1974). Acid hydrolysis of soluble (Macroglycogen) and insoluble glycogen (Proglycogen) was performed as per the methods described before (Jansson 1981). The sum of macro- and pro-glycogen concentrations gave the total

mixed-muscle glycogen concentration. Muscle metabolites (glycogen, glucose, lactate, adenosine triphosphate (ATP), phosphocreatine (PCr), creatine and glucose-6-phosphate) were determined enzymatically (Harris, Hultman et al. 1974).

### **2.8.1 Muscle Powdering Procedure: From Freeze Dried Muscle**

Freeze dried muscle sample was removed from the  $-80^{\circ}\text{C}$  freezer and allowed to reach room temperature in a dessicator (silica gel). All powdering tools were cleaned with 70% methylated spirit and air-dried. Once the muscle tissue had reached room temperature the sample was removed from the eppendorf tube and was placed into an agate mortar. Visible tissue and dried blood were removed and then the sample was cut into small pieces using a scalpel. Using curved, ridged forceps, the muscle was ground and further visible connective tissue was removed. The muscle was then ground to a very fine powder. The sample was then weighed into an eppendorf tube and stored at  $-80^{\circ}\text{C}$  until extraction.

### **2.8.2 Mixed Muscle Metabolites Extraction Procedure**

Powdered muscle sample was removed from the  $-80^{\circ}\text{C}$  freezer and allowed to reach room temperature. The eppendorf tube containing the muscle was centrifuged at high speed for 1 minute to collect the muscle at the bottom of the tube.

A mixture of 0.5M perchloric acid (PCA) and 1mM ethylenediamine tetra-acetic acid (EDTA) was added to the muscle in a ratio of 100  $\mu\text{l}$  per mg of muscle powder. The powder was vortexed frequently for 10 min on ice and care was taken to ensure that the powder stayed in the PCA solution. This was then centrifuged at 14000 rpm for 3 min at  $0-4^{\circ}\text{C}$  and a known volume of supernatant (Extract) was removed to a new eppendorf tube. The pellet was stored at  $-80^{\circ}\text{C}$  for acid hydrolysis to determine glycogen concentrations.

Keeping the extract on ice, a volume of 2.2M potassium hydrogen carbonate ( $\text{KHCO}_3$ ) equal to one quarter of the volume of supernatant was added to neutralize it. The solution was vortexed, uncapped and left on ice for ~ 5 min to allow the generated  $\text{CO}_2$  to escape. The extract was then centrifuged at 14000 rpm for 3 min and the clear supernatant was removed to a new eppendorf tube and stored at  $-80^\circ\text{C}$  until analysis.

### **2.8.3 Acid Hydrolysis of Muscle Extract – Macroglycogen**

The extract was removed from the  $-80^\circ\text{C}$  freezer and allowed to thaw to room temperature. 0.1 ml of 1M hydrochloric acid (HCl) was added to 20 $\mu\text{l}$  of the undiluted neutralized extract. The tube was then capped, mixed and boiled for 2 hours at  $100^\circ\text{C}$  on a dry bath. The extract was then cooled at room temperature and neutralized with 15 $\mu\text{l}$  of 6M sodium hydroxide (NaOH) and stored at  $-80^\circ\text{C}$  until further analysis.

### **2.8.4 Acid Hydrolysis of Muscle Pellet – Proglycogen**

The pellet remaining after the mixed muscle metabolite extraction procedure was used for acid hydrolysis. The pellet was removed from the freezer and allowed to thaw at room temperature. For each sample, 0.1 ml of 1M HCL per mg of muscle powder was added to the pellet and boiled for 2 hours in a tightly screwed eppendorf tube at  $100^\circ\text{C}$  on a heating block. The samples were allowed to cool and then stored at  $-80^\circ\text{C}$  until further analysis.

### **2.8.5 Muscle Metabolite Analysis**

Muscle metabolites (glycogen, glucose, lactate, adenosine triphosphate (ATP), and phosphocreatine (PCr)) were determined enzymatically (Harris, Hultman et al. 1974). Glycogen was determined both as pro- and macro-glycogen (Jansson 1981). The sum of pro- and macro-glycogen concentrations gave the total mixed-muscle glycogen

concentration. Assays were carried out in a 96 well plate using a spectrophotometer (SoftMax Pro.4.7; Spectramax 190, Molecular devices, California, USA) at 340 nm. Assay CV for pro- and macro glycogen was 0.9 and 3% respectively. Undiluted extracts were used for the analysis of ATP, PCr, Cr and lactate. The undiluted hydrolysate was used for the determination of soluble glycogen and a 1:10 dilution of the hydrolysate for insoluble glycogen. Undiluted extracts were used for the analysis of ATP, PCr, Cr and lactate. Assay CV for ATP, PCr, Cr and lactate were 1.1, 1.8, 2.7 and 1.6% respectively. Detailed assay procedures are outlined in appendix 8 to 11.

The neutralised muscle extract was also used for the determination of acetylcarnitine by enzymatic assays using radioisotopic substrates as previously described (Cederblad, Carlin et al. 1990). Briefly, the acetyl group from acetylcarnitine was transferred to CoASH in a reaction catalysed by carnitine acetyltransferase to form acetyl-CoA. The acetyl-CoA was then determined as [ $^{14}\text{C}$ ]-citrate after condensation with [ $^{14}\text{C}$ ]-oxaloacetate by citrate synthase. Assay CV was 3.7%.

PDC activity in muscle tissue was measured using a radioactive assay (Constantin-Teodosiu, Cederblad et al. 1991). 5-10 mg of muscle was homogenised for 50 s, in 200  $\mu\text{l}$  of cold sucrose homogenisation buffer (pH 7.8) containing NaF and dichloroacetic acid. 30  $\mu\text{l}$  of muscle homogenate was added to 720  $\mu\text{l}$  of prewarmed assay buffer at 37<sup>0</sup> (pH 7.8, containing  $\text{NAD}^+$  and CoASH). The reaction was started by the addition of 30  $\mu\text{l}$  pyruvate (26 mM). After a minute, 210  $\mu\text{l}$  of the incubation mix was added to 0.5 M PCA to stop the reaction. This procedure was repeated after 2 and 3 min. The solution was then neutralised with  $\text{KHCO}_3$  and centrifuged at 14000 rpm for 2 min. The supernatant was removed and stored at -80<sup>0</sup>C for subsequent

analysis of acetyl-CoA by the method described in the previous paragraph (Cederblad, Carlin et al. 1990). The assay CV was 6%.

### **2.8.6 Muscle RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from 10-20 mg of skeletal muscle biopsy tissue using Trizol reagent (Invitrogen, Paisley, UK). Each piece of frozen muscle was homogenised on ice with a power homogenizer (PowerGen 700, Fisherbrand; Fisher Scientific) in 800 µl of Trizol reagent and 20 µl of glycogen (10 µg /µl) for 2 x 15 seconds with the sample being placed on ice for 30 seconds in between. The sample was allowed to stand at room temperature for 5 min before the addition of 160 µl of chloroform: isoamyl alcohol (49:1). The sample was then mixed by inversion for 20 seconds, vortexed briefly, allowed to stand at room temperature for 2 min and then centrifuged at 12,000 g for 15 min at 4<sup>0</sup> C. The aqueous phase was then transferred to a fresh tube and 400 µl of ice-cold isopropanol was added. The samples were placed at -20<sup>0</sup>C overnight to precipitate the RNA. The next day, RNA was pelleted by centrifugation at 12,000 g for 15 min at 4<sup>0</sup>C. The supernatant was removed and the pellet was washed with 800 µl of ice cold 75% ethanol and the samples were centrifuged again at 10,000 g for 10 min at 4<sup>0</sup> C. The supernatant was decanted and samples were placed over tissue paper for 5 min to drain off any remaining ethanol. Following a brief pulse in the centrifuge, any last remaining ethanol was removed using a pipette, the RNA pellet was dissolved in 30 µl of RNase free water and the RNA solution was stored at -80<sup>0</sup> C. The concentration of extracted total RNA was quantified using the Ribogreen RNA quantification kit (Molecular probes, Invitrogen, Paisley, UK).

cDNA was synthesised by reverse transcription. Reverse transcription was carried out on 0.5 µg total RNA as described previously (Parr, Sensky et al. 2001). Following reverse transcription, the first strand cDNA was always diluted fourfold. Quantification of reverse transcribed cDNA was performed in real time using an ABI 7700 Sequence Detection System. Human cDNA sequences were obtained from GenBank. Taqman probes and primer sets were designed using Primer Express version 2.0 Software (Perkin-Elmer, Norwalk, CT) (Tsintzas, Jewell et al. 2006). Real-time polymerase chain reaction (PCR) was performed using PCR Universal Master Mix (Applied Biosystems, Warrington, UK). Each reaction contained 5 µl cDNA template, 12.5 µl PCR Universal Master Mix, 300 nmol primers and 125 nmol dual labelled fluorescence probe in a reaction volume of 25 µl. Each RT-PCR reaction was performed in triplicate and all results were normalised to  $\alpha$ -actin mRNA expression using the standard curve method.

#### **2.8.7 Muscle Protein Extraction, Gel Electrophoresis and Western Blotting**

10-30 mg of tissue was homogenized for 30s in 10 volumes of extraction buffer containing 20 mM Tris-HCL, 5 mM EDTA, 2mM DTT (pH 7.5) containing a cocktail of protease inhibitors (P8340, Sigma, Dorset, UK). Whole muscle lysates were centrifuged at 13,000 g for 20 min at 4<sup>0</sup>C and equal volumes of 2 x Laemmli sample buffer (20% glycerol, 125 mM Tris-HCL (pH 6.8), 4% SDS (w/v), 100 mM DTT, 0.02% (w/v) bromophenol blue) was then added to the supernatant. Protein was quantified using the Lowry method (Lowry, Rosebrough et al. 1951).

SDS-PAGE was carried out as described previously (Laemmli 1970). Equal quantities of each protein sample were loaded onto polyacrylamide gels consisting of 5% (v/v) stacking gel and a main gel which ranged in concentration from 8 – 12% (v/v). Gels

were run in SDS running buffer (25 mM Tris, 192 mM glycine and 1% (w/v) SDS at a constant voltage of 200 V for approximately 40 min. Prestained protein markers (Precision Plus Protein Standards, Bio-Rad, UK) were loaded onto each gel to accurately and consistently determine the size of each migrated band.

Western blotting was performed according to previously described methods (Towbin, Staehelin et al. 1979; Burnette 1981). The western blot buffer contained 400 mM glycine, 25 mM Tris and 10% methanol. Whilst SDS-PAGE gels were running, nitrocellulose membranes were cut to size as were four pieces of 3MM paper. When the gel had finished running, it was soaked briefly in Western blot buffer for 5 min. Following this, the Western blot sandwich was assembled; a sponge was laid onto one side of a blotting cassette and placed on top of this was 2 sheets of 3MM paper, the gel, membrane, final 2 pieces of 3MM paper and finally the remaining sponge. Care was taken not to introduce air bubbles into the sandwich. The cassette was clamped together and placed into a western blotting tank. The procedure was carried out for 2h at 150 – 350 mA depending on the size of the transfer tank used. The buffer was always kept cold. The nitrocellulose membrane was removed from the western blot apparatus, briefly washed in methanol and then placed in Ponceau S stain [0.5% (w/v) Ponceau S in 5% (w/v) trichloroacetic acid] for approximately 1 min and membranes were visualised by destaining in methanol. This was to ensure 1) equal transfer efficiency across the gel and 2) that equal quantities of protein were loaded into each well. Ponceau S stain was removed by washing briefly in TBS-T (50 mM NaCl, 100 mM Tris, 1% (v/v) Tween 20). Non-specific binding was then blocked in 5% (w/v) non-fat dried milk (Marvel) in TBS-T for 1h at room temperature before incubating overnight at 4°C with the primary antibody, diluted 1:1000 in 5% (w/v) Marvel TBS-T. The next morning, any unbound antibody was removed by washing with 1% (w/v)

Marvel TBS-T for 12 X 5 min with fresh changes of solution each time. The membrane was then incubated for 1h with the secondary antibody, which was anti-rabbit IgG linked to horseradish peroxidase (HRP) (GE healthcare, little Chalfont, UK).

The membrane was developed with ECL Plus western Blotting Detection reagents (GE healthcare, Little Chalfont, UK). For this, a 40:1 ratio of reagentA to reagent B was mixed together pipetted on to the membrane (0.1 ml/cm<sup>2</sup>) and incubated for 5 min at room temperature. The membrane was blotted using 3MM Whatman chromatography paper to remove the detection reagents and wrapped in cling film before being taped into an autoradiograph cassette. In the dark room, the membrane was exposed to autoradiography film (Hyperfilm ECL, GE healthcare) before being developed in 1 X Kodak X-Ray developer solution (Calumet Photographic Company, Nottingham, UK). Western blot signals were quantified by scanning densitometry and analysed with Quantity-One Multi-analyst Software (Bio-Rad, Hertfordshire, UK).



## **2.9 Stable Glucose Isotope Tracer Technique**

The glucose stable isotope dilution technique was used to determine glucose turnover during basal and insulin stimulated states in the study presented in chapter 3. This technique is a safe, non-invasive way to study substrate fluxes in-vivo in humans. The principle of the method is described below.

Stable isotopes such as  $^1\text{H}$  and  $^2\text{H}$  share the same number of protons but differ in their atomic mass. This property enables us to distinguish between them using mass spectrometry. Naturally occurring  $^2\text{H}$  is rare ( $\sim 0.02\%$ ) whereas  $^1\text{H}$  (99.98%) is common (Wolfe 1984). The background occurrence needs to be taken into account during calculations. Stable isotopes such as  $^2\text{H}$  can be incorporated into organic molecules such as glucose and subsequently applied as tracers to study carbohydrate metabolism. The tracer is infused at a constant rate into the circulation and blood samples are collected at defined intervals. The tracer infusion equilibrates with the entire pool and the rate of flux of the tracer and therefore its tracee can be calculated by the method described by the one-compartment model for steady state kinetics (Steele 1959). This model assumed that the glucose pool functioned as a single compartment in which the infused tracer would mix instantaneously and completely. However, for non-steady state kinetics as in the euglycaemic clamp, in addition to the constant tracer infusion, tracer needs to be added to the exogenous glucose infusion to avoid changes to the enrichment of the glucose pool and thereby underestimate glucose appearance ( $R_a$ ). Modifications to the Steele equation (Finegood, Bergman et al. 1987) need to be made to account for the tracer in the exogenous glucose infusion to calculate glucose disposal ( $R_d$ ) and glucose appearance ( $R_a$ ). The equations used to calculate  $R_d$  and  $R_a$  are given below.

$$R_d(t) = R_a(t) - pV \frac{dG(t)}{dt}$$

And

$$R_a(t) = \{I/Pct_p(t) - pVG(t)[dPct_p(t)/dt]/Pct_p(t) + [Pct_g/Pct_p(t)*Ginf(t)]\} - Ginf(t)$$

Where I is the constant tracer infusion rate (mg/kg/min), t is time, Pct<sub>p</sub>(t) is the percentage enrichment in plasma glucose, p is the pool fraction, V is the volume of distribution for glucose, G(t) is the plasma glucose concentration, dPct<sub>p</sub>(t)/dt is the rate of change of plasma enrichment per min, Ginf(t) is the rate of exogenous glucose infusion, Pct<sub>g</sub> is the percentage enrichment of the glucose infusate and dG(t)/dt is the rate of change in the plasma glucose concentration. V (pv) was set as 165 (Molina, Baron et al. 1990). Implicit assumptions made in the one-compartment model, that the infused tracer would mix instantaneously and completely still apply during these calculations.

It is possible to obtain glucose labeled with stable isotopes in several positions. Commonly used isotopes are [2-d]-, [3d]-, [6, 6-d<sub>2</sub>]-, [1-<sup>13</sup>C]- and U-<sup>13</sup>C. The selection depends on the experiment and type of instrument with which the analysis will be performed. Moreover, the rate of appearance of unlabeled molecules is measured from the dilution of the labeled isotope (Isotope dilution). Therefore, if a labeled isotope leaves the pool, it is assumed that the label does not reenter the pool, i.e. there is no recycling of the labeled moiety. If the label does reenter the pool in the same form, it will not be distinguished from the infusate and could lead to underestimation of the rate of appearance of unlabeled molecules. The issue of recycling is especially pertinent to glucose metabolism since there is an active recycling of glucose carbons. For example, labeled glucose is broken down to <sup>13</sup>C-labeled 3-carbon units such as lactate that can serve as precursors for glucose production in the liver. The newly produced glucose will be labeled with <sup>13</sup>C and

cannot be distinguished from the labeled infusate, leading to underestimation of glucose production. On the other hand, deuterium atoms at positions 2, 3 and 6 are all lost at different stages of glycolysis or gluconeogenesis. Hence, the calculated value depends on the isotope used. All three deuterium atoms are subject to recycling, the least being 6,6-d<sub>2</sub>.

In the study described in chapter 3, a baseline blood sample was taken for determination of glucose and background enrichment of [6, 6-<sup>2</sup>H<sub>2</sub>]-glucose. Subsequently, a stable isotope of glucose ([6, 6-<sup>2</sup>H<sub>2</sub>]-glucose; Cambridge Isotope Laboratories, USA) was administered as an intravenous bolus dose of 4 mg/kg body mass followed by a continuous intravenous infusion of 2.5 mg/kg/hr (IVAC 591 pump; California, USA) 120 min before the commencement of the hyperinsulinaemic clamp. Blood samples were obtained at 5 min intervals during the last 20 min of the continuous infusion for the measurement of isotope enrichment. The isotope infusion continued throughout the 4h insulin clamp. The glucose infusate that was used to maintain euglycaemia was enriched (spiked) with [6, 6-<sup>2</sup>H<sub>2</sub>]-glucose (1%) to minimize fluctuations in plasma enrichment of the stable isotope. Blood samples were collected in EDTA tubes at 20 min intervals, centrifuged immediately at 14000 rpm for 4 min and plasma was separated and stored at – 80°C until analysis for [6, 6-<sup>2</sup>H<sub>2</sub>]-glucose concentrations. The latter analysis was carried out in Dr.L.J.C. van Loon's laboratory, at the Department of Human Biology, Maastricht University, Netherlands. Following derivatisation, plasma [6, 6-<sup>2</sup>H<sub>2</sub>] glucose enrichment was determined by electron ionisation gas chromatography-mass spectrometry (INCOS-XL; Finnigan, Bremen, Germany). Glucose concentrations in the infusates were determined with the COBAS FARA semi-automatic analyzer.

## **2.10 Nuclear Magnetic Resonance Spectroscopy**

Nuclear magnetic resonance spectroscopy (NMR) is a powerful analytical tool that has been widely applied to *in vivo* and *in vitro* metabolic studies. The NMR phenomenon relies on the spin angular momentum and magnetic moment of stable isotopes with odd atomic mass numbers by virtue of their net spin ( $^{13}\text{C}$ ,  $^{31}\text{P}$  or  $^1\text{H}$ ). The combination of spin angular momentum and magnetic moment causes the nuclei to precess in a magnetic field. The NMR technique measures this precessional frequency. This is proportional to the strength of the effective magnetic field at the nucleus and hence is influenced by the local electronic environment. When an atom is placed in a magnetic field, its electrons circulate about the direction of the applied magnetic field. This circulation causes a small magnetic field at the nucleus that opposes the externally applied field. The electron density around each nucleus in a molecule varies according to the types of nuclei and bonds in the molecule. The opposing field and therefore the effective field at each nucleus will vary. This is called the chemical shift phenomenon ( $\delta$ ) that is given by

$$\delta = (f - f_{\text{ref}}) * 10^6 / f_{\text{ref}}$$

Where  $f$  is the observed frequency,  $f_{\text{ref}}$  the reference frequency. The chemical shift is a very precise metric of the chemical environment around the nucleus. For  $^{13}\text{C}$  NMR tetramethylsilane (TMS) is the normal choice of reference for which  $\delta$  is set to zero parts per million (ppm).

The strength of the NMR signal is proportional to the number of spins contributing to it. The sensitivity of the technique also depends on the natural abundance of the stable isotope. Hence, for  $^{13}\text{C}$  NMR, the sensitivity is poor due to its low abundance ( $\sim 1.1\%$ ) and only metabolites such as triacylglycerols or glycogen, that are highly concentrated in certain tissues can be directly observed *in vivo* (Alger, Sillerud et al. 1981). The

technique has provided new insights into glycogen metabolism and has been extensively used to study glycogen metabolism in healthy volunteers (Shulman, Rothman et al. 1985; Casey, Mann et al. 2000) and in patients with type 1 diabetes (Cline, Rothman et al. 1994; Hwang, Perseghin et al. 1995). There are several advantages to this technique. The wide chemical shift ( $>200$  ppm) of the  $^{13}\text{C}$  nucleus permits metabolites to be identified directly from the  $^{13}\text{C}$  NMR spectra and repeated measurements can be made without undue discomfort or risk to subjects. The technique is non-invasive and obviates the need for procedures such as liver and muscle biopsy.

All  $^{13}\text{C}$  MR spectra were acquired on a 3.0-Tesla whole body magnetic resonance scanner with a 1-m diameter bore. A surface coil was used with a carbon coil for transmission and reception, and quadrature proton coils for  $^1\text{H}$  decoupling. This consisted of a 7cm diameter circular  $^{13}\text{C}$  coil and two 13cm  $^1\text{H}$  coils. Subjects were positioned in the scanner in the supine position with the surface coil placed over the liver region of the torso. Care was taken to reposition subjects and the coil as accurately as possible for subsequent measurements. Manual shimming was performed on the water resonance and the broadband decoupling frequency was centered on the glycogen resonance. The coils were all tuned and matched using a Network Analyser (HP model 8751A, 5-500 KHz).

$^{13}\text{C}$  spectra were acquired with a simple pulse-acquire (decouple) sequence. The excitation pulse was a  $100\mu\text{s}$  pulse at a peak power of 390 (10) W with CYCLOPS phase cycling. Broadband decoupling was achieved using three WALTZ-8 cycles during acquisition with a peak power of 50 (2) W. A repetition time of 360 ms was used and spectra were collected in blocks of 1000 acquisitions giving a temporal

resolution of 6 min. Two blocks were collected and summed for each time point. The sampling time was 142  $\mu$ s, and 512 data points were collected during the acquisition time. RF powers were monitored throughout acquisition period to ensure that we did not exceed the maximum values allowed according to specific absorption rate limits recommended by the National Radiological Protection Board. No adjustments to the spectrometer were made between measurements, except for tune and matching the  $^{13}\text{C}$  surface coil before each spectroscopic measurement.

All spectra were analyzed using the Matlab version of Magnetic Resonance User Interface (MRUI). Spectral peaks were selected using the AMARES algorithm and were fitted to Lorentzian lineshapes. The signal of interest arises from the C-1 position of glycogen at 100.5 ppm (relative to tetramethylsilane = 0). The lipid peaks obscured resonances from other glycogen carbons (C-2 to C-6). The coefficient of variation, calculated for repeated analysis of a single spectrum, was 3.8% ( $n = 15$ ). The integral of the glycogen peak was expressed as a fraction of the formate peak derived from a phantom containing formate at the centre of the coil. Quantification was done using a liver shaped phantom containing a solution of 184 mmol/l oyster glycogen and 150 mmol/l KCL. Glycogen concentrations were calculated using the formula:  $[R_{\text{gly (s)}} * (\text{Glyc})] / R_{\text{gly (ph)}}$ , where  $R_{\text{gly (ph)}}$  and  $R_{\text{gly (s)}}$  are the ratios of glycogen to formate in the phantom and subject, respectively and [Glyc] is the concentration of glycogen in the phantom in mmol/l.

## **2.11 Statistics**

Statistical analysis was carried out using SPSS, Version 11.5 software package. All data are presented as mean  $\pm$  standard error of the mean (SEM) unless stated otherwise. Two-way analysis of variance (ANOVA) was used to compare means of

repeated measures (Chapter 3 and 4). Student's t test was used for paired data. A mixed factor ANOVA was used to compare repeated measures data derived from different groups (Chapter 5). A P value less than 0.05 was considered as significant.

## **Chapter 3**

**High fat/low carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates non-oxidative glucose disposal in humans: an important role for skeletal muscle PDK4.**

### **3.1 Introduction**

Understanding the aetiology of insulin resistance is of major clinical importance, not least because this is the main feature of type 2 diabetes (DeFronzo, Bonadonna et al. 1992). One potentially modifiable nutritional determinant of insulin sensitivity is diet composition. Several studies have shown that when non-esterified fatty acid (NEFA) availability was profoundly increased by intralipid infusions, impaired whole body insulin sensitivity was observed in humans (Ferrannini, Barrett et al. 1983; Boden and Jadali 1991; Boden, Jadali et al. 1991; Yki-Jarvinen, Puhakainen et al. 1991; Boden, Chen et al. 1994). However, high fat feeding in humans has produced contradictory results. High fat diets for just 3 days have been shown to induce whole body insulin resistance (Bachmann, Dahl et al. 2001; Pehleman, Peters et al. 2004). In contrast, high fat feeding for 11 to 21 days does not induce whole-body insulin resistance although the partitioning of glucose metabolism is altered with decreased oxidation and increased non-oxidative glucose disposal (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001). Further detailed *in-vivo* studies are therefore required to investigate the potentially more subtle changes in insulin-mediated muscle metabolism that are associated with a high dietary fat intake in humans.

The cellular mechanisms by which increased availability of NEFA may induce insulin resistance are unclear. The mitochondrial pyruvate dehydrogenase enzyme complex (PDC) occupies a central role in muscle intermediary metabolism and has been



proposed to play a primary role in the development of insulin resistance (Randle, Priestman et al. 1994; Randle, Priestman et al. 1994). The activity of this complex is down regulated when there is an increased availability of NEFA, which promotes fat oxidation and suppresses glucose metabolism and this is mediated through changes in the activity of pyruvate dehydrogenase kinase (PDK) (Gudi, Bowker-Kinley et al. 1995; Huang, Gudi et al. 1998). Administration of a high fat diet is associated with significant increases in muscle PDK4 expression in healthy humans (Peters, Harris et al. 2001). Insulin down regulates transcript levels of PDK2 and PDK4 in insulin resistant non-diabetic Pima Indians (Majer, Popov et al. 1998), but no study has examined the effect of insulin on their expression in healthy humans. Furthermore, the signalling mechanisms by which NEFA and insulin regulate these kinases *in vivo* are not very well characterised in humans.

This study examined the effect of 6 days of isoenergetic high fat/low CHO diet compared to a control diet on insulin-mediated whole-body and muscle intermediary metabolism. It was hypothesised that high fat diet would impair insulin-mediated whole body glucose metabolism via impairment of PDC activity and upregulation of PDK4. In addition it was also hypothesised that high fat diet induced PDK 4 upregulation was mediated through PPAR signalling.

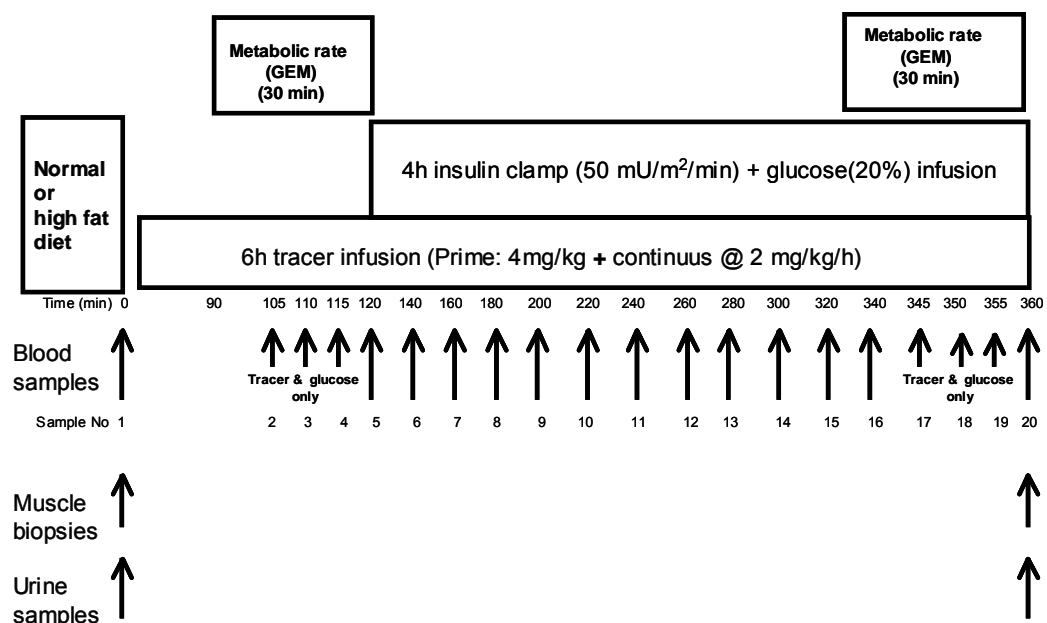
### 3.2 Subjects

Ten healthy males [age 25.6 (2.5) yr and BMI 23.7 (0.9) kg/m<sup>2</sup>] were recruited and informed of all procedures and risks associated with the experimental procedures prior to obtaining informed consent. All procedures used in this study were performed according to the Declaration of Helsinki and approved by the University of Nottingham Medical School Ethics Committee.

### 3.3 Study Design and Protocol

#### 3.3.1 Study Design

All subjects underwent two 7-day trials, at least 2 weeks apart, in a randomised cross over design as represented below. On each occasion, subjects consumed for 6 days either a high fat [(HF) 75% Energy as Fat] or normal diet [(CON) 32% Fat]. On day 7, subjects were infused with [6, 6-<sup>2</sup>H<sub>2</sub>]-glucose for 2h before and during a 4h hyperinsulinaemic euglycaemic clamp to quantify insulin sensitivity. Muscle biopsies were obtained from the *vastus lateralis* before and after each diet and after the clamps.



### 3.3.2 Study Protocol

On day 1, subjects reported to the laboratory at 0800 h after an overnight fast and after recording the body weight a baseline blood sample was drawn. Subsequently, under local anaesthetic, the *vastus lateralis* muscle was biopsied as described previously (Bergstrom 1962). One piece of fresh muscle tissue was immediately used for mitochondrial extraction and the remaining part was snap frozen in liquid nitrogen for subsequent enzyme, RNA and protein extraction. Subjects then consumed either a normal (CON) or a high fat (HF) diet for 6 consecutive days. A minimum 2-week interval between the 2 diets was allowed in each subject. The HF and CON diets were designed based on the subjects' usual dietary habits and energy intake to ensure palatability and adherence to the study protocol. The HF diet was designed to provide 75% of energy from fat (of which 35% from saturated fat) and 10% of energy as carbohydrate (CHO). The CON diet was designed to provide 50% of energy from CHO and 35% from fat. Both diets and menus for the 6 days were designed using a dietary analysis program (Microdiet; Version 1.1, Downlee Systems Limited, UK) and diet composition is detailed in table 3.7.1. All food, beverage (non-caffeinated) and snack requirements for both diets were purchased and delivered to the study participants. Written instructions on cooking methods and ingredients were also provided. Ready-made meals with known nutritional content were provided to subjects who were unfamiliar with cooking methods. Subjects were requested to adhere to the items on the menu and to record intake. Subjects were also requested to abstain from alcohol consumption, smoking and intense exercise for 3 days before and during each 6-day dietary intervention. Food records were analysed at the end of the study using the dietary analysis program.

On day 7 of both treatments, subjects reported to the laboratory at 0800 h after an overnight fast. Subjects were weighed; a baseline urine sample was acquired and subjects then rested on a reclined couch. Resting  $\dot{V}\text{CO}_2$  and  $\dot{V}\text{O}_2$  were measured for 30 min using a ventilated hood system (GEM, Nutren Technologies, Manchester, UK) and values were used for the calculation of energy expenditure and substrate oxidation rates. Measurements were made while the subjects were lying supine, undisturbed and awake. A muscle biopsy was then obtained and at 0900 h, a Teflon catheter was inserted into the antecubital vein of one arm for glucose tracer, 20% glucose (spiked with glucose tracer, 1%) and insulin infusion. Another catheter was placed into a dorsal hand vein in a retrograde fashion to obtain arterialized blood samples. The hand was placed in a heated box (55°C) for blood sampling. Subjects were infused with [6, 6-<sup>2</sup>H<sub>2</sub>] glucose in a primed (22 µmol/kg body mass) and continuous (13.75 µmol/kg/h) fashion for 2h before (basal period), and during a 4h hyperinsulinaemic (50 mU/m<sup>2</sup>/min) euglycaemic (4.5 mmol/l) clamp for the determination of glucose appearance ( $R_a$ ) and glucose disappearance ( $R_d$ ) rates. Total glucose tracer infusion rate averaged  $677 \pm 34$  nmol/kg/min. A second indirect calorimetry measurement was made during the last 30 min of the insulin clamp and a muscle sample was obtained at the end of the clamp. Blood samples were collected at 20 min intervals during the study. Urine samples were collected during the study day for glucose and nitrogen measurements and were stored at -20°C in 10% thymol until analysis.

### **3.4 Methods**

A brief description of the methods is given below. Please refer to Chapter 2: Methods for detailed description of analytical methodology.

#### **3.4.1 Blood and Urine Analysis**

Blood and urine glucose, and blood lactate were measured shortly after collection. Whole blood  $\beta$ -hydroxybutyrate was measured in perchloric acid (PCA) treated blood samples. Serum insulin was measured using a radioimmunoassay kit. Plasma NEFA and urea were measured using kits from WAKO Chemicals (Germany) and Randox laboratories (UK), respectively. Following derivatisation, plasma [6, 6-<sup>2</sup>H<sub>2</sub>] glucose enrichment was determined by electron ionisation gas chromatography-mass spectrometry (INCOS-XL, Finnigan, Bremen, Germany). Urine nitrogen was determined using the Kjeldahl method.

#### **3.4.2 Muscle Metabolites**

Ten to twenty mg of frozen muscle was freeze-dried and washed with 40 % petroleum ether to remove fat. Muscle metabolites (ATP, PCr, Creatine, lactate and glucose-6 phosphate) were extracted using PCA and determined enzymatically (Harris, Hultman et al. 1974). Acetylcarnitine was determined using a radioimmunoassay (Cederblad, Carlin et al. 1990). Acid hydrolyses of the muscle extract and the muscle pellet left over after PCA extractions were carried out to measure macro- and pro-glycogen, respectively (Jansson 1981). Five to ten mg of frozen muscle was used to determine the active form of PDCa (Constantin-Teodosiu, Cederblad et al. 1991).

### **3.4.3 RNA Extraction and Real-time Quantitative PCR**

Total RNA was extracted from 10-20 mg of frozen muscle tissue as described previously (Chomczynski and Sacchi 1987) using TRIzol reagent (Invitrogen, Paisley UK). Please refer to chapter 2 (Section 2.8.6) for detailed methods.

### **3.4.4 Protein Extractions and Western Blotting**

Total protein extracts were prepared from 20-30 mg of frozen muscle as previously described and used for the determination of HKII, SREBP1c, PPAR $\alpha$  and PPAR $\delta$  protein expression. Mitochondria were extracted from 20-40 mg of fresh muscle tissue as previously described (Wibom and Hultman 1990) and used for the determination of PDK2 and PDK4 protein expression. Proteins were separated, blocked, western blotted and quantified and are described in detail in chapter 2 (section 2.8.7).

### **3.4.5 Calculations**

Calculations of glucose disposal and substrate oxidation were made at steady state during the clamp (last 30 min). Carbohydrate ( $C_{ox}$ ) and fat ( $F_{ox}$ ) oxidation rates were calculated from the  $\dot{V}CO_2$  and  $\dot{V}O_2$  measurements (Frayn 1983) after correcting for protein oxidation. Rates of protein oxidation were estimated from urinary nitrogen excretion after correction for changes in blood urea nitrogen pool size. It was assumed that for each gram of nitrogen excreted in the urine, 6.04 l of  $O_2$  were consumed and 4.89 l of  $CO_2$  were produced. The glucose stable isotope dilution technique was used to determine glucose turnover during basal and insulin stimulated states. Modified Steele equations (Finegood, Bergman et al. 1987) were used to calculate  $R_d$  and  $R_a$ . Glucose disposal was also calculated from the glucose infusion rate during the clamp (DeFronzo, Tobin et al. 1979). Hepatic glucose output (HGO) was calculated as the

difference between  $R_a$  and glucose infusion rate (GIR) during the clamp. Non-oxidative glucose disposal (NOGD) was calculated as the difference between  $R_d$  and  $C_{ox}$ . Homeostatic Modelling Assessment – Insulin resistance index (HOMA-IR) was also determined using fasting insulin and glucose concentrations (Levy, Matthews et al. 1998). Fasting insulin and glucose concentrations, and the HOMA-2 calculator was used to determine insulin resistance. The calculator was downloaded from the Oxford trials unit website (<http://www.dtu.ox.ac.uk>).

### **3.4.6 Statistics**

Repeated measures were analyzed using two-way (treatment x time) analysis of variance (SPSS, Version 11.5). Bonferroni multiple comparisons post-hoc tests were used to compare paired data where appropriate. P values less than 0.05 were considered as significant. All data are expressed as mean (SEM). Statistical comparisons were made at steady state during the clamps.

## **3.5 Results**

### **3.5.1 Diet Analysis**

There was a small but significant ( $P < 0.01$ ) excess energy intake in the HF treatment compared to CON [11.9 (0.2) vs. 11.0 (0.2) MJ/d] although this did not result in significant changes in body mass and resting energy expenditure. All subjects experienced symptoms of lethargy and hunger during the HF dietary treatment. The mean daily proportion of energy as CHO was 7.4 (0.2) % in the HF diet and 49.8 (0.8) % in the CON diet ( $P < 0.01$ ), whereas the fat intake was 76.7 (0.4) vs. 32.3 (0.7) % ( $P < 0.01$ ), respectively. There was no difference in protein intake between the 2 diets. Furthermore, it should be noted that there was no difference in daily energy intake and macronutrient composition between the subjects' prescribed CON diet and their

recorded habitual diet [10.8 (1.2) MJ/d, of which 47.0 (1.5) % was derived from CHO, 34.3 (1.7) % from fat and 18.7 (1.7) % from protein].

### **3.5.2 Circulating metabolites and hormones**

There were no differences in fasting blood glucose and serum insulin concentrations before and after each diet (Table 3.6.2). There was no difference in Homeostatic Modelling Assessment – Insulin resistance index (HOMA-IR) between dietary interventions [CON: Pre-diet 0.99 (0.18) vs. post 0.89 (0.11) and HF: Pre-diet 0.77 (0.06) vs. post 0.63 (0.07)]. There were no differences in insulin concentrations at steady state during the hyperinsulinaemic clamp [CON: 71.8 (3.5) vs. HF: 70.0 (3.5) mU/l]. There were no diet-induced differences in fasting plasma NEFA concentrations but they were markedly suppressed during both clamps. Fasting  $\beta$ -hydroxybutyrate concentrations were increased after the HF diet compared to the CON diet ( $P = 0.05$ ; Table 3.6.2). During the clamp,  $\beta$ -hydroxybutyrate concentrations were completely suppressed with no differences between diets.

### **3.5.3 Whole body substrate metabolism**

There was a difference between diets in non-protein respiratory exchange ratio at basal [CON 0.79 (0.01) vs. HF 0.75 (0.01),  $P < 0.05$ ] and insulin-mediated conditions [CON 0.91 (0.01) vs. HF 0.87 (0.01),  $P < 0.01$ ]. There was no diet-induced difference in resting metabolic rate [CON 5.65 (0.17) vs. HF 5.65 (0.17) kJ/min]. Insulin increased ( $P < 0.01$ ) the metabolic rate after both diets but there were no differences between diets [CON 5.99 (0.17) vs. HF 6.07 (0.17) kJ/min].

There was no difference in  $R_d$  between diets under basal conditions [CON 10.6 (2.6) vs. HF 8.8 (2.9)  $\mu\text{mol/kg/min}$ ]. HGO was comparable under basal conditions following both diets [CON: 10.9 (0.9) vs. HF 8.8 (0.8)  $\mu\text{mol/kg/min}$ ] and was



completely suppressed during clamp conditions in both trials. Under clamp conditions  $R_d$  was higher during the last 30 min of the clamp after the HF diet [CON 57.5 (3.8) vs. HF 64.5 (4.9)  $\mu\text{mol/kg/min}$ ,  $P < 0.05$ , Fig. 3.6.1].  $C_{ox}$  was reduced ( $P < 0.05$ ) after HF when compared to CON under basal [CON 8.0 (1.2) vs. HF 4.6 (1.4)] and clamp conditions [CON 21.5 (2.4) vs. HF 17.2 (1.0)  $\mu\text{mol/kg/min}$ ]. NOGD under clamp conditions was greater after the HF diet [CON 36.0 (2.5) vs. HF 47.3 (4.6)  $\mu\text{mol/kg/min}$ ,  $P = 0.01$ ].  $F_{ox}$  was higher under clamp conditions after the HF diet compared with CON diet (CON  $3.3 \pm 0.5$  vs. HF  $5.3 \pm 0.3$   $\mu\text{mol/kg/min}$ ,  $P < 0.05$ ), with a trend for a difference under basal conditions (CON  $7.2 \pm 0.2$  vs. HF  $8.7 \pm 0.7$   $\mu\text{mol/kg/min}$ ,  $P = 0.06$ ).

#### **3.5.4 Muscle metabolism**

There was no difference in baseline PDCa activity between treatments (Fig. 3.6.2). The HF diet induced a 65% reduction in PDCa activity ( $P < 0.05$ ) when compared to CON. The insulin-mediated increase in PDCa activity was blunted after the HF diet when compared to CON diet ( $P < 0.05$ ). Muscle glycogen concentrations were similar prior to both diets (Fig. 3.6.3). The HF diet caused a 26% decline in muscle glycogen content, which was different from the change after the CON diet ( $P < 0.05$ ). Insulin-mediated muscle glycogen deposition was greater ( $P < 0.05$ ) after the HF diet when compared to CON. There were no differences between diets in muscle ATP, PCr, lactate, G-6-P and creatine concentrations (Table. 3.6.3). Muscle acetylcarnitine concentrations were unchanged after the CON diet but increased after the HF diet and remained high during the clamp when compared to CON (Table. 3.6.3)

#### **3.5.5 Muscle expression of metabolic genes and proteins**

The HF diet induced a ~2-fold increase ( $P < 0.05$ ) in both PDK4 mRNA and protein content when compared to CON [Fig. 3.6.4 (B & E)]. Insulin infusion down regulated

the expression of PDK4 mRNA, but not protein, after both diets. There were no diet- or insulin-induced changes in PDK2 mRNA and protein expression [Fig. 3.6.4 (A, C and D)]. There were significant increases in insulin-mediated HKII and SREBP-1c mRNA [Fig. 3.6.4 (F)] expression after the CON diet, whereas non-significant increases were observed after the HF diet. There were no diet- or insulin-induced changes in mRNA expression of PK and ChREBP [Fig. 3.6.4 (G)], PPAR $\alpha$  and  $\delta$ , PGC1 $\alpha$ , CPT1, FAT/CD36, LCAD and the protein expression of HKII, SREBP1c, PPAR $\alpha$  and  $\delta$  (data not shown).

### **3.6 Figures and Tables**

**Table 3.6.1 Diet Composition**

	<b>High Fat (HF)</b>	<b>Control (CON)</b>
<b>Energy Intake (MJ/day)</b>	11.9 (0.2)*	11.0 (0.2)
<b>Fat (g)</b>	258.8 (6.2)	94.8 (3.1)
<b>Fat (%)</b>	76.4 (0.5)	32.3 (0.7)
<b>Saturated Fat (g)</b>	121.7 (4.2)	25.5 (1.2)
<b>Monounsaturated Fat (g)</b>	88.1 (2.4)	22.8 (0.7)
<b>Polyunsaturated Fat (g)</b>	17.7 (0.7)	13.4 (1.2)
<b>Carbohydrate (g)</b>	62.1 (3.5)	350.6 (12.0)
<b>Carbohydrate (%)</b>	7.6 (0.3)	49.8 (0.8)
<b>Sugar (g)</b>	28.1 (1.5)	143.7 (11.1)
<b>Sugar (%)</b>	3.4 (0.1)	20.5 (1.3)
<b>Starch (g)</b>	9.6 (0.8)	24.3 (2.0)
<b>Starch (%)</b>	1.2 (0.1)	171.4 (16.6)
<b>Protein (g)</b>	120 (4.1)	115.9 (2.9)

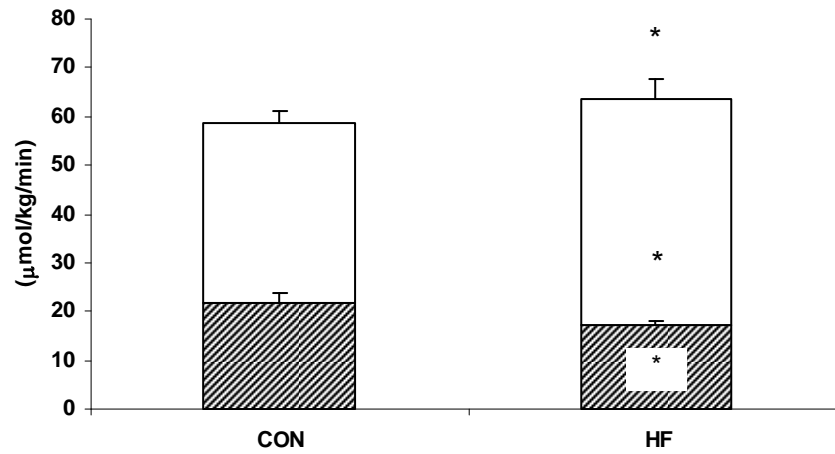
Mean (SEM). \* P < 0.01 compared to control value.

**Table 3.6.2 Blood metabolites and hormones**

	CON		HF	
	Pre	Post-Diet	Pre	Post-Diet
<b>Blood Glucose (mmol/l)</b>	4.8 (0.1)	4.5 (0.1)	4.5 (0.1)	4.4 (0.2)
<b>Serum Insulin (mU/l)</b>	7.4 (1.3)	6.4 (0.8)	6.1 (0.6)	5.1 (0.7)
<b>Plasma NEFA (mmol/l)</b>	0.47 (0.08)	0.48 (0.04)	0.42 (0.04)	0.53 (0.04)
<b>Blood <math>\beta</math>-hydroxybutyrate (mmol/l)</b>	0.08 (0.02)	0.13 (0.03)	0.07 (0.01)	0.56 (0.19)*

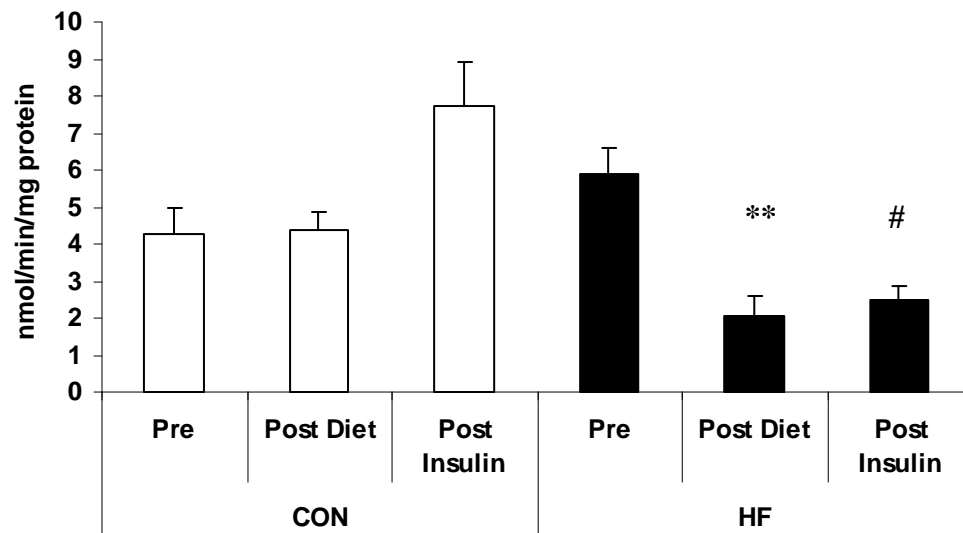
Data are mean (SEM); n = 10. \*P = 0.05 from Pre HF.

**Figure.3.6.1 Insulin-mediated whole-body glucose disposal ( $R_d$ )**



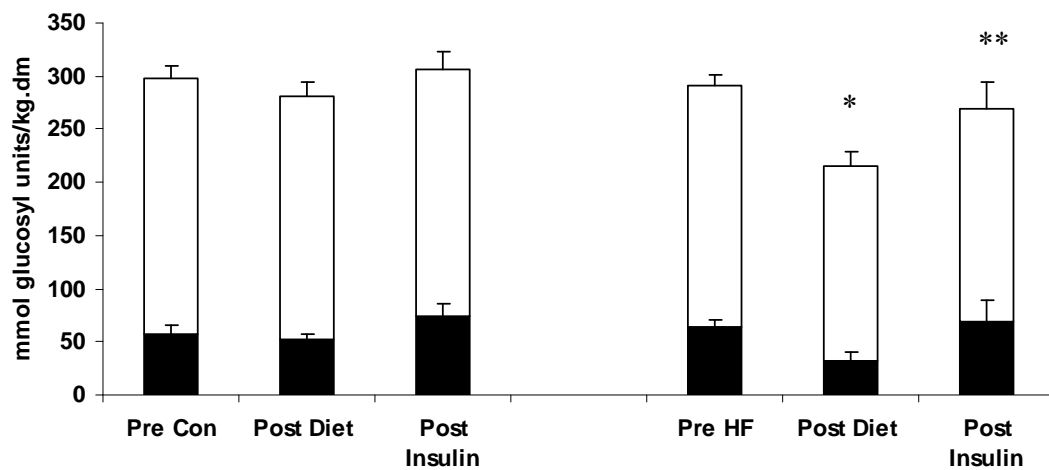
Whole-body glucose disposal ( $R_d$ ) after the control (CON) and high fat (HF) diets. Open part of column denotes non-oxidative disposal and hatched part denotes CHO oxidation. \*  $P < 0.05$  when compared to CON diet for differences in  $R_d$ , non-oxidative disposal and CHO oxidation.

**Figure.3.6.2 Muscle PDCa activity**



Muscle PDCa activity at baseline, post-diet and post-insulin infusion. \*\* $P < 0.05$  for HF diet-induced reduction in PDCa and # $P < 0.05$  for blunted PDCa activity post-insulin when compared to CON diet (treatment x time interaction, 2 way ANOVA). Data are mean (SEM);  $n = 10$  for  $R_d$  and  $n = 8$  for PDCa.

**Figure.3.6.3 Muscle Glycogen**



Muscle glycogen concentrations pre-, post-diets and post insulin clamp. Open columns represent proglycogen and closed columns represent macroglycogen. Data are mean (SEM); n = 10. \*P < 0.05 for reduction in glycogen concentrations post HF diet when compared to CON. \*\*P<0.05 for increase in glycogen concentrations post-insulin in HF treatment.

**Table 3.6.3 Muscle metabolite concentrations**

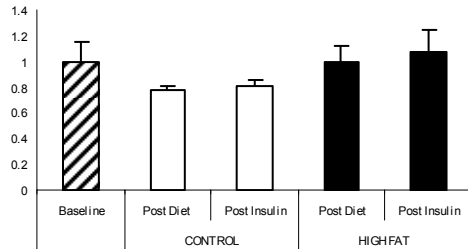
	CON			HF		
	Pre	Post-Diet	Post-Insulin	Pre	Post-Diet	Post-Insulin
<b>ATP</b>	23.4 (0.7)	26.2 (1.4)	24.2 (0.8)	25.4 (1.9)	23.5 (0.6)	25.7 (0.9)
<b>PCr</b>	71.9 (3.4)	70.8 (4.9)	61.3 (3.8)	64.7 (4.2)	65.4 (3.2)	70.1 (3.5)
<b>Creatine</b>	43.9 (4.6)	40.6 (4.9)	45.3 (4.5)	41.1 (4.6)	45.0 (3.8)	37.6 (4)
<b>Lactate</b>	7.0 (0.7)	7.2 (0.9)	9.0 (1.0)	7.2 (1.3)	5.5 (0.8)	11.2 (1.4)
<b>Acetylcarnitine</b>	4.3 (0.4)	4.5 (0.5)	4.7 (0.6)	4.7 (0.5)	7.9 (1.3)*	5.3 (1.0)**
<b>Glucose-6-Phosphate</b>	3.4 (0.9)	4.7 (1.7)	1.2 (1.1)	3.9 (1.1)	3.9 (1.2)	2.9 (1.1)

Data are expressed as mean (SEM) as mmol/kg dry muscle. n = 10 for ATP, PCr, creatine and G-6-P, and n = 9 for lactate and acetylcarnitine. \* P<0.05 for increase in acetylcarnitine concentrations post-HF diet, \*\*P<0.05 for elevated acetylcarnitine concentrations post-insulin after HF compared with CON (Treatment\*time interaction, 2 way ANOVA).

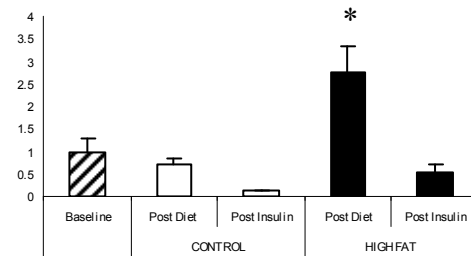


**Figure.3.6.4 Messenger RNA and protein expression.**

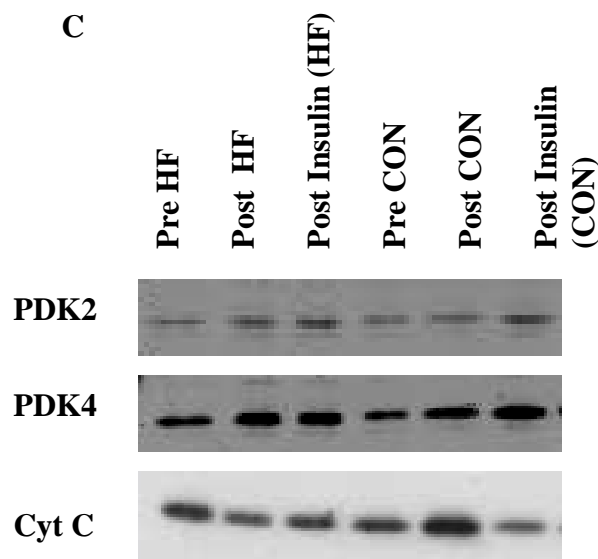
**A: PDK2 mRNA**



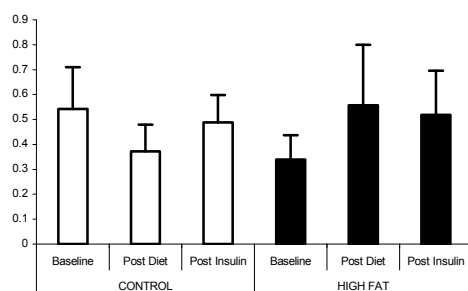
**B: PDK4 mRNA**



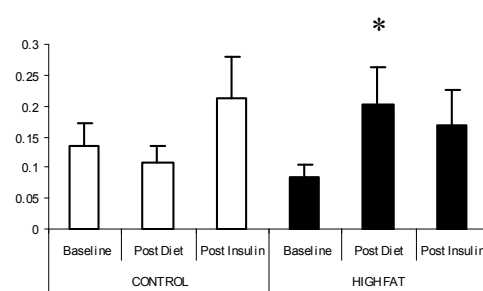
**C**



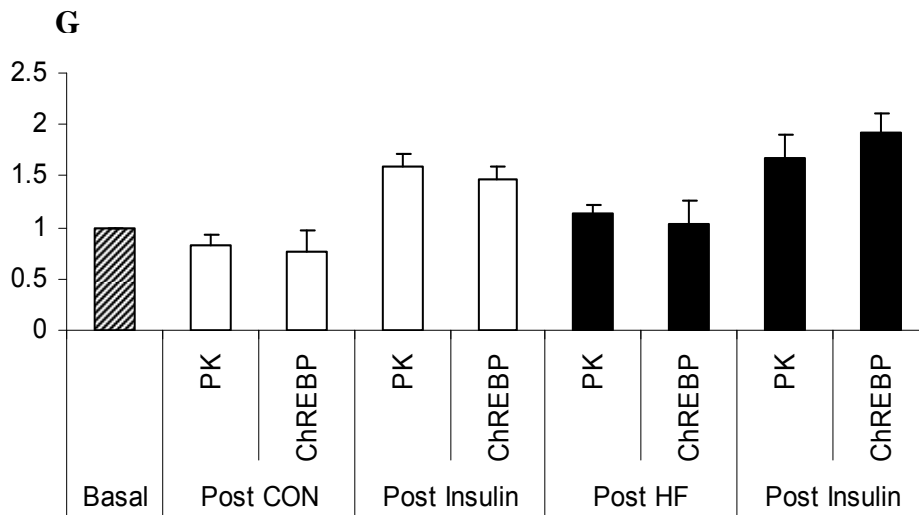
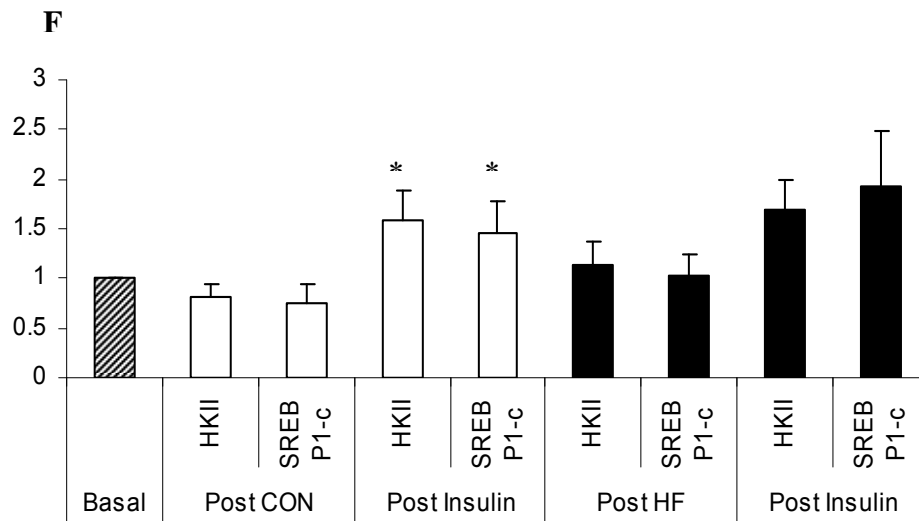
**D: PDK2 protein**



**E: PDK4 protein**



3.6.4 (A & B): Muscle PDK 2 and 4 mRNA expressions, 3.6.4 (C): representative western blots of PDK2, PDK4 and cytochrome C (control) and 3.6.4 (D & E): PDK2 and PDK4 mitochondrial protein expression. Open columns denote CON diet and closed columns denote HF diet. All mRNA changes are relative to baseline value = 1 (denoted by the hatched column). Data are mean (SEM); A: n = 10, B: n = 9, C: n = 8, D: n = 7. \* P < 0.05 (Treatment effect, ANOVA).



3.6.4 (F): Baseline, post-diet and post-insulin mRNA expression changes of HKII and SREBP-1c and 3.6.4 (G): PK and ChREBP. All mRNA changes are relative to basal value = 1 (denoted by the hatched column). Open columns denote CON diet and closed columns denote HF diet. Data are mean (SEM); n = 10. \* P < 0.05 when compared to the post diet value.

### **3.7 Discussion**

In the present study 6 days of high fat feeding led to a reduction of basal and insulin-mediated CHO oxidation without inducing whole-body insulin resistance. In fact, an increase in  $R_d$  during the last 30 min of the clamp was observed after the HF diet compared to the CON diet. As a consequence there was an increase in insulin-mediated non-oxidative glucose disposal both at the whole body and skeletal muscle level. These responses were associated with impaired muscle PDCa activity, most likely as a result of selective up regulation of PDK4 expression. Furthermore, it is shown for the first time that insulin infusion can suppress PDK4 but not PDK2 gene expression *in vivo* in human skeletal muscle.

High fat feeding in humans has produced contradictory results. In agreement with the findings from the present study, high fat dietary treatment for 11 to 21 days did not induce whole body insulin resistance although the partitioning of glucose metabolism was altered with decreased oxidation and increased non-oxidative glucose disposal (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001). Furthermore, a study employing a high fat diet for 16 days reported no effect on insulin sensitivity during a 3h hyperinsulinaemic clamp although 12 out of 25 subjects had greater GIRs during the last hour of the infusion after the high fat diet than after the control diet (Yost, Jensen et al. 1998). Similarly, a slight increase in  $R_d$  during the last 30 min of the clamp was observed after the HF than the CON diet. In contrast, high fat feeding for just 3 days appears to induce whole body insulin resistance (Bachmann, Dahl et al. 2001; Pehleman, Peters et al. 2004). It is possible that acute changes in dietary fat availability (several hours up to 3 days) might induce insulin resistance because of a greater imbalance between plasma NEFA availability and their muscle oxidation, whereas after several days an increase in NEFA availability can be compensated by a

greater intramuscular lipid storage and/or utilisation. On the other hand, this difference may also be due to methodological differences in determining insulin resistance in the above studies (oral glucose tolerance test vs. insulin clamps; clamp duration and ambient insulin concentrations). Therefore, further studies are required to elucidate the precise sequence of adaptations to high fat diets in humans.

In the present study, the differential partitioning of intracellular glucose metabolism (with decreased oxidative and increased non-oxidative glucose disposal) observed in the high fat dietary treatment extends previous findings at the whole-body level (Cutler, Gray et al. 1995; Bisschop, de Metz et al. 2001) and may constitute one of the earliest intramuscular adaptations to high fat diet in healthy humans. Moreover, the reduction in CHO oxidation was not readily reversible even after 4h of insulin and glucose infusion. Glucose transport and oxidation, along with glycogen synthesis, are also impaired in patients with type 2 diabetes (Del Prato, Bonadonna et al. 1993). Interestingly, when the defect in glucose transport was normalised by hyperglycaemia and hyperinsulinaemia, only glucose oxidation remained impaired (Del Prato, Bonadonna et al. 1993). Therefore, it is likely that the impairment in insulin-mediated glucose oxidation is an early adaptation in metabolic states characterized by elevated lipid metabolism.

In the present study, a reduction in muscle glycogen after the high fat diet was observed as shown previously (Putman, Spriet et al. 1993; Zderic, Davidson et al. 2004) and this is likely to have increased muscle glycogen synthase activity (Cutler, Gray et al. 1995). This, in turn, may account for the greater insulin-mediated muscle glycogen storage observed after the HF diet in the present study. Indeed, the increase in muscle glycogen content was comparable to the increase in insulin-mediated whole

body glucose disposal observed after the HF diet. In particular, non-oxidative glucose disposal in the control and HF trials were 36 and 47  $\mu\text{mol/kg/min}$ , respectively, whereas the increase in muscle glycogen storage was 31 and 56  $\mu\text{mol glucosyl units/kg/min}$ , respectively. Perhaps this is not surprising if one considers that under conditions of impaired CHO oxidation when glucose is made available intracellularly, either by feeding or during a hyperinsulinaemic clamp, it will be directed towards either glycogen synthesis or non-oxidative glycolysis (Yki-Jarvinen, Puhakainen et al. 1991; Cutler, Gray et al. 1995). However, under hyperinsulinaemic conditions the contribution of non-oxidative glycolysis is rather small (3-5%) although it may double under conditions of elevated fat availability and impaired CHO oxidation (Yki-Jarvinen, Puhakainen et al. 1991; Cutler, Gray et al. 1995). In the current study, muscle lactate concentration tended to increase more during the clamp after the high fat diet. In the absence of changes in muscle glucose-6-phosphate concentrations (an index of non-oxidative glycolysis) the higher accumulation of muscle lactate was likely to be due to the impairment in PDC activation observed in the HF trial. Furthermore, it should be noted that under hyperinsulinaemic conditions glucose disposal is not restricted to skeletal muscle tissue. Thus, the increase in insulin-stimulated whole body non-oxidative metabolism after the high fat diet would also be expected to replenish non-muscle e.g. hepatic glycogen stores, although the latter was not determined in the present study. Further studies are required to examine the detailed partitioning of non-oxidative glucose disposal in humans after consumption of a high-fat diet.

The changes in whole-body oxidative metabolism were also mirrored by corresponding changes in intracellular oxidative metabolism. Following the HF diet, muscle PDCa activity was reduced, indicating reduced CHO oxidation, and muscle

acetylcarnitine concentrations were increased reflecting increased fat oxidation. During the hyperinsulinaemic clamp, muscle PDCA activity remained lower whereas both muscle lactate and glycogen concentrations increased (reflecting an increase in intracellular non-oxidative glucose disposal). Thus, it would appear that at the cellular level the reduced flux through PDCA facilitates the increase in both non-oxidative glucose metabolism and oxidative fat metabolism.

Skeletal muscle PDC activity is inhibited by PDK, and four PDK isoenzymes have been identified in skeletal muscle, but only PDK4 expression is increased in starvation and diabetes in animal models (Wu, Sato et al. 1998; Wu, Blair et al. 2000) and starvation in healthy humans (Tsintzas, Jewell et al. 2006). Similarly, in the present study administration of a high-fat diet for 6 days was associated with significant increases in muscle PDK4 mRNA and protein expression, which is in agreement with a previous 3-day study in healthy humans (Peters, Harris et al. 2001). However, there was no diet or insulin-induced changes in gene and protein expression of PDK2, the other major isoform in human skeletal muscle. Insulin was shown to down regulate transcript levels of PDK2 and PDK4 in insulin resistant non-diabetic Pima Indians (Majer, Popov et al. 1998). In contrast, this is the first study to show that in healthy humans insulin can readily suppress PDK4 but not PDK2 gene expression in skeletal muscle although there was no effect on protein expression, most likely due to the short-term nature of the insulin infusion.

The selective increase in PDK4 expression was associated with a decrease in muscle PDCA activity following the high fat diet. The fact that insulin infusion did not affect PDK4 protein expression indicates no suppression of PDK activity by insulin, thus explaining the blunted PDCA activity after 4h of insulin infusion. Moreover, this data

confirms that in addition to allosteric regulation, PDK activity is regulated by transcriptional mechanisms. Contrary to evidence from cell lines and animal studies that high fat feeding induced up regulation of PDK4 is mediated by PPAR signalling (Muoio, MacLean et al. 2002; Muoio, Way et al. 2002; Abbot, McCormack et al. 2005), there were no changes observed in this study in gene expression of PPAR $\alpha$ , PPAR $\delta$ , their coactivator PGC1 $\alpha$  or some of their known transcriptional targets (CD36, CPT1 and LCAD). This is in line with the findings from a recent study indicating that the selective up regulation of skeletal muscle PDK4 expression in fasted humans occurs in a novel manner distinct, at least in part, from the PPARs pathway (Tsintzas, Jewell et al. 2006). Although not measured in the present study, other signalling pathways potentially involved in high dietary fat mediated upregulation of PDK4 could include activation of FOXO (Kwon, Huang et al. 2004) and/or activation of PGC-1 $\alpha$ / Estrogen-related receptor (ERR  $\alpha$ ) signalling systems (Wende, Huss et al. 2005; Araki and Motojima 2006). This would require further investigation in future human studies.

In conclusion, short-term high fat/low CHO dietary intake does not induce whole-body insulin resistance in healthy humans but causes a shift in intracellular glucose metabolism by reducing insulin-mediated glucose oxidation and stimulating non-oxidative glucose disposal. Up regulation of muscle PDK4 expression is likely to be responsible for the inhibition of muscle PDK $\alpha$  and the subsequent reduction in glucose oxidation. The latter appears to precede changes in glucose uptake further highlighting a key role for PDK4 in substrate metabolism and insulin action in human skeletal muscle.

## **Chapter 4**

**Exercise under hyperinsulinaemic conditions increases whole body glucose disposal without affecting muscle glycogen utilisation in type 1 diabetes.**

### **4.1 Introduction**

Exercise can play an integral role in the management of type 1 diabetes due to its blood glucose lowering effect (Lawrence 1926). Adjustments to insulin dose and carbohydrate (CHO) intake are generally necessary to reduce the risk of exercise-induced hypoglycaemia (Kemmer 1992), and patients need to be proactive with their diabetes management to accommodate planned exercise (Schiffrin and Parikh 1985). Current recommendations are for patients to reduce insulin doses prior to exercise (Rabasa-Lhoret, Bourque et al. 2001), although, in practice, many patients continue with customary insulin doses and take additional CHO. The average pre-prandial and peak therapeutic insulin concentrations in patients using a basal-bolus insulin regimen are typically 20 to 30 and 70 to 80 mU/l respectively (Gulan, Gottesman et al. 1987; Nielsen, Jorgensen et al. 1995). By contrast, in non-diabetic subjects, circulating insulin normally declines considerably to 5 mU/l or below during exercise, and glucagon concentration increases in order to facilitate substrate mobilization (Felig, Wahren et al. 1972; Wasserman, Geer et al. 1991; Coggan, Raguso et al. 1997). Depending on the exercise timing and its temporal relationship to the last insulin injection, patients with diabetes will therefore generally be carrying out exercise under markedly hyperinsulinaemic conditions with an increased risk of hypoglycaemia (Rabasa-Lhoret, Bourque et al. 2001).

In healthy volunteers exercising under physiological hyperinsulinaemia (DeFronzo, Ferrannini et al. 1981; Wasserman, Geer et al. 1991), exercise and insulin interact synergistically to increase glucose uptake. In contrast, patients with type 1 diabetes



exercising at 50%  $\dot{V}O_2$  max under basal insulin concentrations of about 10 mU/l show reduced glucose uptake and greater reliance on fat oxidation when compared to controls (Raguso, Coggan et al. 1995). However, when the intensity of exercise was increased to 70%  $\dot{V}O_2$  max, these differences were no longer present. In reality, typical everyday therapeutic insulin concentrations are often significantly greater than the concentrations employed in the above study (Nielsen, Jorgensen et al. 1995) and not all patients with type 1 diabetes are able to carry out such high intensity exercise. The impact of moderate exercise (60%  $\dot{V}O_2$  max) at normal therapeutic hyperinsulinaemic conditions on whole-body substrate and muscle substrate metabolism in patients with type 1 diabetes has not been investigated.

The underlying cellular mechanisms involved in regulating both insulin and exercise mediated glucose uptake are only partly understood. The serine phosphorylated forms of protein kinase B (PKB/Akt), glycogen synthase kinase 3 (GSK-3)  $\alpha$  and  $\beta$  isoforms and extracellular signal-regulated protein kinase (ERK 1/2) are intracellular signalling targets potentially involved in the regulation of both insulin- and exercise-mediated glucose uptake (Christ-Roberts, Pratipanawatr et al. 2003; Sakamoto, Arnolds et al. 2004). However, the impact of hyperinsulinaemia and exercise in patients with type 1 diabetes on these insulin-signalling targets is not known.

The aims of this study were to examine the effects of hyperinsulinaemia approximating to pre-prandial and peak therapeutic insulin concentrations during moderate exercise on, a) whole body and muscle substrate metabolism in patients with type 1 diabetes and b) key insulin-signalling targets involved in the regulation of glucose uptake in muscle in patients with type 1 diabetes. It was hypothesized that

exercise under peak therapeutic insulin concentrations would increase glucose utilization and oxidation and reduce reliance on intramuscular glycogen as a metabolic fuel.

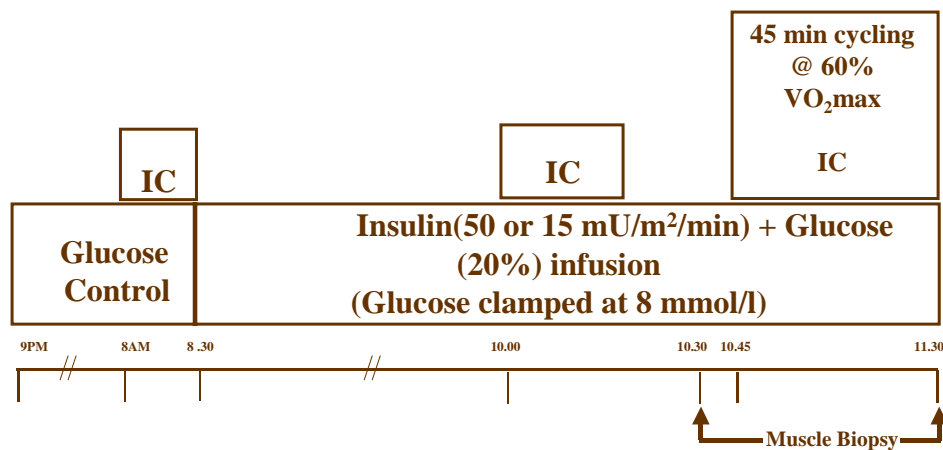
#### **4.2 Patients and Preliminary Measurements**

Eight recreationally active, C-Peptide negative men with type 1 diabetes were recruited. The mean (SEM) age and BMI were 36.4 (1.5) years and 24.6 (0.7) kg/m<sup>2</sup> respectively. The mean duration of diabetes was 11.3 (1.4) years and they had reasonably well controlled diabetes with a glycated haemoglobin of 7.9 (0.2) %. Six patients were on a basal bolus subcutaneous insulin regimen and two were on twice a day premixed insulin injections. All patients were screened to exclude micro and macro vascular complications. The patients' mean  $\dot{V}O_2$  peak was 44.5 (1.2) ml.kg<sup>-1</sup>.min<sup>-1</sup> as determined during a preliminary visit using an incremental cycling test (Lode Excalibur cycle ergo meter, Groningen, Netherlands). The criteria used for attainment of  $\dot{V}O_2$  peak were; heart rate response greater than 90% of predicted maximum heart rate, RER > 1.10 (British Association of Sport and Exercise Sciences criteria). Patients were familiarized with the exercise protocol during pre-study visits to the laboratory, and were informed of all procedures and risks associated with the experimental procedures prior to obtaining informed consent. All procedures used in this study were performed according to the Declaration of Helsinki and approved by the Nottingham NHS Research Ethics Committee.

## **4.3 Study Design and Protocol**

### **4.3.1 Study Design**

Studies were carried out in the fasted state on 2 occasions in random order with a 2-week interval between visits and the design is schematically represented below. Indirect calorimetry measurements are abbreviated as IC. Volunteers maintained an isocaloric diet and monitored their capillary blood glucose closely prior to the study. They were requested to avoid smoking, alcohol and exercise for three days prior to each study visit.



#### 4.3.2 Study Protocol

The subjects omitted their medium/long acting insulin for 24 hours prior to the study. On the evening prior to study, subjects consumed a standardised high CHO (2g/kg) meal, injected their usual short acting soluble insulin and were admitted to the hospital for a low dose intravenous insulin infusion to maintain euglycaemia overnight. An ante grade cannula was inserted into a cubital fossa vein for insulin infusion. A retrograde cannula was inserted into a dorsal hand vein for blood sampling to measure glucose during the overnight stay and also for drawing arterialised blood samples the following day during the study with the hand placed in a hot air box (55<sup>0</sup>C). At 8AM the following morning, subjects were transferred to the exercise laboratory for the study protocol. Resting  $\dot{V}CO_2$  and  $\dot{V}O_2$  were measured for 20 min using a ventilated hood system (GEM, Nutren Technologies, UK) and values were used for the calculation of resting energy expenditure and substrate oxidation rates. Measurements were made while the subjects were lying supine, undisturbed and awake. Intravenous insulin infusions were given at steady-state rates of either 15 or 50 mU.m<sup>-2</sup>.min<sup>-1</sup> (with an appropriate prime) in random order for a total of three hours. The 15 and 50-mU clamps were designed to approximate typical pre-prandial (LO) and post-prandial (HI) therapeutic insulin concentrations respectively (Gulan, Gottesman et al. 1987; Nielsen, Jorgensen et al. 1995). Blood glucose was clamped at 8 mmol/l to approximate the average 24 hour blood glucose levels observed in reasonably well controlled patients with type 1 diabetes (Nielsen, Jorgensen et al. 1995). This also reflects a realistic blood glucose concentration that patients would aim for prior to exercise. Subjects rested in the supine position for the first 120 min of the study and blood samples were collected at regular intervals. A second resting indirect calorimetry measurement was made during the last 20 min of the resting

period. Following this, patients assumed an upright position on the ergometer and cycled at 60%  $\dot{V}O_2$  peak for 45 min and the hyperinsulinaemic clamp was continued during cycling. Five min expired gas samples (Sensormedics Vmax 29, CA, USA) and blood samples were collected every 15 min during cycling. Heart rate and rating of perceived exertion using a Borg scale (Borg 1982) were also measured at 15 min intervals during exercise. A muscle biopsy was obtained from the vastus lateralis under local anaesthetic before and after cycling as described previously (Bergstrom 1962), but with suction applied to the end of the biopsy needle to improve tissue sampling. Two different biopsy sites from the same leg were used for muscle sampling on one visit and the contra lateral leg during the second visit. The post exercise biopsy was taken at least 3 cm distal to the first site to reduce experimental variability (Costill, Pearson et al. 1988). The samples were immediately frozen in liquid nitrogen. Urine samples were collected during the overnight admission and during the study day for glucose and urea measurements. The urine samples were stored at  $-20^{\circ}\text{C}$  in 10% thymol until analysis.

## **4.4 Methods**

A brief description of the methods is given below. Please refer to Chapter 2: Methods for detailed description of analytical methodology.

### **4.4.1 Blood and Urine**

Whole blood and urine glucose, and blood lactate were measured during the experiments. Serum insulin, glucagon and NEFA were measured using commercially available kits. Whole blood  $\beta$ -hydroxybutyrate was measured in perchloric acid (10%) treated blood samples. Plasma catecholamines were measured using high performance liquid chromatography with electrochemical detection (Forster and

Macdonald 1999). Plasma and urine urea were determined using a commercially available enzymatic kinetic method.

#### **4.4.2 Muscle Metabolites**

Muscle powdering, extraction and measurement of metabolites [free glucose, glucose-6-phosphate, lactate, adenosine triphosphate (ATP), phosphocreatine (PCr) and creatine] were carried out as described previously (Harris, Hultman et al. 1974). Acid hydrolyses of small aliquots of the muscle extract and of the muscle pellet left over after perchloric acid extractions were carried out to measure macro- and pro-glycogen respectively. The resultant hydrolysates were used to determine the glucose residues enzymatically (Jansson 1981). Acetylcarnitine was determined enzymatically using a radioisotope assay (Cederblad, Carlin et al. 1990).

#### **4.4.3 Protein Extraction and Western Blots**

Total protein extracts were prepared from 10-20 mg of frozen biopsy tissue. Please refer to chapter 2 for detailed methods (section 2.8.7). Proteins were separated using 5-20% gradient gels and then transferred overnight to Hybond-C Nitrocellulose membranes (Amersham Biosciences, UK). After blocking with 5% BSA for 1h at room temperature, the membranes were incubated overnight with primary antibodies for phospho-Akt serine<sup>473</sup>, phospho-GSK3alpha/beta serine<sup>21</sup>/ serine<sup>9</sup>, phospho-ERK1 threonine<sup>202</sup>, phospho-ERK2 tyrosine<sup>204</sup> (Cell Signaling, USA) and desmin (Sigma, USA) was used as a control. This was followed by incubation for 1h at room temperature with secondary antibodies (Goat anti-rabbit HRP for Akt and GSK3, Amersham Biosciences, UK; goat anti-mouse HRP for ERK 1/2, DakoCytomation, Denmark). All immunoreactive proteins were visualised using ECL plus (Amersham Biosciences, UK) and quantified by densitometry using the Gene Tools version 3.0 software (SynGene, Division of Synoptics Ltd., Cambridge UK).

#### **4.4.4 Calculations**

Exogenous glucose utilization was calculated from the glucose infusion rates (GIR) achieved during the insulin-glucose clamp (DeFronzo, Tobin et al. 1979). Calculations were made at steady state during the resting period of the clamp (90-120min) and during the entire 45 min period of the exercise. The GIR was corrected for glycosuria. Fasting-, insulin- and exercise- mediated CHO and fat oxidation rates were calculated from the  $\dot{V}O_2$  and  $\dot{V}CO_2$  measurements (Frayn 1983). Substrate oxidation at rest was corrected for protein oxidation using urinary urea concentration and correcting for changes in the blood urea pool during the experiment (Jequier, Acheson et al. 1987).

#### **4.4.5 Statistics**

Statistical analysis was carried out using SPSS, Version 11.5 software package. Two-way ANOVA (treatment and time) was used to compare data from repeated measures. Student's t test (two tailed) was used to compare paired data within each infusion rate or corresponding time points between infusion rates where appropriate. A P value less than 0.05 was considered as significant. All data are expressed as Mean (SEM).

## **4.5 Results**

All 8 patients completed the resting part of the experimental protocol and 7 completed the exercise protocol on both visits. The 8<sup>th</sup> subject was unable to complete the exercise protocol on his HI visit. He managed to cycle for 25 min and had to stop because of undue exhaustion. Data acquired in the resting state was included in the data analysis but the incomplete exercise data was excluded from the analysis. Hence for statistical treatment,  $n = 8$  for data acquired at rest whereas  $n = 7$  for the exercise data. This is highlighted in the text, figures and tables. The subjects cycled at a mean workload of 140 (9) watts during both the LO and HI trials. The mean oxygen consumptions during exercise were 29 (2) and 29 (1)  $\text{ml.kg}^{-1}.\text{min}^{-1}$  in the LO and HI trials respectively, being almost exactly 60% of the predetermined maximal aerobic capacity.

### **4.5.1 Blood Metabolites and Hormones**

Blood glucose concentrations at rest were 8.8 (0.4) and 8.3 (0.3) mmol/l during the LO and HI trials respectively. During exercise, glucose concentrations were 8.1 (0.5) and 8.1 (0.4) mmol/l respectively. The serum insulin concentrations at rest were 28 (3) and 76 (5) mU/l during the LO and HI trials respectively (Fig. 4.6.1). During exercise, there were further modest increases ( $P < 0.05$ ) to 35 (3) and 96 (7) mU/l respectively despite the insulin infusion rates remaining constant. Fasting plasma NEFA concentrations were comparable in the two interventions and were suppressed with the insulin infusions at rest and during exercise (Fig. 4.6.2). However, plasma NEFA concentrations were lower in the HI trial ( $P < 0.01$ ). Baseline  $\beta$ -hydroxybutyrate concentrations were comparable [LO: 0.4(0.1) mmol/l vs. HI: 0.4(0.1)] and were suppressed to concentrations below 0.1 mmol/l during both interventions at rest and during exercise. Blood lactate concentrations increased



during insulin infusion with higher values achieved during the 50-mU clamp at rest [LO: 0.5 (0.1) mmol/l vs. HI: 0.7 (0.1);  $P < 0.05$ ]. There was a further increase during exercise but there was no significant difference between trials [LO: 2.3 (0.5) vs. HI: 2.7 (0.6);  $P = \text{NS}$  for treatment effect]. Serum glucagon concentrations were suppressed by insulin infusions after 30 min in the HI trial ( $P < 0.05$ ) and did not increase during exercise, with no differences between studies (Fig. 4.6.3). There was a marked exercise-induced increase in plasma noradrenaline concentrations during both interventions ( $P < 0.05$ ) but only a non-significant increase in plasma adrenaline was observed. However, there was no difference in catecholamine concentrations between studies at rest or during exercise (Fig. 4.6.4).

#### **4.5.2 Whole body substrate metabolism**

During both studies, the exogenous glucose utilisation rate increased substantially to maintain concentrations at  $\sim 8$  mmol/l when patients changed posture from the supine to the ambulant state. The exogenous glucose utilisation rate increased considerably further ( $P < 0.001$ ) during exercise in both studies but more so in the HI trial ( $\Delta 11.3 \pm 1.5 \text{ mg.kg}^{-1}.\text{min}^{-1}$ ) when compared to the LO trial ( $\Delta 4.6 \pm 1.1 \text{ mg.kg}^{-1}.\text{min}^{-1}$ , Table 4.6.1). Exercise also induced a substantial increase in CHO oxidation during both trials but it was 15% greater during the HI trial when compared to the LO trial ( $\Delta 3.7 \pm 1.4 \text{ mg.kg}^{-1}.\text{min}^{-1}$ ,  $P < 0.05$ , Table 4.6.1). During exercise, fat oxidation contributed to  $\sim 23$  and 15% of total energy expenditure in the LO and HI trials respectively (NS, Table 4.6.1). There was no difference in the mean rating of perceived exertion (Borg scale) during exercise between interventions [LO: 13.3(0.6) vs. HI: 13.1(0.6)].

#### **4.5.3 Muscle metabolism**

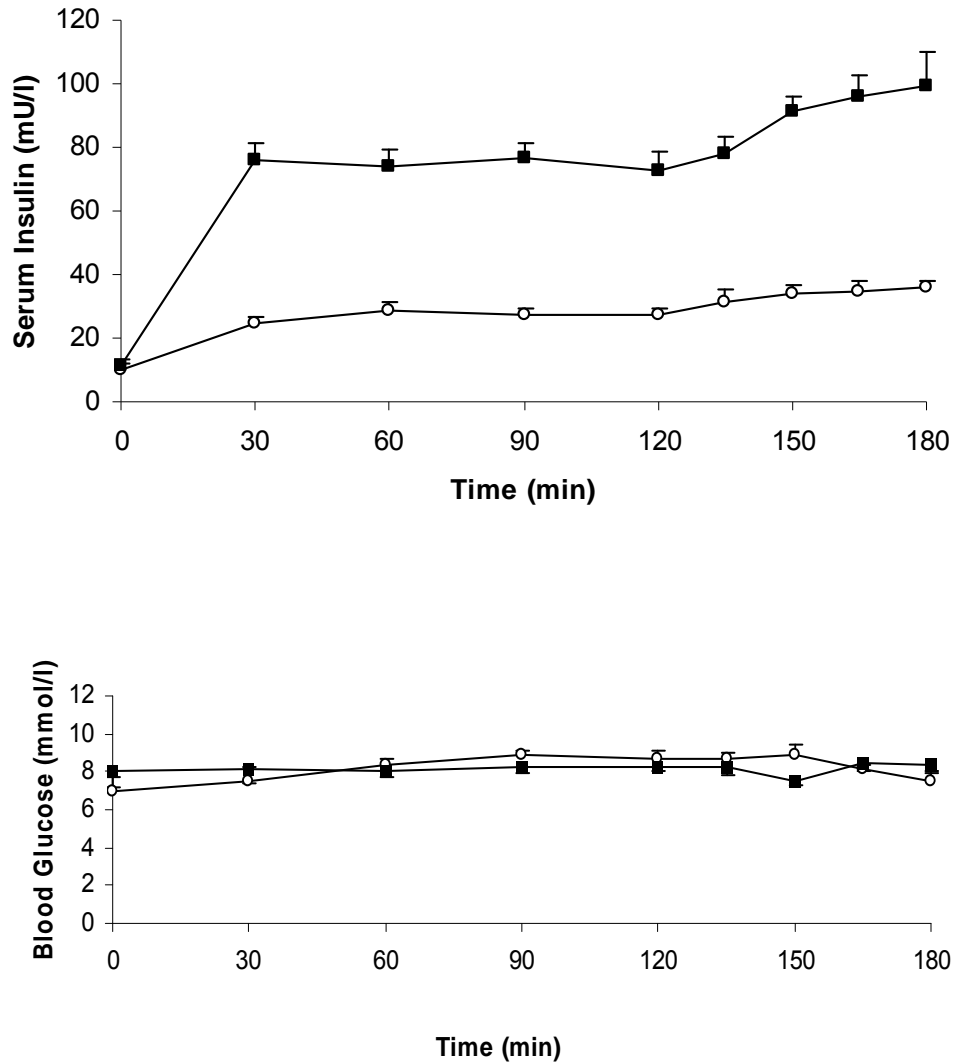
Pre-exercise muscle glycogen concentrations were comparable between trials and were reduced by 40 – 43% after exercise (Table 4.6.2). The rate of glycogen utilization during exercise was similar during both interventions [LO: 3.9 (0.6) vs. HI: 4.1(0.6) mmol glucosyl units.kg dry muscle<sup>-1</sup>.min<sup>-1</sup>]. There were no differences between interventions in either pro- or macro-glycogen utilization rates. There was no increase in intramuscular free glucose accumulation during either intervention. There was a 3 to 4 fold increase in muscle lactate and acetylcarnitine concentrations during exercise with no differences between interventions (Table 4.6.2). The PCr/ATP ratio before and after exercise during the LO [2.7(0.1) and 2.9(0.2)] and HI clamps [2.9(0.2) and 2.8(0.2)] were no different.

#### **4.5.4 Muscle Protein Phosphorylation**

Serine phosphorylation of Akt, GSK 3  $\alpha$ , GSK 3  $\beta$ , ERK1 and ERK2 were similar at the end of the 2h insulin infusions with no significant differences between the LO and HI trials (Fig 4.6.5). Superimposition of exercise over the hyperinsulinaemic conditions did not alter the phosphorylation of these signalling targets and there were no differences between trials (Fig 4.6.5).

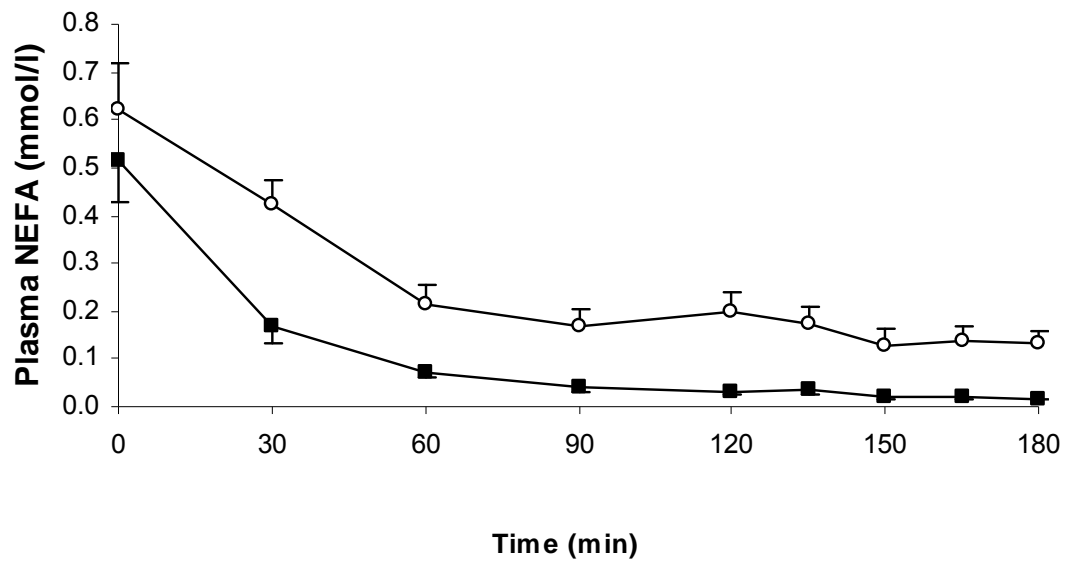
## 4.6 Figures and Tables

Figure 4.6.1 Serum Insulin and Blood Glucose



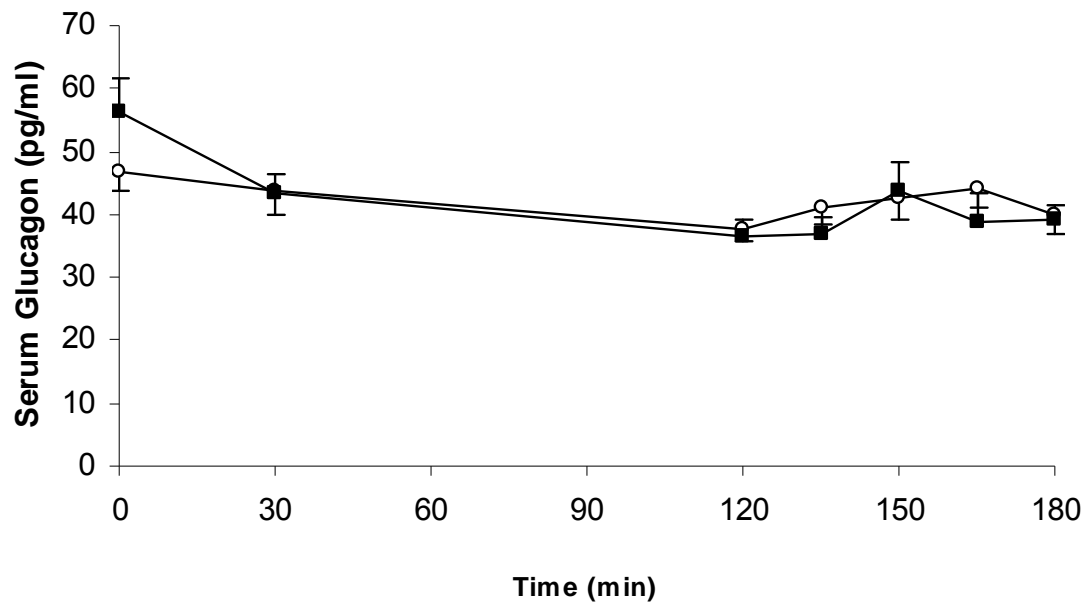
Serum insulin and glucose concentrations during the resting period of the clamp and during exercise in the HI (■) and LO (○) trials. Data are expressed as Mean  $\pm$  SEM. A:  $n = 7$  for resting period and 6 for exercise period. Value immediately before exercise denotes measurement made in sitting position. \*  $P < 0.001$  for differences in concentrations between trials.

**Figure 4.6.2 Plasma NEFA**



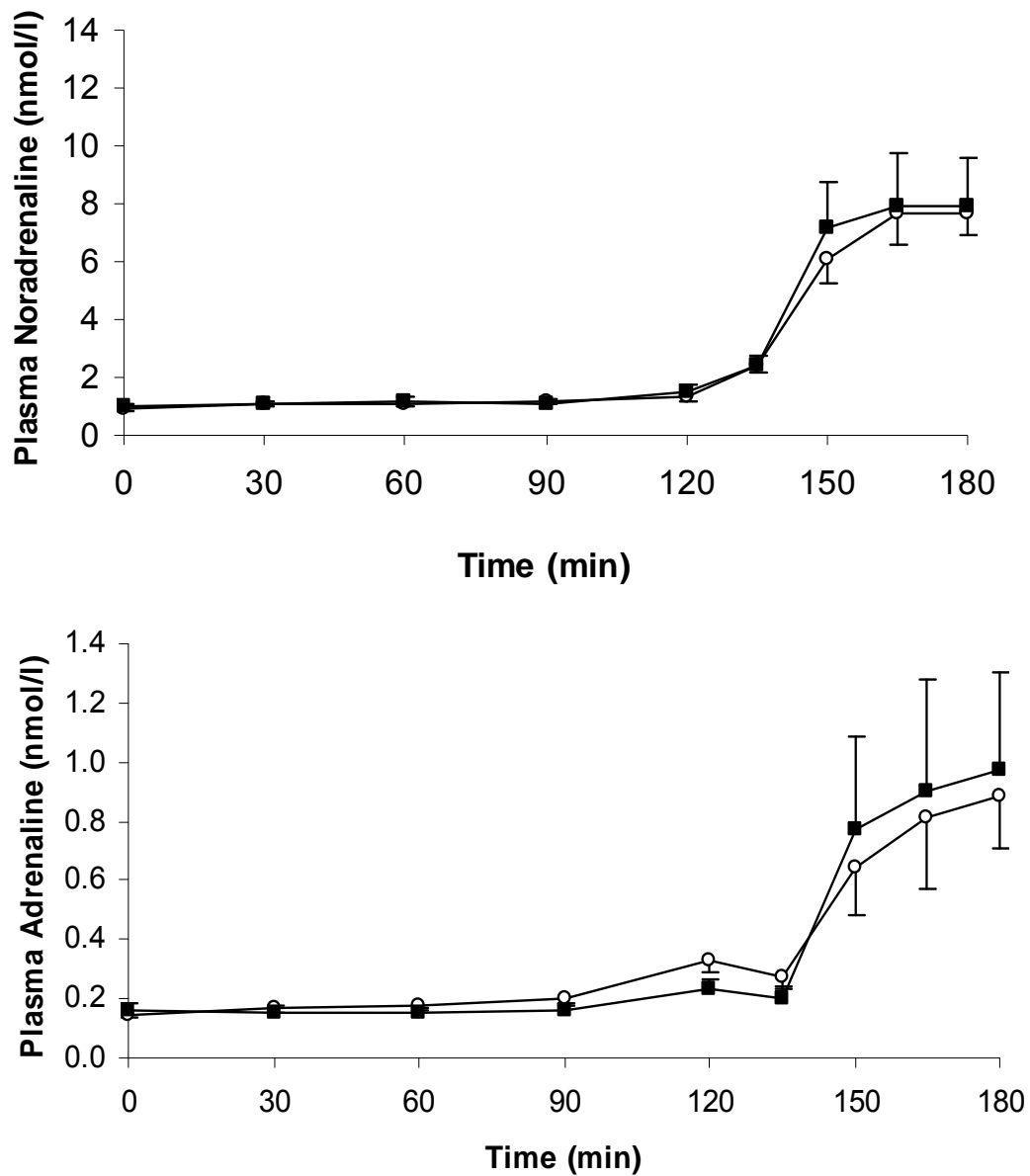
Plasma NEFA concentrations during the resting part of the clamp and during exercise in the HI (■) and LO (○) trials. Data are expressed as Mean  $\pm$  SEM.  $n = 8$  for resting period and 7 for exercise period. Value immediately before exercise denotes measurement made in sitting position. \* $P < 0.01$  for differences between trials.

**Figure 4.6.3 Serum Glucagon**



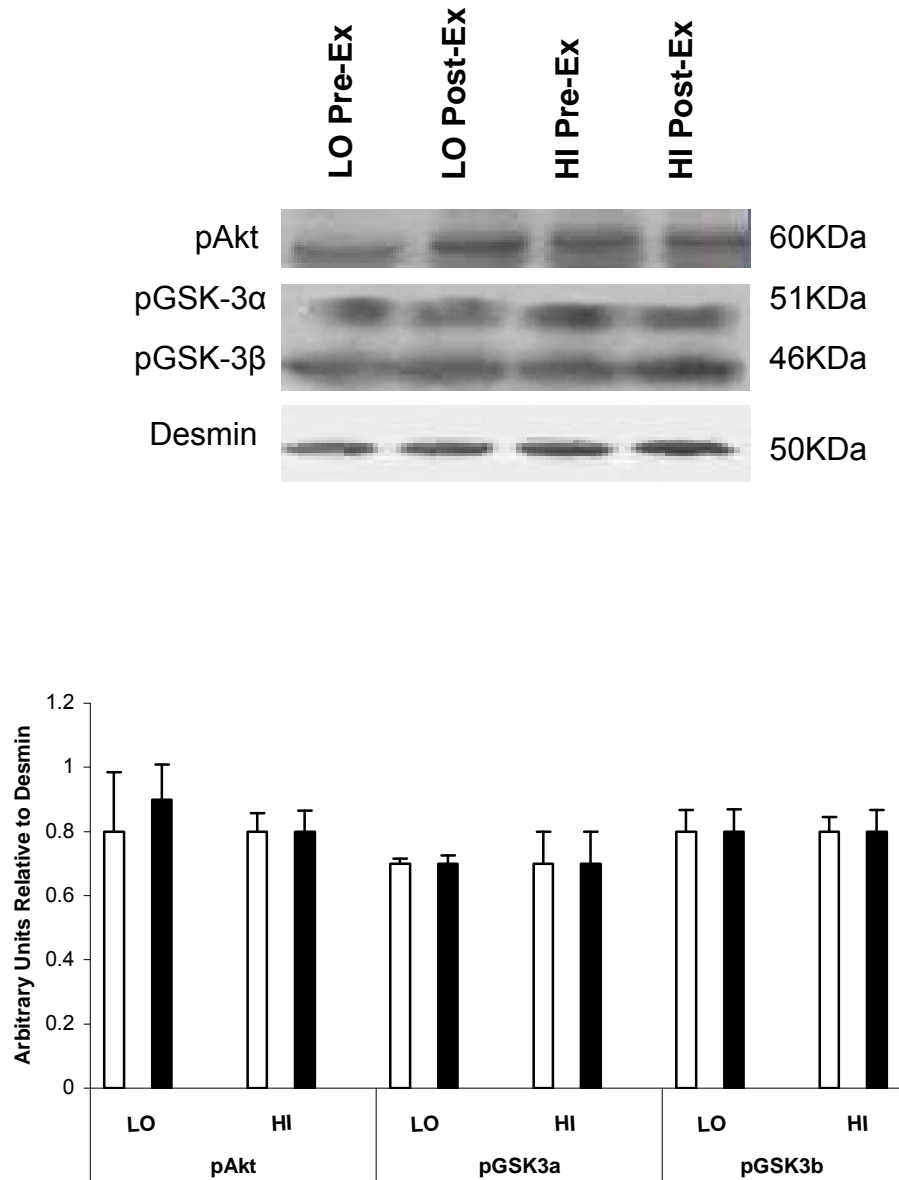
Serum glucagon concentrations during the resting period of the clamp and during exercise in the HI (■) and LO (○) trials. Data are expressed as Mean  $\pm$  SEM.  $n = 8$  for resting period and 7 for exercise period. \*\*  $P < 0.05$  for differences in glucagon concentrations from baseline in the HI trial.

**Figure 4.6.4 Plasma Catecholamines**

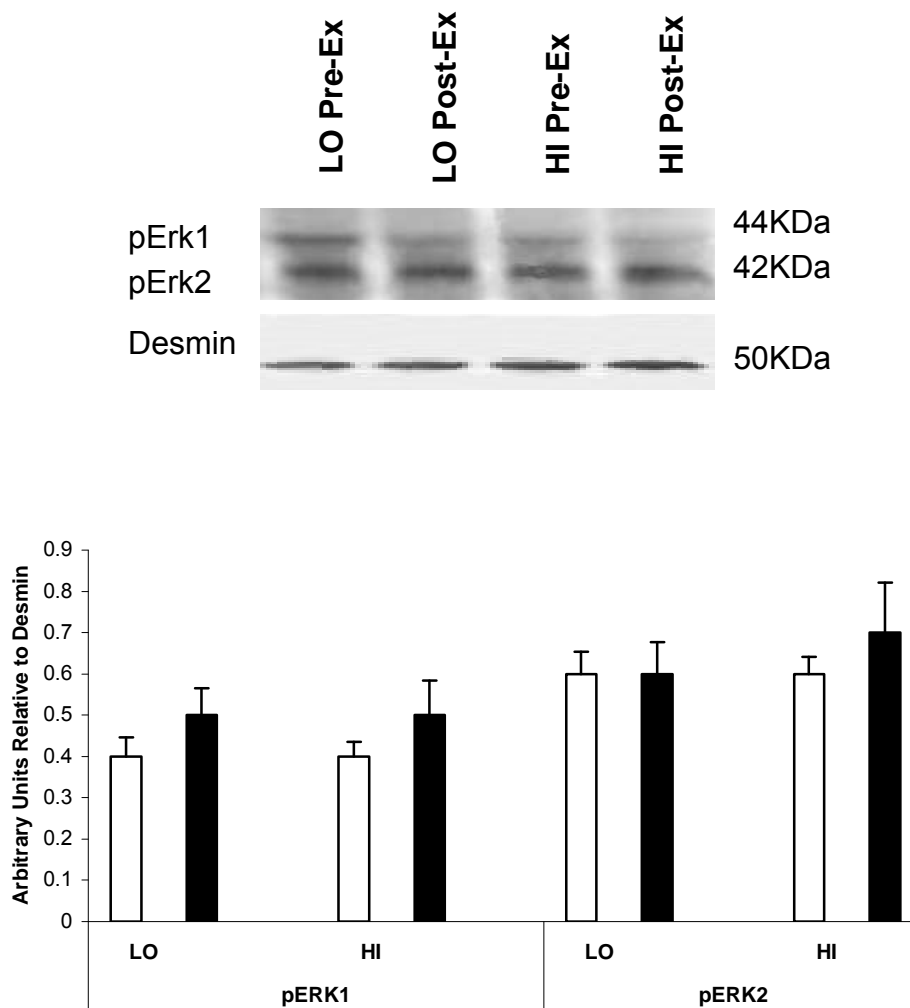


Plasma catecholamine concentrations during the resting period of the clamp and during exercise in the HI (■) and LO (○) trials. Data are expressed as Mean  $\pm$  SEM.  $n = 8$  for resting period and 7 for exercise period. Value immediately before exercise denotes measurement made in sitting position. There were no differences in catecholamine responses between interventions either at rest or during exercise but there was a significant increase in noradrenalin during exercise ( $P < 0.05$ ).

**Figure 4.6.5 Phosphorylated Proteins**



Comparisons of the effect of insulin and exercise on phosphorylation of Akt, GSK3 alpha/beta. Open columns represent pre-exercise and closed columns represent post-exercise. Data are expressed as Mean  $\pm$  SEM. N = 7 for Akt. N= 5 for GSK.



Comparisons of the effect of insulin and exercise on phosphorylation of ERK 1/2.

Open columns represent pre-exercise and closed columns represent post-exercise.

Data are expressed as Mean  $\pm$  SEM. N = 7



**Table 4.6.1 Basal, insulin and exercise induced substrate oxidation rates, respiratory exchange ratio (RER) and exogenous glucose utilization rate.**

	LO			HI		
	Basal	Ins+ Rest	Ins+Ex	Basal	Ins+Rest	Ins+Ex
	N = 8	N = 8	N = 7	N = 8	N = 8	N = 7
<b>Carbohydrate Oxidation</b> (mg/kg/min)	1.09 (0.4)	1.9 (0.3) <sup>a</sup>	27.8(1.9)	1.4(0.4)	2.7(0.2) <sup>a</sup>	31.7(2.7) <sup>b</sup>
<b>Fat Oxidation</b> (mg/kg/min)	0.8(0.2)	0.5(0.1)	4.1(0.7)	0.8(0.2)	0.1(0.1)	2.5(0.6)
<b>RER</b>	0.80(0.03)	0.88(0.02) <sup>a</sup>	0.92(0.01)	0.84(0.04)	0.98(0.02) <sup>a</sup>	0.95(0.01) <sup>b</sup>
<b>Exogenous glucose utilization</b> (mg/kg/min)	0	2.3(0.4)	6.9(1.2)	0	7.1(1.1) <sup>c</sup>	18.4(2.1) <sup>c</sup>

Data are expressed as Mean (SEM). <sup>a</sup> P<0.05 when compared to the respective baseline, <sup>b</sup> P<0.05 when compared to the LO trial. <sup>c</sup> P<0.001 when compared to the LO trial. (Ins = Insulin and Ex=Exercise). Values for Ins + Rest are the means for the final 30 min of the clamp at rest and values for Ins + Ex are the means for the whole 45 min of exercise period.

**Table 4.6.2 Muscle metabolite concentrations.**

	<b>LO</b>		<b>HI</b>	
	<b>Pre-Exercise</b>	<b>Post-Exercise</b>	<b>Pre-Exercise</b>	<b>Post-Exercise</b>
<b>Total Glycogen</b> (n= 7)	406 (21)	236 (30) *	403 (22)	227 (24) *
<b>Pro Glycogen</b> (n=7)	324 (17)	194 (25)	323 (20)	176 (20)
<b>Macro Glycogen</b> (n = 7)	82 (9)	42 (6)	80 (7)	51 (10)
<b>Free Glucose</b> (n = 6)	4.4 (0.9)	6.9 (1.3)	4.2 (2.0)	7.6(2.3)
<b>Gluc-6-Phosphate</b> (n = 6)	1.6(1.0)	1.1(0.2)	1.5(0.3)	0.8(0.2)
<b>Lactate</b> (n = 7)	6.3 (1.5)	17.9 (3.9) *	5.6 (1.2)	23.8 (3.5) *
<b>Acetylcarnitine</b> (n = 6)	5.2 (0.3)	16.9 (1.0) *	4.2 (0.5)	17.0 (2.4) *

Data are expressed as Mean (SEM) mmol glucosyl units/kg.dry muscle for glycogen and mmol/kg. dry muscle for metabolites. \*Values were different when compared to their respective baseline (P<0.05).

## **4.7 Discussion**

The principal finding of this study was that exercise under peak therapeutic hyperinsulinaemic conditions in patients with type 1 diabetes did not spare muscle glycogen utilisation at a time of high exogenous glucose utilization and oxidation. Muscle intermediary metabolism during exercise was not affected by changes in therapeutic insulin concentrations seen routinely in clinical practice (25-90 mU/l). The insulin-mediated phosphorylation of key signalling targets during exercise did not differ between the two insulin concentrations used.

In patients with type 1 diabetes, exercise and hyperinsulinaemia conditions have a synergistic influence on whole-body glucose utilisation. During exercise, the exogenous glucose infusion rate was substantially increased in both studies to prevent a fall in blood glucose. The amount of glucose infused was substantially higher in the HI trial (68g) when compared to the LO trial (25g) to maintain blood glucose at 8 mmol/l. Hence, it might have been expected that muscle glycogen would have been spared during the HI trial, but no such differences were observed. Moreover, there was no difference in either intramuscular free glucose or glucose-6-phosphate concentrations. Intramuscular lactate accumulation (an index of rate of glycolysis) and acetylcarnitine concentrations [an index of pyruvate flux through pyruvate dehydrogenase complex (Constantin-Teodosiu, Carlin et al. 1991)] were similar during both trials. Moreover, superimposition of exercise over the hyperinsulinaemic conditions did not influence the phosphorylation of Akt, GSK3  $\alpha/\beta$  and ERK 1/2. This is contrary to previously reported findings in healthy volunteers and rats (Wojtaszewski, Hansen et al. 2000; Chen, Bandyopadhyay et al. 2002; Christ-Roberts, Pratipanawatr et al. 2003; Sakamoto, Arnolds et al. 2004). Indeed, previous studies have shown that both insulin and contraction can activate Akt in skeletal

muscle (Bruss, Arias et al. 2005). It has been shown that skeletal muscle Akt phosphorylation returns to baseline after 15 min of muscle contraction (Sakamoto, Hirshman et al. 2002). The duration of exercise in the present study was 45 min and it is possible Akt phosphorylation had returned back to baseline by the time of the second biopsy. Alternatively, it is possible that the hyperglycaemic conditions could have had an inhibitory effect on Akt activation as observed in patients with type 2 diabetes (Christ-Roberts, Pratipanawatr et al. 2003). The above data indicate that at least some of the additional increase in glucose uptake during exercise especially in the HI trial might not have entered contracting muscle. This is in agreement with a previous study which assessed glucose disposal in patients with type 1 diabetes during one-legged isometric exercise under hyperinsulinaemic conditions using 5 fluoro-deoxy glucose positron emission tomography (Peltoniemi, Yki-Jarvinen et al. 2001). These results suggest the presence of impaired skeletal muscle insulin stimulated glucose uptake in patients with type 1 diabetes and further investigations are required to confirm this hypothesis.

In the present study, exercise induced a substantial increase in CHO oxidation in both trials. However, the rate of CHO oxidation in the HI trial was only slightly greater (~15%) when compared to the LO trial. Under fasting conditions, fat oxidation can contribute up to 40% of energy needs during exercise at moderate intensity in healthy volunteers (Ahlborg, Felig et al. 1974). However, in the present study the hyperinsulinaemic conditions appear to have impaired the exercise-induced increase in catecholamine mediated lipolysis (Wahrenberg H 1989). As a result, fat oxidation contributed to less than a quarter of the total energy used to meet the needs of the exercising muscle. Since plasma NEFA availability was suppressed under these conditions, the source of fat oxidised was likely to have been intramuscular

triglyceride stores as seen in other studies (Standl, Lotz et al. 1980; Wahren J 1980). Hence, hyperinsulinaemia limits fat utilisation, thereby increasing CHO oxidation and hence glucose requirements.

Exercising with type 1 diabetes is not straightforward. There were significant increases in insulin concentrations during exercise in both trials without any changes to the insulin infusion rates. This has been reported in previous studies and it is likely that a combination of systemic insulin infusion and reduced portal blood flow during exercise could lead to reduced hepatic insulin clearance (Dela, Mikines et al. 1999; Tsintzas, Simpson et al. 2003). Hence, under real life conditions, exercise not only increases absorption from subcutaneously administered injection depots but also might decrease insulin clearance leading to high circulating insulin levels during exercise and increase the risk of exercise-induced hypoglycaemia. Current recommendations are to reduce short-acting insulin doses immediately prior to exercise and to take modest amounts of CHO during exercise to prevent hypoglycaemia. However, even with reductions in soluble insulin dosages, patients will often be exercising at insulin concentrations similar to those observed in the LO study ( $\sim 25$  mU/l), well in excess of insulin levels seen in non-diabetic subjects during exercise ( $\sim 5$  mU/l). Some patients do not reduce insulin doses prior to exercise, but choose instead to take larger amounts of CHO to forestall hypoglycaemia. Furthermore, patients do not always find it possible to plan meals, insulin doses and exercise rigorously. In practise, exercise is therefore often undertaken at higher post-prandial insulin concentrations (similar to the HI study) requiring additional CHO intake. The comparative physiology of these two situations has not previously been described in patients with type 1 diabetes.

The present study has shown for the first time that in patients with type 1 diabetes, exercise under differing insulin concentrations is associated with similar energy expenditure and muscle glycogen utilisation rates despite the substantial increase in exogenous glucose utilisation in the HI trial when compared with the LO trial. Since it is difficult to precisely estimate the mass of working muscle it is difficult to quantify the contribution of muscle glycogen to whole body CHO oxidation. Nevertheless, the amount of muscle glycogen utilized was similar in the two trials and there was a significant increase in CHO oxidation on both occasions with a slightly greater value observed in the HI trial. Interestingly, the increase in exogenous glucose utilization relative to the increase in CHO oxidation was much greater in the HI trial compared with the LO. This suggests that some of the additional increase in whole body glucose uptake during exercise in the HI trial might have been directed away from the contracting muscle towards non-oxidative pathways in other tissues. Further studies are required to confirm whether glucose storage occurs in the liver and/or non-contracting muscle tissue during exercise under such conditions. If the site of deposition of excess glucose was indeed the liver, this might potentially reduce the risk of 'late' hypoglycaemia. However, there would be a considerable risk of hypoglycaemia occurring during exercise at high insulin concentrations if CHO intake is not increased to match energy expenditure.

This study illustrates that even at typical pre-prandial insulin concentrations (as seen in the LO trial) substrate metabolism during exercise in patients with type 1 diabetes is far from normal, with a low rate of fat oxidation, marked muscle glycogen utilization and a need for considerable quantities of exogenous glucose to meet energy needs. Exercising at higher, typically postprandial insulin concentrations, increases exogenous glucose utilisation, but does not spare muscle glycogen breakdown. In

order to further understand the physiological responses to exercise in patients with type 1 diabetes, additional studies are required to investigate the impact of peak insulin concentrations on exercise capacity and muscle fatigue and the role of liver glycogen in maintaining glycaemia during and after exercise.

## **Chapter 5**

**Hyperinsulinaemia during exercise does not suppress hepatic glycogen concentrations in patients with type 1 diabetes.**

### **5.1 Introduction**

Fear of exercise-induced hypoglycaemia poses a significant barrier to active participation in recreational sports and games in patients with type 1 diabetes (Kemmer 1992; Tuominen, Karonen et al. 1995; Dube, Valois et al. 2006). The incidence of hypoglycaemia during an acute bout of moderate intensity exercise in children and adolescents with type 1 diabetes has been recently reported to be up to 30% (Tansey, Tsalikian et al. 2006). Although there is no direct evidence in adults, the incidence of exercise-induced hypoglycaemia can be expected to be similar in adults with type 1 diabetes. However, the physiological responses to exercise and hence the factors contributing to exercise-induced hypoglycaemia in patients with type 1 diabetes are not fully understood.

During exercise in non-diabetic individuals, either increased carbohydrate intake and/or increased hepatic glucose production are required to match the increased rate of muscle glucose uptake during exercise (Wahren, Felig et al. 1971; Jenkins, Chisholm et al. 1985). To facilitate increased hepatic glucose output, the hormonal response to exercise performed in the post-absorptive state is characterized by reciprocal changes in plasma insulin and glucagon concentrations (Wahren, Felig et al. 1971). An increase in both hepatic glycogenolysis and gluconeogenesis contribute to augmented hepatic glucose output during exercise with a greater contribution from the former (Felig and Wahren 1975). However, during prolonged exercise under



fasting conditions a greater contribution of hepatic glucose output is derived from gluconeogenesis (Bjorkman and Eriksson 1983; Lavoie, Ducros et al. 1997).

In patients with type 1 diabetes, the hormonal milieu is disturbed and this may affect hepatic glycogen metabolism. Firstly, it is clearly impossible to replicate the fall in insulin concentration normally seen during exercise using exogenous insulin injections in patients with type 1 diabetes. During exercise, systemic and to a lesser extent portal insulin concentrations will be higher in patients with type 1 diabetes treated with exogenous insulin than in normal subjects. Therefore, when patients exercise typically with high insulin concentrations (Tuominen, Karonen et al. 1995; Rabasa-Lhoret, Bourque et al. 2001), this may suppress hepatic glucose output (Zinman, Murray et al. 1977) with a consequent risk of hypoglycaemia both during and after exercise (Zinman, Murray et al. 1977). Secondly, in addition to the fall in insulin a rise in glucagon is required for the full increment in hepatic glucose output (Wasserman, Spalding et al. 1989). Prevention of this physiological response results in attenuation of hepatic glucose output (Hirsch, Marker et al. 1991). It has also been suggested that the increased risk of hypoglycaemia in patients with type 1 diabetes could be due to impaired hepatic glucose output in response to infused glucagon (Orskov, Alberti et al. 1991). Thirdly, patients with poorly controlled diabetes have low fasting hepatic glycogen concentrations and impaired net hepatic glycogen synthesis following mixed meal ingestion (Cline, Rothman et al. 1994; Hwang, Perseghin et al. 1995). This in turn may predispose these patients to hypoglycaemia during subsequent exercise.

A large body of evidence concerning hepatic glucose metabolism during exercise in diabetes has been derived from animal experiments (Sindelar, Balcom et al. 1996;

Sindelar, Chu et al. 1997; Sindelar, Chu et al. 1997; Cherrington, Edgerton et al. 1998; Sindelar, Chu et al. 1998) but there is a lack of *in vivo* human data. Human studies using magnetic resonance spectroscopy (MRS) have reported defects in mobilization of hepatic glycogen stores in patients with type 1 diabetes (Petersen, Price et al. 2004; Kishore, Gabriely et al. 2006). However, these studies were performed either under very low circulating insulin concentrations (5-10 mU/l) or after hypoglycaemia. The impact of real-life therapeutic insulin concentrations on hepatic glycogen metabolism during exercise in patients with type 1 diabetes is not known. In chapter 4, it was shown that exercise under peak therapeutic insulin concentrations increases exogenous glucose utilisation and that this increase was disproportionate to the relative increase in carbohydrate oxidation suggesting an increase in glucose flux through non-oxidative pathways, perhaps towards hepatic glycogenesis.

In the previous chapter, it was hypothesised that during exercise under hyperinsulinaemic and hyperglycaemic conditions, excess glucose disposal observed in the HI trial was directed towards non-oxidative pathways such as liver glycogen synthesis. The aim of this study was to investigate *in vivo* changes in liver glycogen and whole body substrate metabolism both before and after a meal, and during subsequent moderate exercise in patients with type 1 diabetes under therapeutic insulin concentrations. It was hypothesized that, when compared to non-diabetic controls, in patients with type 1 diabetes, exercise under relative hyperinsulinaemic conditions would suppress hepatic glycogen mobilization and thereby increase the risk of exercise-induced hypoglycaemia.

## **5.2 Methods**

A brief description of the methods is given below. Please refer to Chapter 2: Methods for detailed description of analytical methodology.

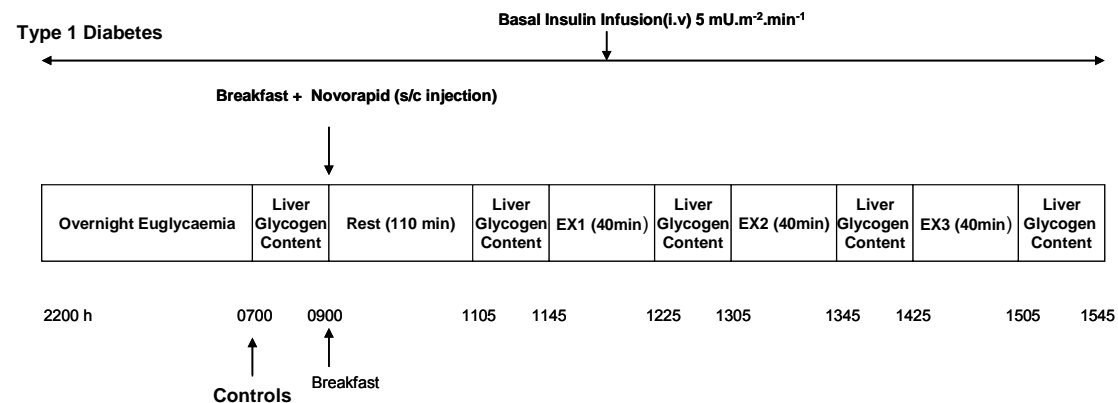
### **5.2.1 Patients and Preliminary Measurements**

I recruited 7 physically active men with uncomplicated type 1 diabetes from our hospital diabetes clinics [DM: Mean (SEM) diabetes duration 10 (2) yr] and 5 non-diabetic physically active controls [CON]. The baseline characteristics of the volunteers are outlined in table 5.4.1. Five patients were on a basal-bolus subcutaneous insulin regimen and two were on twice a day premixed insulin injections. All patients were screened to exclude micro and macro vascular complications. The control subjects were medically screened and were in good health. Patients and controls were informed of all experimental procedures and the associated risks prior to obtaining informed consent. All procedures used in this study were performed according to the Declaration of Helsinki and approved by the Nottingham NHS Research Ethics Committee and The University of Nottingham Research Ethics Committee.

$\dot{V}O_2$  peak was determined during a preliminary visit using an incremental cycling test (Excalibur cycle ergometer; Lode, Groningen, Netherlands).

### 5.2.2 Experimental Design and Protocol

An outline of the study design is depicted here below. For 3 days prior to the trials, controls and patients maintained an iso-energetic diet and patients monitored their capillary blood glucose closely to avoid episodes of hypoglycaemia. All participants were requested to avoid smoking, alcohol and exercise for three days prior to each study visit. Fat free mass was determined using bioelectrical impedance (Bodystat Quadscan 4000, Isle of Man, UK).



Patients (DM) omitted their medium/long acting insulin for 24-36 hours prior to the study. On the evening prior to study, all subjects consumed a standardised high carbohydrate (2.5g of CHO/kg fat free mass) meal and the patients injected their usual short acting soluble insulin with this meal and were admitted to the hospital. An ante grade cannula was inserted into a cubital fossa vein for a low dose intravenous insulin infusion to maintain euglycaemia overnight. A retrograde cannula was inserted into a dorsal hand vein for blood sampling to measure glucose concentrations during the overnight stay. On the day of the study this cannula was also used for drawing

arterialised blood samples for metabolite and hormone measurements, with the hand being placed in a hot air box (55°C). At 8AM the following morning, subjects were transferred to the Magnetic Resonance (MR) centre for the study protocol. Intravenous insulin infusion was continued at a constant rate of 5 mU.m<sup>-2</sup>.min<sup>-1</sup> to cover basal insulin requirements until the end of the study.

The non-diabetic controls (CON) reported directly to the MR centre after an overnight fast from 10PM and a retrograde cannula was inserted into a dorsal hand vein for drawing arterialised blood samples as above. A baseline <sup>13</sup>C MR spectroscopy measurement of liver glycogen was carried out for both patients and controls. All subjects then consumed a high glycaemic index (GI 82.9) carbohydrate meal (2.5 g of CHO/kg fat free mass) as described in table 5.4.2. Patients with diabetes injected their fast acting insulin (Novorapid; NovoNordisk, Denmark) 5 min before breakfast, subcutaneously in the abdominal wall. The insulin dose prescribed was 0.5 U for every 10g of carbohydrate ingested (a 50% dose reduction from what these patients would have normally administered for this meal). All subjects were requested to consume the meal within 15 min and subsequently rested on a bed for 110 min to replicate a typical post-prandial pre-exercise state. Further spectroscopy measurements of liver glycogen were acquired 110 min after consumption of the meal. All subjects (DM & CON) then carried out three bouts of cycling at 60% $\dot{V}O_2$  peak for 40 min each. The cycling sessions were interrupted for 40 min between bouts to obtain further spectroscopy measurements of liver glycogen. Hydration was maintained during cycling by ingesting water at a rate of 10-ml/kg-body weight every 20 min. Heart rate and rating of perceived exertion using a Borg scale (Borg 1982) were measured at 20 min intervals during exercise. Blood samples were drawn at the

same intervals for metabolite and hormone measurements. Urine samples were collected throughout the experiment for glucose measurements.

On a separate day to the liver glycogen data acquisition visit, the above exercise and feeding protocol was replicated in each non-diabetic volunteer and patient with diabetes to acquire indirect calorimetry data. The presence of a strong magnetic field and working space limitations precluded us from making these measurements on the same visit as when MR scanning was taking place. During this visit there was a slight variation to the insulin treatment protocol. On the evening prior to this visit, patients had the same high carbohydrate meal and their usual insulin treatment regime (Quick acting insulin followed by their long acting insulin). Patients were advised to monitor their capillary glucose regularly to avoid hypoglycaemia and were not admitted overnight. Moreover, they did not receive the intravenous basal insulin infusion during the study since patients administered their usual long acting insulin the previous night. Resting  $\dot{V}CO_2$  and  $\dot{V}O_2$  were measured for 20 min using a ventilated hood system (GEM, Nutren Technologies, Manchester, UK) before and 110 minutes after consumption of the same meal to allow calculation of energy expenditure and substrate oxidation rates (Frayn 1983). Subsequently, five min expired gas samples (Vmax 29; Sormedics Yorba Linda, CA, USA) were collected every 20 min whilst cycling and the measured  $\dot{V}CO_2$  and  $\dot{V}O_2$  values were used to determine substrate oxidation rates during exercise.

### **5.2.3 Blood and Urine Metabolites**

Whole blood and urine glucose, and blood lactate were measured during the experiments. Serum insulin, glucagon and NEFA were measured using commercially available kits. Whole blood  $\beta$ -hydroxybutyrate was measured in perchloric acid

(10%) treated blood samples. Plasma catecholamines were measured using high performance liquid chromatography with electrochemical detection (Forster and Macdonald 1999).

#### **5.2.4 $^{13}\text{C}$ Magnetic Resonance Spectroscopy**

All  $^{13}\text{C}$  MR data were acquired on a 3.0-Tesla dual-channel whole body spectrometer, with a 1-m bore. Please refer to chapter 2 in section 2.10 for full protocol.

### **5.3 Results**

Patients and controls cycled at a mean workload of 153 (12) and 169 (17) W. The respective mean oxygen consumptions were 26.6 (1.1) and 29.8 (2.5) ml. [kg lean body mass] $^{-1} \cdot \text{min}^{-1}$  being almost exactly 60% of the predetermined  $\dot{V}\text{O}_2$  peak. Two patients developed hypoglycaemic symptoms (blood glucose < 2.5 mmol/l) at the end of the second bout of exercise and did not proceed to the third bout. A further 2 patients developed asymptomatic hypoglycaemia (defined as blood glucose < 3.5 mmol/l) at the end of the third bout of exercise. All control subjects completed the three exercise bouts with no evidence of hypoglycaemia.

#### **5.3.1 Blood metabolites and hormones**

Blood glucose concentrations were higher in patients than controls at rest and during EX1 & EX2 (Fig 5.4.1A,  $p < 0.05$ ) but similar at the end of EX2 and during EX3. Fasting serum insulin concentration was greater in patients than controls (Fig 5.4.1B,  $p < 0.05$ ). During all three-exercise bouts, serum insulin concentrations were greater in patients than controls (Fig 5.4.1B,  $p < 0.05$ ). Fasting plasma NEFA concentrations were comparable between groups. Plasma NEFA concentrations were suppressed after breakfast and during EX1 in both groups (Table 5.4.3,  $p < 0.05$ ). NEFA concentrations started rising during EX2 and peaked during EX3 with no differences

between groups. There was no difference in fasting blood  $\beta$ -hydroxybutyrate (BOHB) concentrations between groups (Table 5.4.3). BOHB concentrations were suppressed to  $\sim 0.05$  mmol/l during the first 2 exercise sessions ( $p < 0.05$ ) but increased during the last session of exercise with no difference between groups. Fasting blood lactate concentrations were also comparable between groups (Table 5.4.3). Exercise induced a significant ( $p < 0.05$ ) increase in blood lactate concentrations in both groups with no significant differences between groups. Baseline plasma noradrenalin and adrenaline concentrations were comparable in both groups (Table 5.4.3). Exercise induced a significant ( $p < 0.05$ ) increase in both plasma adrenaline and noradrenalin concentrations in both groups during EX1, EX2 and EX3 with no difference between groups. Fasting serum glucagon concentrations were significantly ( $p < 0.05$ ) lower in patients with type 1 diabetes when compared to controls (Table 5.4.3). Following the meal there was a significant ( $p < 0.05$ ) increase in serum glucagon concentrations in patients with diabetes but not in controls resulting in similar pre-exercise values between groups. Furthermore, there was no difference in glucagon concentrations during exercise between groups.

### **5.3.2 Whole body substrate metabolism**

There were no significant differences in either fasting or postprandial RER values between patients and controls (Table 5.4.4). A substantial increase in whole body carbohydrate oxidation was observed with the onset of exercise but there was no difference between groups (Table 5.4.4). Similarly, a substantial increase in whole body fat oxidation was observed during exercise, again with no differences between groups (Table 5.4.4).



### **5.3.3 Liver glycogen**

There were no significant differences in fasting liver glycogen between groups [DM: 178 (54) mmol/l; CON: 212 (63), Fig.5.4.2]. Following the high carbohydrate breakfast, there was a two to three fold increase in hepatic glycogen concentration in both patients and controls but there was no difference between groups [DM: 589 (138) mmol/l; CON: 463 (77), Fig.5.4.2]. Hepatic glycogen concentrations decreased after EX1 [DM: 298 (67) mmol/l; CON: 303 (59)], EX2 [DM: 267 (41) mmol/l; CON: 210 (51)] and EX3 [DM: 195 (80) mmol/l; CON: 145 (63)] in both groups (Fig.5.4.2). There were no differences in hepatic glycogen concentrations during exercise between patients and controls.

## **5.4 Figures and Tables**

**Table 5.4.1 Baseline Characteristics**

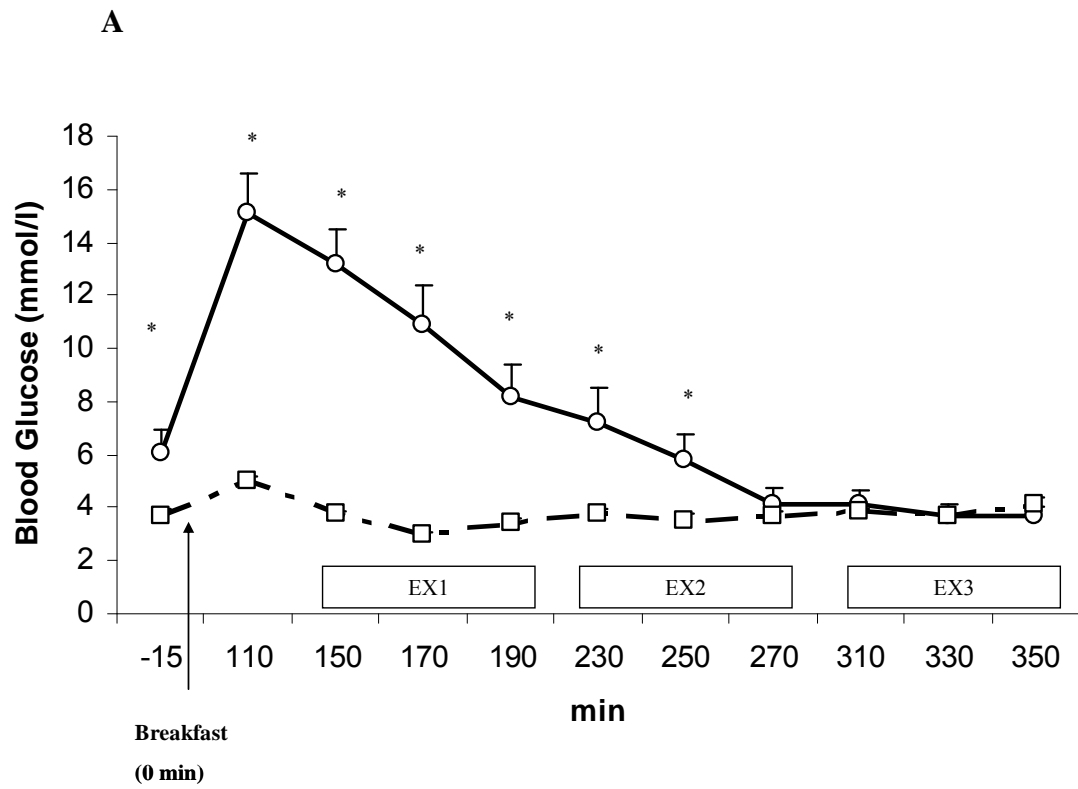
	<b>DM</b>	<b>CON</b>	<b>p value</b>
<b>Age (yr)</b>	33 (3)	30 (3)	0.509
<b>BMI (kg/m<sup>2</sup>)</b>	24 (1)	22 (1)	0.054
<b>Lean body mass (kg)</b>	68 (3)	65 (5)	0.548
<b>HbA<sub>1c</sub> (%)</b>	8.1 (0.2)	5.4 (0.1)*	0.000
<b><math>\dot{V}O_2</math> Peak (ml.kg<sup>-1</sup> lean body mass.min<sup>-1</sup>)</b>	43 (2)	52 (4)	0.09

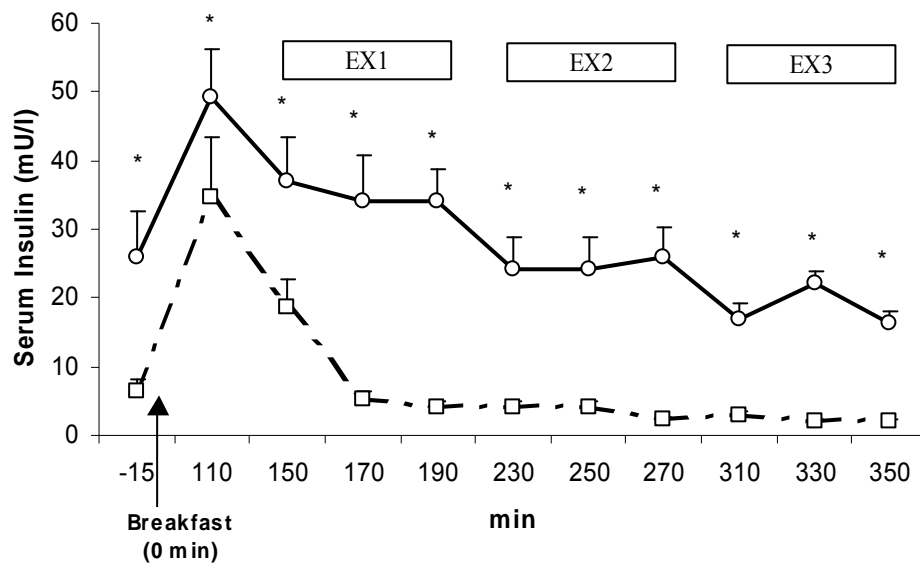
**Table 5.4.2 Breakfast Content**

<b>Product</b>	<b>Food GI</b>	<b>Meal Glycaemic Load</b>	<b>Carbohydrate g/kg lean mass</b>
Kellogg's Cornflakes	81	23.1	0.71
Skimmed milk	32	2.1	0.17
White bread	100	26.3	0.66
Strawberry Jam	51	5.9	0.29
Lucozade (GSK)	95	25.4	0.67
<b>Total</b>	-	<b>82.9</b>	<b>2.5</b>

The GI of the meal was calculated as previously described (Wolever, Jenkins et al. 1991) and the GI values of individual components were taken from published tables (Foster-Powell, Holt et al. 2002).

**Figure 5.4.1 Blood glucose and serum insulin**



**B**

Blood glucose (A) and serum insulin (B) concentrations in patients with type I diabetes ( $\circ$ ) and controls ( $\square$ ). EX1, 2, 3 represent the first second and third bouts of exercise respectively. \*  $P < 0.05$  for differences in blood glucose and serum insulin concentrations vs. controls.  $n = 7$  and  $n = 5$  in the patient and control group, respectively.  $n = 5$  at time points 330 and 350 min in the patient group.

**Table 5.4.3 Blood metabolite and hormone concentrations before and after breakfast, and at the end of each session.**

	Fasting		After Meal		End of EX1		End of EX2		End of EX3	
	DM	CON	DM	CON	DM	CON	DM	CON	DM	CON
<b>Plasma NEFA</b> (mmol/l)	0.31 (0.05)	0.32 (0.07)	0.05 (0.02)	0.06 (0.01)	0.05 (0.02)	0.06 (0.02)	0.45 (0.17)	0.48 (0.10)	0.87 (0.13)	1.29 (0.18)
<b>Blood <math>\beta</math>-Hydroxybutyrate</b> (mmol/l)	0.32 (0.19)	0.05 (0.01)	0.06 (0.01)	0.02 (0.01)	0.10 (0.01)	0.09 (0.01)	0.06 (0.01)	0.08 (0.02)	0.46 (0.12)	0.42 (0.18)
<b>Blood Lactate</b> (mmol/l)	1.0 (0.2)	0.9 (0.1)	1.2 (0.1)	1.0 (0.1)	2.5* (0.6)	1.6* (0.3)	1.9* (0.3)	1.2* (0.2)	1.6* (0.3)	1.2* (0.2)
<b>Plasma Noradrenalin</b> (nmol/l)	3.4 (0.2)	2.8 (0.3)	2.0 (0.1)	2.3 (0.2)	9.4* (0.6)	8.5* (1.1)	7.8* (1.6)	8.9* (1.1)	10.5* (1.1)	8.0* (1.6)
<b>Plasma Adrenaline</b> (nmol/l)	0.2 (0.1)	0.1 (0.03)	0.2 (0.04)	0.2 (0.03)	1.1* (0.2)	1.4* (0.5)	2.2* (0.8)	2.2* (1.0)	5.7* (1.6)	2.1* (0.5)
<b>Glucagon</b> (pg/ml)	50.3 (3.1)	70.9** (9.4)	78.6 (5.0)	77.4 (17.5)	77.5* (6.6)	90.2* (14.1)	79.1* (5.0)	87.4* (12.9)	78.3* (3.9)	92.0* (11.5)

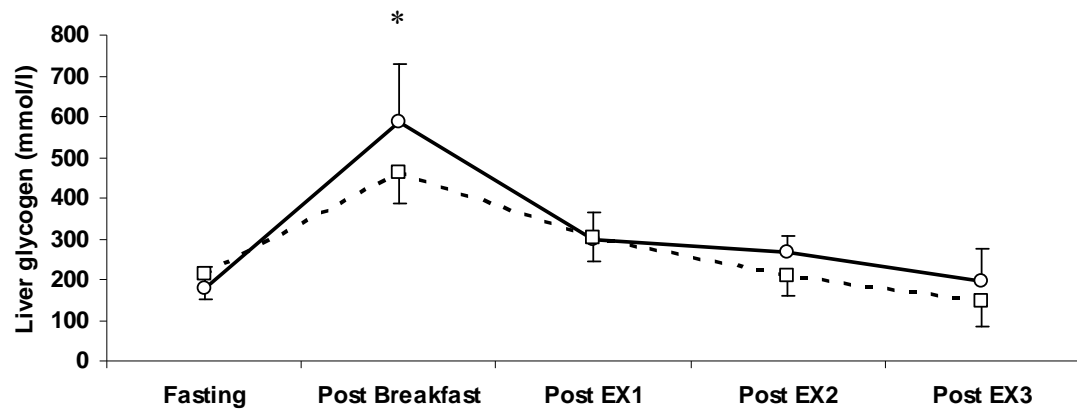
Data are expressed as mean (SEM). DM: n = 7 and CON: n = 5 for all metabolites except for  $\beta$ -Hydroxybutyrate (n = 4). During EX3, n = 5 for all metabolites and hormones in both groups. \*  $p < 0.05$  for difference from fasting value within the same trial and \*\*  $p < 0.05$  from DM.

**Table 5.4.4 RER and substrate oxidation rates before and after breakfast, and during exercise.**

	Carbohydrate Oxidation		Fat Oxidation		RER	
	(mg.min <sup>-1</sup> .kg lean body mass <sup>-1</sup> )		(mg.min <sup>-1</sup> .kg lean body mass <sup>-1</sup> )			
	DM	CON	DM	CON	DM	CON
<b>Fasting</b>	0.17 (0.06)	0.28 (0.04)	0.14 (0.02)	0.09 (0.02)	0.79 (0.03)	0.87 (0.03)
<b>1h Post Breakfast</b>	0.44 (0.07)	0.49 (0.10)	0.06 (0.02)	0.06 (0.05)	0.91 (0.03)	0.94 (0.05)
<b>2h Post Breakfast</b>	0.46 (0.03)	0.55 (0.06)	0.06 (0.01)	0.02 (0.03)	0.93 (0.02)	0.98 (0.03)
<b>EX1</b>	3.47 (0.16)	3.59 (0.23)	0.49 (0.04)	0.50 (0.06)	0.92 (0.01)	0.92 (0.01)
<b>EX2</b>	3.11 (0.20)	3.78 (0.28)	0.56 (0.06)	0.43 (0.12)	0.90 (0.01)	0.93 (0.02)
<b>EX3</b>	2.92 (0.31)	3.21 (0.17)	0.72 (0.13)	0.73 (0.10)	0.88 (0.02)	0.89 (0.01)

Data are expressed as mean (SEM). EX1, 2, 3 represent the first, second and third bout of exercise respectively. Values for EX1, EX2 and EX3 represent the average during each exercise bout. n = 7 and n = 5 in the patient and control group respectively. n = 6 during EX3 in the patient group.

**Figure 5.4.2 Liver glycogen**



Liver glycogen concentrations in patients with type 1 diabetes (○) and controls (□) before and after a standardized breakfast, and after 3 bouts of 40 min sub maximal exercise. EX1, 2, 3 represent the first second and third bouts of exercise, respectively.  $n = 7$  and  $n = 5$  in the patient and control group, respectively.  $n = 5$  in the patient group at time point post EX3. \*  $P < 0.01$  for postprandial increase in liver glycogen in both groups.



## **5.5 Discussion**

There were 3 main findings from this study. Firstly, following a standardized evening meal and overnight control of glycaemia fasting hepatic glycogen concentrations in patients with type 1 diabetes were comparable to non-diabetic controls. Secondly, similar post-prandial liver glycogen deposition occurred after consumption of a high glycaemic index breakfast and injection of a lower than normal dose of subcutaneously administered rapid acting insulin analogue. Thirdly, changes in liver glycogen content during exercise were similar in patients with type 1 diabetes and in controls despite higher relative systemic hyperinsulinaemia (3 fold) present in the former compared to the latter group.

Insulin, glucagon and catecholamines have varying roles during exercise and appear to respond in a hierarchical fashion to regulate hepatic glucose output and prevent exercise-induced hypoglycaemia (Hoelzer, Dalsky et al. 1986; Tuttle, Marker et al. 1988; Hirsch, Marker et al. 1991; Cryer, Davis et al. 2003). In non-diabetic individuals a fall in serum insulin concentration occurs during exercise. This allows increased hepatic glucose output through a combination of glycogenolysis and gluconeogenesis to match the increased muscle glucose uptake which occurs during exercise (Wasserman, Williams et al. 1989). In patients with type 1 diabetes managed with subcutaneous insulin injections, a fall in serum insulin concentration cannot occur during exercise. Hence, if the physiological augmentation of hepatic glucose output is thereby inhibited, the blood glucose concentration may fall. However, the present study show that changes in glycogen content during exercise in patients with type 1 diabetes was comparable to that of the non-diabetic controls despite the prevailing hyperinsulinaemic and hyperglycaemic conditions. This is in contrast to previous healthy human (Petersen, Price et al. 2004) and some (Wasserman, Lacy et

al. 1991) but not all (Cherrington, Edgerton et al. 1998; Camacho, Pencek et al. 2004) animal studies which demonstrated insulin-induced suppression of hepatic glucose output. It could be argued that despite the prevailing hyperinsulinaemic conditions, a preserved glucagon response to exercise could have accounted for the lack of differences in exercise-induced changes in liver glycogen content between groups. Indeed, a rise in glucagon is required during post-absorptive exercise for the full increment in hepatic glucose output (Wasserman, Spalding et al. 1989; Lavoie, Ducros et al. 1997). On the contrary, it has been shown in patients with type 1 diabetes that during exercise under fasting conditions, hepatic glucose output is comparable to that of controls, and this is despite higher circulating insulin concentrations and lower glucagon responses in the patient group (Raguso, Coggan et al. 1995). The role of glucagon in the regulation of hepatic glucose output during exercise performed in the postprandial state is not clear. In chapter 4, it has been shown that moderate intensity exercise performed during a hyperinsulinaemic clamp did not induce a significant increase in glucagon concentrations in patients with type 1 diabetes. Furthermore, in the current study there was no difference in serum glucagon concentrations during exercise performed in the postprandial state between patients with type 1 diabetes and controls. This resulted in a lower glucagon to insulin ratio in the patient group when compared to the control group. Therefore, the parallel reduction in hepatic glycogen content between groups despite the obvious differences in the glucagon to insulin ratio suggests that other factors are important for the regulation of hepatic glucose output during exercise performed in the postprandial state in patients with type 1 diabetes. It is also plausible that a preserved exercise-induced increase in counter regulatory response (catecholamines) in this patient cohort enabled continuous substrate mobilization from the liver (Simonson, Koivisto

et al. 1984; Hoelzer, Dalsky et al. 1986; Hirsch, Marker et al. 1991; Purdon, Brousson et al. 1993). It is also known that pre-exercise hypoglycaemia is associated with blunted counter-regulation and impaired hepatic glycogenolysis (Galassetti, Tate et al. 2003; Kishore, Gabriely et al. 2006). Since in the present study, patients were admitted overnight and hypoglycaemia was avoided prior to the experiment, counter-regulatory mechanisms were likely to have been preserved. Hence, counter-regulatory mechanisms may play a more prominent role in hepatic glycogen metabolism during exercise than the suppressive effect of relative hyperinsulinaemia in patients with type 1 diabetes.

Hepatic carbohydrate metabolism has been reported to be defective in patients with poorly controlled type 1 diabetes (Cline, Rothman et al. 1994; Hwang, Perseghin et al. 1995). It is likely that portal hypoinsulinaemia due to poorly controlled type 1 diabetes contributed to the lower fasting hepatic glycogen concentrations observed in these studies (Sindelar, Chu et al. 1998). Since in the present study, patients were infused with intravenous insulin overnight, portal hypoinsulinaemia would have been alleviated which may explain the absence of any difference in fasting glycogen concentrations. Patients with poorly controlled type 1 diabetes have impaired net hepatic glycogen synthesis following a mixed meal ingestion and show an increase in hepatic gluconeogenesis (Hwang, Perseghin et al. 1995). However, these defects can be corrected after combined long-term and overnight control of glycaemia with intravenous insulin (Bischof, Bernroider et al. 2002) and the results from this study support this observation. Postprandial, pre-exercise liver glycogen concentration in DM was comparable to control despite the relative lower estimated portal insulin concentration (~25-35 mU/l) when compared to controls (~100 mU/l). This is most likely to be the result of hyperglycaemic conditions in the postprandial period.

Combined hyperglycaemic and hyperinsulinaemic conditions results in increased hepatic glycogen synthesis due to the stimulatory effect on glycogen synthase flux and inhibition of glycogen phosphorylase (Petersen, Laurent et al. 1998).

Previous studies demonstrated a preferential utilization of free fatty acids over carbohydrate in patients with type 1 diabetes during moderate exercise in the fasting state (Wahren, Hagenfeldt et al. 1975; Wahren, Sato et al. 1984). However, these experiments were performed after insulin withdrawal for 24h. When moderate exercise was performed during fasting insulin conditions ( $\sim 10$  mU/l), patients with type 1 diabetes had comparable carbohydrate but higher fat oxidation rates when compared to controls (Zinman, Murray et al. 1977; Raguso, Coggan et al. 1995). It is likely that in the present study, the higher circulating systemic insulin concentrations ( $\sim 25$  mU/l) corrected the previously reported differences in substrate oxidation between patients with type 1 diabetes and controls.

A typical carbohydrate rich pre-exercise meal and reduced subcutaneous insulin dose was used in this study. The patients were hyperglycaemic at the start of exercise and despite the 50% insulin dose reduction; patients were still hyperinsulinaemic ( $>30$  mU/l). Routine clinical care would recommend that patients do not carry out exercise at these levels of hyperglycaemia for the fear of inducing diabetic ketoacidosis. However, this study has demonstrated that as long as adequate insulinisation is maintained the risk of inducing significant ketosis is minimal. However, there is a dearth of clinical evidence as to the ideal glycaemic level at which patients should exercise to derive both health benefits and prevent exercise-induced hypoglycaemia. A greater fall in blood glucose concentrations from 13 to 8 mmol/l during EX1, whereas only a modest fall from 8 to 5 mmol/l was observed during EX2 and little or

no change during EX3. It is likely that in addition to contraction-induced increase in muscle glucose uptake, hyperinsulinaemia and hyperglycaemia induced increase in muscle glucose uptake during EX1 were responsible for this difference.

In chapter 4, it was shown that exercise under differing insulin concentrations (25 vs. 80 mU/l) was associated with similar energy expenditure and muscle glycogen utilisation despite a substantial increase in exogenous glucose utilisation. This suggested that glucose was possibly being diverted during exercise to non-oxidative pathways in the liver and/or non-contracting muscle. However, in the present study, there was no sparing of hepatic glycogen utilisation in patients with type 1 diabetes under hyperinsulinaemic conditions when compared to controls. It remains to be tested whether the increase in non-oxidative glucose disposal observed in chapter 4 was directed towards non-contracting muscle tissue. Interestingly, four patients developed hypoglycaemia at the end of the second and third bout of exercise despite the lack of suppression of hepatic glycogen utilisation. This was possibly related to either the duration of exercise (Wasserman and Zinman 1994), or the suppression of gluconeogenesis at these high insulin concentrations or differences in the relative contributions of blood glucose and muscle glycogen to muscle carbohydrate oxidation during the second and third bouts of exercise.

In conclusion, in patients with type 1 diabetes, hyperinsulinaemic and hyperglycaemic conditions during moderate exercise did not suppress hepatic glycogen utilisation when compared to healthy controls. These findings do not support the hypothesis that exercise-induced hypoglycaemia in patients with type 1 diabetes is due to suppression of changes in hepatic glycogen content. It is possible that diminished gluconeogenesis

and/or increased peripheral glucose uptake might be important factors contributing to exercise-induced hypoglycaemia.

## **Chapter 6**

### **6.1 General Discussion**

The overall objective of this thesis was to further the understanding of the effect of diet, insulin and exercise on the regulation of carbohydrate metabolism. Three studies were undertaken in both non-diabetic healthy volunteers and patients with type 1 diabetes. A range of well established *in vivo* techniques (indirect calorimetry, insulin-glucose clamps, stable glucose isotopes, muscle biopsies and magnetic resonance spectroscopy) were employed. Key observations from these studies and limitations are discussed in the following sections.

#### **6.1.1 Impact of diet composition on insulin-mediated substrate metabolism**

Skeletal muscle is the major site for insulin-mediated glucose disposal (DeFronzo, Jacot et al. 1981). Hence, insulin-mediated glucose uptake and the fate of intracellular glucose are thought to be responsive to the macronutrient composition of dietary intake. Data presented in chapter 3 confirms earlier observations made by others (Borkman, Campbell et al. 1991; Cutler, Gray et al. 1995; Louheranta, Turpeinen et al. 1999; Bisschop, de Metz et al. 2001; Lovejoy 2002) that short-term high fat diet interventions do not induce whole body insulin resistance. However, a partitioning effect is seen in insulin-mediated intracellular glucose metabolism. A shift from carbohydrate oxidation to muscle glycogen storage was observed. Moreover, an increase in fatty acid oxidation was observed as evidenced by the lower respiratory exchange ratio (RER) and increased muscle acetylcarnitine (an index of muscle acetyl CoA accumulation) concentrations when compared to controls.

Insulin infusion and glucose availability was unable to correct the deficits in carbohydrate oxidation induced by 6 days of a high fat diet. This observation is

supported at a cellular level since the activity of PDCa was reduced after the high fat diet and was not fully restored with insulin. It is not clear whether this is an adaptive change, or an early stage of mal adaptation which, if prolonged, would lead to the development of whole body insulin resistance. In addition, the small sample size of this study limits generalisation to a wider population. This can only be resolved with longer duration multicentre intervention studies to elucidate the complex maladaptive responses to dietary changes. However, designing longer duration intervention studies is fraught with difficulties and they are resource intensive. These studies need to overcome difficulties in ensuring dietary compliance, preventing weight gain and develop reliable tools to accurately assess dietary intake under free-living conditions.

Many of the enzymes involved in glucose oxidation are regulated allosterically and through covalent modification during short-term changes in macronutrient availability. However, long-term changes to nutrient availability appear to invoke the transcriptional machinery. In chapter 3, the whole body physiological changes were associated with inhibition of skeletal muscle PDCa activity and significant increases in muscle PDK4 mRNA and protein expression after the high fat diet, which is in agreement with previous studies (Sugden, Kraus et al. 2000; Peters, Harris et al. 2001; Pehleman, Peters et al. 2004). The important role of PDK4 in the regulation of PDCa activity is thus highlighted. It is crucial to understand the regulation of PDC activity since its activity state is reduced in diabetes and starvation (Sugden, Holness et al. 1989; Wu, Sato et al. 1998; Tsintzas, Jewell et al. 2006). Peroxisome proliferator-activated signalling (PPARs) has been proposed to play a key role in high fat feeding induced up regulation of PDK4 (Huang, Wu et al. 2002; Muoio, MacLean et al. 2002; Muoio, Way et al. 2002; Abbot, McCormack et al. 2005). However, contrary to evidence from cell lines and animal studies there were no changes in gene and protein



expression of PPAR $\alpha$ , PPAR $\delta$ , their coactivator PGC1 $\alpha$  or some of their known transcriptional targets (CD36, CPT1 and LCAD). Alternatively, it is possible that other factors such as acyl-CoA derivatives of NEFA might be responsible for the up regulation of muscle PDK4 expression. The transcriptional mechanisms by which increased free fatty acids up regulate the expression of the PDK4 gene in human skeletal muscle is not clear but may be mediated by activation of the PPAR gamma coactivator (PGC-1 $\alpha$ )/ Estrogen-related receptor (ERR  $\alpha$ ) signalling systems (Wende, Huss et al. 2005; Araki and Motojima 2006). Moreover, the exact mechanism by which insulin mediates the activity of the pyruvate dehydrogenase complex is still unresolved and further research is required.

#### **6.1.2 Impact of type 1 diabetes on exercise induced substrate metabolism**

Patients with type 1 diabetes are advised to adjust insulin therapy proactively according to anticipated intensity and duration of exercise and thereby avoid exercise-induced hypoglycaemia (Schiffrin and Parikh 1985; Sonnenberg, Kemmer et al. 1990; Rabasa-Lhoret, Bourque et al. 2001). The results from this thesis have shown that the rate of glucose utilisation during exercise was dependent on circulating insulin concentrations and glycaemia. Patients would have developed hypoglycaemia if it were not for either the exogenous glucose supply during exercise (chapter 4) or the hyperglycaemic conditions at the onset of exercise (Chapter 5). However, muscle and liver glycogen utilisation were unaffected by changes in circulating insulin concentrations or the availability of blood glucose. In the first exercise study (chapter 4), the exogenous glucose infusion rate was substantially increased in the HI trial to maintain blood glucose at 8 mmol/l. It might have been expected that muscle glycogen would have been spared during the HI trial, but there was no difference in glycogen utilization between trials. In the second exercise study (chapter 5), changes

in liver glycogen concentration during exercise were comparable in non-diabetic controls and patients with type 1 diabetes despite the hyperinsulinaemic conditions in the patient group. These findings are contrary to the results observed during resting studies. Acute increase in peripheral insulin in patients with type 1 diabetes diminished liver glycogenolysis (Boden, Cheung et al. 2003). This suggests that the regulation of substrate mobilisation during exercise is insensitive to islet hormones in patients with type 1 diabetes.

During exercise, insulin, glucagon and catecholamines appear to respond in a hierarchical fashion to regulate hepatic glucose output and prevent exercise-induced hypoglycaemia (Hoelzer, Dalsky et al. 1986; Tuttle, Marker et al. 1988; Hirsch, Marker et al. 1991; Cryer, Davis et al. 2003). A fall in insulin and a rise in glucagon are necessary to allow increased hepatic glucose output during exercise. In patients with type 1 diabetes managed with subcutaneous insulin injections, a fall in serum insulin concentration cannot occur during exercise. Moreover, the studies described in chapter 4 and 5 demonstrated that when the circulating insulin concentration is between 30-80mU/l, there was no significant increase in glucagon concentrations during exercise. This resulted in a lower glucagon to insulin ratio in patients with type 1 diabetes when compared to non-diabetic individuals and could have affected hepatic glucose output. However, the study described in chapter 5 showed that changes in liver glycogen concentration during exercise in patients with type 1 diabetes were comparable to that of the non-diabetic controls despite differences in glucagon to insulin ratio between groups. The lack of reciprocal hormonal changes in serum insulin and glucagon concentrations did not appear to negatively influence changes in hepatic glycogen content in patients with type 1 diabetes. It is plausible that in such patients other factors such as catecholamines regulate hepatic glucose output during

exercise under those conditions. This is in agreement with previous studies which suggested that catecholamines play a more prominent role in hepatic glucose metabolism during exercise than the suppressive effect of relative hyperinsulinaemia (Simonson, Koivisto et al. 1984; Hoelzer, Dalsky et al. 1986; Hirsch, Marker et al. 1991; Purdon, Brousson et al. 1993).

In chapter 5, patients with type 1 diabetes were becoming near hypoglycaemic during the third bout of exercise despite comparable glycogen concentrations. It is most likely that glucose utilisation outstripped glucose production during this exercise bout. During prolonged exercise, gluconeogenesis can account for as much as 30 to 40% (Ahlborg, Felig et al. 1974). It is likely that the higher circulating insulin concentrations during the third bout of exercise resulted in suppression of gluconeogenesis and gluconeogenic precursor uptake by the liver (Boden, Cheung et al. 2003). One of the key limitations of this study was that direct measurements of gluconeogenesis or precursor uptake were not carried out and this merits further investigation.

A novel observation of interest in the first exercise study (chapter 4) was that the additional increase in whole body glucose utilisation during exercise under hyperinsulinaemic-hyperglycaemic conditions appeared to be directed towards non-oxidative pathways rather than oxidative metabolism. It is possible that the exercise-induced increase in blood flow and substrate delivery to non-contracting muscle and/or liver could explain this observation. Further studies are required to confirm whether glucose storage indeed occurs during exercise under such conditions in patients with type 1 diabetes. This might have implications in the prevention of hypoglycaemia following the cessation of exercise.

Previous studies demonstrated a preferential utilization of free fatty acids over carbohydrate in patients with type 1 diabetes during moderate exercise in the fasting state (Wahren, Hagenfeldt et al. 1975; Wahren, Sato et al. 1984). However, these experiments were performed after insulin withdrawal for 24h. When moderate exercise was performed with basal insulin replacement ( $\sim 10$  mU/l), patients with type 1 diabetes had comparable carbohydrate but higher fat oxidation rates when compared to controls (Zinman, Murray et al. 1977; Raguso, Coggan et al. 1995). These differences disappeared during high intensity exercise. It is likely that in the study described in chapter 5, the higher circulating insulin concentrations during exercise ( $\sim 20$ - $30$  mU/l) corrected the previously reported differences in substrate oxidation between patients with type 1 diabetes and controls.

Plasma NEFA concentrations were almost totally suppressed due to high circulating systemic insulin concentrations in the study described in chapter 4 and during the first bout of exercise in the study described in chapter 5. It is therefore reasonable to assume that lipolysis was suppressed, although tracing NEFA and glycerol release using stable isotopes would have been required to confirm this. Despite this, there was no difference in fat oxidation under different circulating insulin concentrations (Chapter 4). These results suggest that during short duration ( $< 1$ h) moderate intensity exercise in patients with type 1 diabetes, intramuscular triglyceride oxidation may contribute to whole body fat oxidation under hyperinsulinaemic conditions. During prolonged moderate intensity exercise (chapter 5), plasma NEFA concentrations rose above resting values in patients with type 1 diabetes and were comparable to control subjects despite differences in hyperinsulinaemia. Neurohormonal regulation of substrate mobilisation therefore varies depending on exercise intensity and duration.

Since the catecholamine responses to exercise in patients with type 1 diabetes were comparable to the control subjects, it is possible that this overrode the suppressive effect of hyperinsulinaemia on lipolysis.

### **6.1.3 Limitations of thesis and directions for future work**

There are generic limitations to both study design and research methodology in all of the three studies described in this thesis. All three studies were interventional in nature and conducted in a small cohort of carefully selected volunteers during a short time frame. Therefore, a key limitation is the generalisation of the results to a wider group of individuals. Secondly, indirect calorimetry was used to determine substrate oxidation in all three studies. The results obtained by this technique are only indicative of the underlying metabolism. Tracer methodology would need to be applied for truly quantitative results. Moreover, indirect calorimetry results should be interpreted with caution in the presence of gluconeogenesis, lipogenesis and acid-base disorders. Thirdly, changes in gene expression and muscle metabolism were studied in the vastus lateralis muscle, and are assumed to be representative of a typical skeletal muscle. Moreover, muscle biopsy samples could be subject to sampling and processing errors. Finally, MR spectroscopy is a relatively novel and expensive technique and can be conducted only in a few centres around the world. The primary data are subject to extensive mathematical processing and could explain the wide variability in the results. It is still debatable as to whether this technique is truly quantitative. Despite these limitations, all three studies provide an important insight into factors affecting energy metabolism both in health and type I diabetes.

Several key observations require further investigation. Exercise under hyperinsulinaemic hyperglycaemic conditions did not appear to suppress liver glycogen utilisation. This is contrary to findings in healthy volunteers and needs to be investigated further. Tracing liver glucose output using stable glucose isotopes would help to delineate the role of glycogenolysis and gluconeogenesis in liver glucose output under these conditions. Furthermore, it is likely that overriding counterregulatory responses in the patients studied augmented liver glycogen mobilisation. It would be interesting to replicate these studies in patients with and without autonomic neuropathy and compare the physiological responses to exercise. The information gained from these studies would help to tailor insulin and diet in patients with type 1 diabetes.

An intriguing finding from this thesis was that during exercise, exogenous glucose was perhaps directed towards non-contracting muscle. This also raises the possibility of impaired glucose uptake in contracting muscle. One legged cycling using arteriovenous differences and muscle biopsies could be used as a model to test this hypothesis. Furthermore, an unexpected finding was the failure to observe changes in key phosphorylation targets promoting insulin and exercise mediated glucose uptake. The lack of a baseline muscle biopsy prior to insulin infusion (chapter 4) makes it difficult to offer an adequate explanation for this finding. A further study is required to confirm or refute this observation. The study protocol needs to include a baseline muscle biopsy prior to starting the insulin infusion and another biopsy after 10-15 min of cycling. This would determine whether there is a relationship between phosphorylated events and glucose disposal under those conditions. Furthermore, the protocol needs to be carried out under both euglycaemic and hyperglycaemic conditions to rule out a negative effect of hyperglycaemia. The study protocol could

also benefit from the inclusion of age-, body mass- and physical training-matched non-diabetic volunteers as a comparative group.

Finally, in order to translate the observations from these studies into real clinical practice, the experiments would need to be replicated using subcutaneous insulin therapy. The unpredictable nature of subcutaneous insulin depots could be bypassed by using the latest insulin pump devices to deliver continuous insulin infusion. This would provide an added advantage of omitting an overnight admission and less cumbersome experimental procedures.

## **6.2 Conclusions**

Increasing dietary fat intake in healthy humans influences glucose oxidation through mechanisms other than substrate competition via Randle's glucose-fatty acid cycle. A short-term increase in NEFA availability invokes the transcriptional machinery regulating carbohydrate metabolism via PDK4 up regulation but not the regulators of fat metabolism. However, the precise signaling pathway mediating the effect of NEFA remains to be elucidated.

It has been shown in this thesis for the first time that hyperinsulinaemic conditions in patients with type 1 diabetes do not repress exercise-induced changes in either muscle or liver glycogen concentrations. Exercise under hyperinsulinaemic-hyperglycaemic conditions increases whole body glucose utilization. Interestingly, a proportion of the increased glucose utilization appears to be directed towards non-oxidative glucose disposal. It has also been demonstrated that in patients with type 1 diabetes, the absence of reciprocal physiological changes in insulin and glucagon concentrations does not prevent changes in hepatic glycogen content as long as the catecholamine response to exercise is preserved.

In patients with type 1 diabetes, lipolysis appears to be inhibited during short-term moderate intensity exercise under hyperinsulinaemic conditions as evidenced by the suppressed plasma NEFA concentrations. Intramuscular triglyceride stores appear to be the main contributor to fat oxidation under these conditions. However, during progressive moderate intensity exercise under hyperinsulinaemic conditions there was a continuous rise in NEFA concentration over basal values and was comparable to non-diabetic individuals.



Several clinically relevant inferences can be made from the data presented in this thesis and are highlighted below.

1) In patients with type 1 diabetes, liver and muscle glycogenolysis during exercise can be insensitive to therapeutic insulin concentrations. However, this is quite dependent on intact autonomic responses to exercise and has to be taken into account before planning exercise under therapeutic insulin concentrations. Glycogen stores can be normalised by a combination of high carbohydrate meals and intensive control of glycaemia for 24-h prior to planned activity.

2) During moderate intensity exercise under hyperinsulinaemic conditions, patients require around 30 to 60 g (during a 40 min period) of exogenous glucose to maintain glycaemia at 8 mmol/l. This information could provide guidance to patients who carry out regular unplanned exercise. This would have to be consumed at regular intervals during the course of any activity. Patients could have the choice of consuming less should they aim to achieve a tighter degree of overall glycaemic control. Patients would still need to have a carbohydrate rich post-exercise meal to replenish the endogenous liver and muscle glycogen stores and prevent post-exercise hypoglycaemia.

3) It has been recommended that patients with type 1 diabetes refrain from exercise if significantly hyperglycaemic due to the risk of inducing keto-acidosis. However, the study described in chapter 5 showed that ketosis during exercise under hyperglycaemic conditions did not occur provided that sufficient insulin was administered with the pre-exercise meal. Hence, appropriate insulin dose reduction (by 25 to 50%) prior to exercise depending on the duration and intensity of the activity would reduce the risk of ketosis.

Achieving tight glycaemic control can be difficult in patients with type 1 diabetes with current therapies. Exercise makes this even more difficult and there is relatively a limited evidence base on the metabolism in exercise in patients with type 1 diabetes. Data presented in this thesis adds further evidence on the complex multifactorial regulation of carbohydrate metabolism both in health and type 1 diabetes. However, a lot more work needs to be done to further the understanding of carbohydrate and fat metabolism during exercise and thereby refine patient advice to permit safe participation in sport and games.

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## **Appendix**

### **Appendix 1: Hyperinsulinaemic Clamp Calculations**

**Calculations of Prime and Continuous Insulin infusion Rate for the Hyperinsulinaemic Clamps (e.g. 50 mU/m<sup>2</sup>/min)**

#### **Prime**

$$0-2 \text{ min} = 7.52 * F$$

$$2-4 \text{ min} = 3.28 * F$$

$$4-6 \text{ min} = 2.99 * F$$

$$6-8 \text{ min} = 2.82 * F$$

$$8-10 \text{ min} = 2.57 * F$$

#### **Continuous Infusion**

$$10 \text{ min} = 2.40 * F$$

Where  $F = (\text{Body Surface Area (m}^2\text{)} / \text{Insulin Syringe Concentration}) * (50/40)$

#### **Calculation of Glucose Infusion Rate**

Glucose is started 4 min after the insulin primer has begun, at a rate of 2mg/kg/min and increased at 10 min to a rate of 2.5 mg/kg/min.

Therefore with a 20% glucose infusion,

$$\text{Infusion rate at 4 min} = (2 \text{ mg/kg.min} * \text{kg bodyweight}) * (1/200 \text{ ml/mg}) = \text{ml/min}$$

Multiply by 60 for ml/hr.

## **Appendix 2: Calculation of whole body protein oxidation using urine urea concentration and correcting for possible changes in the body's urea pool**

1a) Multiply urine urea concentration by 0.47 (which is the fraction of Nitrogen in urea). (If concentration is in mM convert to g/ml). Urea accounts for ~90% of total N in urine, so your calculation will be a good index of protein oxidation rather than an accurate one.

1b) For a more accurate calculation of protein oxidation, measurements of total urinary nitrogen (in g/ml) are required.

2) Multiply the above number (from either 1a or 1b) by the total urine volume (in ml) to derive total g of N over the recovery period.

3) Divide the above number by the duration (in min) of the urine collection period to derive g of N/min.

4) Multiply the above value by 6.25 to derive the protein oxidation rate in g/min.

5) The above value has to be corrected for changes in urea pool during the experiment and hence plasma urea before and after the experiment needs to be measured and the following calculation used:

Correction factor: (Plasma urea conc. at start - plasma urea conc. at the end of observation period) x 60 x 0.47 x (0.57 x Body mass of subject) / (1000 x time between samples in min) where, 0.47 is fraction of N in urea, 60 is molecular weight of urea, and 0.57 is the urea pool as fraction of body mass.

6) The outcome of the above calculation is change in urea N expressed in g/min and could be a positive or negative number (depending of course whether the plasma urea concentration at start is lower or higher than the concentration at the end).

7) Multiply the above value (maintaining the negative or positive sign) by 6.25 to derive the protein oxidation rate in g/min.

8) Add the above (positive or negative) number to the number obtained from step 4 above. This will give you the total calculated protein oxidation rate in g/min.

10/ Use the equations from Frayn 1983 to calculate the nonprotein RQ and then the fat and CHO oxidation rates.

## **References**

Frayn 1983 JAP 55: 628-634

Jequier et al. 1987 Ann Rev Nutr 7: 187-208.

### **Appendix 3: Glucagon Assay**

#### **Principle**

Double antibody glucagon procedure is a sequential radioimmunoassay. After preincubation of the sample with anti-glucagon antibody,  $^{125}\text{I}$ -labeled glucagon competes with glucagon in the sample for antibody sites. After incubation for a fixed time, separation of bound from free is achieved by the PEG-accelerated double-antibody method, followed by centrifugation. The precipitate containing the antibody-bound fraction is then counted, and patient's concentrations are read from a standard curve.

#### **Procedure**

All components except the precipitating solution must be at room temperature before use. Glass tubes should be used for the assay to prevent adsorption of glucagon to plastic tubes.

##### **1) Making up the standards**

Just before the assay, dilute the 584 pg/ml Glucagon calibrator F in the Glucagon Zero calibrator (A), using glass tubes as described in the table below. Vortex each dilution thoroughly.

Add this		To This		Yielding this Calibrator		
$\mu\text{l}$	Calibrator	$\mu\text{l}$	Calibrator		Pg/ml	Pmol/l
50	F	950	Zero	B	25	7
100	F	900	Zero	C	50	14
200	F	800	Zero	D	100	29
500	F	500	Zero	E	250	72

- 2) Label 16 glass tubes in duplicate: T (Total counts), NSB (non-specific binding) and B through F. label additional tubes for samples and controls.
- 3) Pipet 200  $\mu$ l of the Zero calibrator A into the NSB and A tubes, and 200  $\mu$ l of each of the remaining calibrators B through E into correspondingly labelled tubes. Pipet 200  $\mu$ l of each patient plasma sample and each control into the tubes prepared.
- 4) Add 100  $\mu$ l of glucagon antiserum to all tubes except the NSB and T tubes. Vortex, cover the tubes and incubate for 24 hours at 2-8<sup>0</sup> C. The following day add 100  $\mu$ l of <sup>125</sup>I Glucagon to all tubes. The T tubes need no further processing. Cover the tubes and incubate for a further 24 hours at 2-8<sup>0</sup> C. The next day add 1 ml of cold precipitating solution to all tubes except T tubes.
- 5) Centrifuge for 15 min at 3000 rpm (1500 x g). Decant the supernatant and retain the precipitate for counting. Let the tubes stand inverted for atleast 10 min to dry. Blot the rims for any residual droplets. Count each tube for 1 min in a gamma counter.

### **Calculations**

The counts per minute (cpm) for the standards are converted to log to the base 10. A Pearson correlation was derived between the log transformed values and the known concentrations of the calibrators. The log<sub>10</sub> cpm values were substituted in the regression equation and the antilog of the calculated values then gave the actual concentration of glucagon in the samples.

#### **Appendix 4: Free Fatty Acid (NEFA) Assay – Plate reader version**

Materials: NEFA C kit by WAKO Chemicals (GmbH). Order from Alpha Labs, catalogue number 994-75409. Price £193 per kit (spring 2001).

96-well plate, multiple channel pipette, yellow pipette tips, 25 µl positive displacement pipette and tips, 5 ml Gilson and tips, beaker of double-distilled/UHQ water.

**Colour reagent A.** Puncture the cap before opening to equilibrate the pressure inside the bottle. Add 10 ml of solvent A to each bottle of dry colour reagent A. Mix gently.

**Colour reagent B.** Puncture the cap before opening to equilibrate the pressure inside the bottle. Add 20 ml of solvent B to each bottle of dry colour reagent B. Mix gently.

**Note:** Only make up as many bottles as you need for your current assay, as the reagents will go off after 10 days. Each individual bottle provides enough for an assay with 8 standards and up to 75 samples.

Thaw and gently vortex samples before use. Gently swirl the 1 mM oleic acid standard provided in the kit, to ensure it is evenly mixed.

Make sure 96-well plate is clean and dry before use. If in doubt rinse with double-distilled water, then methanol, and dry with compressed air. Make sure “A1” is in the top left-hand corner.



Working in vertical rows, top to bottom, left to right, pipette out the assay as follows:

	Volume 1 mM oleic acid (μl)	Volume sample	FFA concentration in well (mM)
Blank	0	0	0
Std 1	1	0	0.0044
Std 2	2	0	0.0088
Std 3	3	0	0.0132
Std 4	4	0	0.0176
Std 5	5	0	0.022
Std 6	7	0	0.0308
Std 7	9	0	0.0396
Sample	0	4	unknown

Make sure the incubator of the plate reader is switched on and preheat to 37°C.

Using the 8-channel pipette, add 75 μl of colour reagent A to each well. (Reverse pipetting helps to avoid bubbles). Shake gently (using the plate shaker, if the plate reader has one) then put in the 37°C incubator for 10 minutes.

Add 150 μl of reagent to each well using the multi-channel pipette. Shake and incubate at 37°C for 10 minutes.

Make sure the plate reader is set to read at 550 nm and that it is reading in vertical columns from top to bottom and left to right. Define A1 as the blank and the rest of the first column as the standards. Ensure that the path check is switched on.

After reading, print out the readings and/or save to disc.

**To calculate the results:**

The plate reader should subtract the absorbance of the blank from all the other absorbances. If not, do this first.

Plot the absorbances of the standards on the y axis against their calculated concentrations on the x axis. Fit a linear curve and find the gradient or slope (n).

To find the concentration of the sample in the well: Divide the absorbance of the sample by the slope. Multiply by 57.25 to allow for the dilution of the sample in the well by the other reagents. (Total volume in well/sample volume in well =  $229/4 = 57.25$ .)

## **Appendix 5: Insulin assay procedure Coat -a- Count**

### **Making up the standards (or “Calibrators”)**

Take one set of “calibrators” A to G out of the fridge in E34. Make a note of the concentrations stated on the labels – they could vary from one batch to another.

Be careful when you uncap them – powder can fly out.

Using 1 ml (and 5 ml) Gilson pipettes, add 6 ml of de-ionised water to vial “A” and 3 ml to each vial “B” to “G”.

Re-cap vials and mix by gentle swirling.

Leave them to stand for 30 minutes. Meanwhile, label up 6 Eppendorfs for each calibrator.

When ready, divide each calibrator up into 6 aliquots, i.e. pipette 1 ml of “A” into each “A” Eppendorf, 0.5 ml of each “B” into each “B” Eppendorf, etc.

Label up a snap-top freezer bag with your name, “Insulin calibrators A – G”, the date and the expiry date (30 days later). Put in the Eppendorfs and freeze at -20°C. (E4 or B14).

### **Insulin Assay Procedure**

Take 1 set of calibrators A to G, 1 set of QCs 4,5, and 6, and your samples out of the fridge. Ideally, do the samples in batches of about 100. Put in a rack and leave to thaw. Label the sample tubes with numbers in the order that you are going to do them. Make a note of this order. Take green antibody-coated tubes out of the fridge in E34. They come in bags of 25. You will need 1 per sample, plus 20 for the standard curve and QCs. Label the assay tubes up as follows:

2 plain LP4's: TC, 2 plain LP4's: NSB, 2 green tubes each for A, B, C, D, E, F, and G, 1 green tube each for QCs 4,5, and 6, 1 green tube for each sample, 1 green tube each for QCs 4,5, and 6 (we put some at the end of the assay to check for “assay

drift”). Next get the radioactive label ready. Take one or more vials of  $^{125}\text{I}$ -Insulin out of the fridge in E34 (One vial gives you enough for about 108 assay tubes). Measure out 100 ml de-ionised water in the graduated cylinder for each vial and add to the gel concentrate. Mix by gentle swirling. Label up a beaker with radioactive tape for pipetting the radioactive label out of. When the samples have thawed, mix by vortexing. Mix the calibrators and QCs by gentle inversion.

Using the 200  $\mu\text{l}$  Gilson, pipette out the assay as follows:

Nothing in the “TC” tubes, 200  $\mu\text{l}$  of A into each “NSB” and each “A” tube

(N.B. A is the “zero standard” and measures “zero binding” or “total binding”)

200  $\mu\text{l}$  of each calibrator B to G, QCs 4 to 6, and samples into their corresponding tubes.

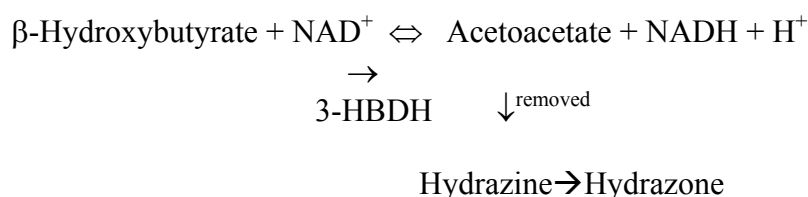
N.B. When pipetting, make sure it goes down to the bottom of the tube. Use a clean pipette tip for each tube. Now add the radioactive label to each assay tube. Pour the label into the beaker, use the 1 ml Gilson and pipette 1 ml of  $^{125}\text{I}$ -Insulin into each assay tube. Do it slowly and carefully to avoid splashing. Now cap the TC tubes. Mix all the other tubes by vortexing. Cover the whole rack in cling film and leave at room temperature for 18 – 24 hours.

The following day, split the assay. Take the TC tubes out and put them in a spare rack. Get plenty of blue bench roll ready, and lay out several thicknesses on the radioactive work area. Have a bit of running water draining through the sink. Decant the tubes by tipping the rack upside-down over the sink. Keeping them upside-down, blot them on the blue bench roll and sharply tap them a few times. Leave them standing upside down for 2-3 min. Tap again, turn the right way up and blot off any visible water you can see. Put the TCs back in the rack and count the tubes on the

gamma-counter in E48. Use programme 24. Check the programme to make sure that the standards are correct – if not, edit the programme before you start counting.

## **Appendix 6: $\beta$ -Hydroxybutyrate assay**

### **Principle**



3-HBDH = 3-Hydroxybutyrate dehydrogenase

$\text{NAD}^+$  = Nicotinamide adenine dinucleotide  
(Coenzyme that serves as an electron acceptor for dehydrogenase)

NADH absorbs light at 340nm; hence formation of NADH and extinction of light at  $\lambda$  340nm is a measure of reaction. Equilibrium constant of the reaction is  $1.45 \times 10^{-9}$  at 25 °C. At pH 8.5, approx 40% of 3-hydroxybutyrate is oxidised to acetoacetate. Hydrazine is added to the reaction mixture to trap acetoacetate (forming the hydrazone) enabling the reaction to proceed quantitatively left  $\rightarrow$  right. The enzyme 3-HBDH, has low activity, but is most efficient between 25 °C and 30 °C.

### **Sample collection**

Add 50 or 100 $\mu$ l whole blood to 50 or 200 $\mu$ l 10% PCA. Mix and allow precipitate to form. Centrifuge at 1000g for 5 mins. Remove supernatant  $\geq$  600 $\mu$ l  $\rightarrow$  clean vial. (Precise volume not necessary at this stage, it will be measured accurately for assay).

Freeze at -20°C

### **Samples**

Centrifuge all samples at 1000g for 5 min. Remove 300 $\mu$ l supernatant into a separate tube. To this tube add 110 $\mu$ l 20% KOH. A white precipitate (potassium perchlorate) forms. Mix and check pH of supernatant using a tiny dot on pH indicator paper. Aim

for pH 7-8. As long as it is not acid or strongly alkaline the assay will work.  
Centrifuge at 1000g for 5 mins.

### **Standards**

To each 300µl std mix (d), add 65µl 20% KOH and 45µl H<sub>2</sub>O. 110µl in total, same volume as samples. Precipitate forms, check pH of supernatant as for samples.  
Centrifuge at 1000g for 5 mins.

### **Loading the plate**

A) Per well, either 100µl standard (acidified and neutralized, including blanks.) Do standard curve in duplicate or 100µl sample (acidified and neutralized). 3 wells per sample. Immediately before adding to the plate, mix 200µl NAD solution with 2ml hydrazine tris buffer (per 40 wells required on plate)

B) Per well add 55µl of this freshly prepared NAD hydrazine tris buffer using a multichannel pipette.

C) Set plate-reader incubation temp to 30°C. Mix and read plate at  $\lambda$  340nm.

D) Add 1µl enzyme per well except one well per sample. This is the sample blank, since each sample is tainted by the blood precipitate initially but clears during incubation to varying degrees (bleached by either the KOH or the hydrazine)

Mix and read at  $\lambda$  340nm to check enzyme reaction beginning as expected.

E) Incubate plate at 30°C for 1hour. Mix and read plate at  $\lambda$  340nm.

## CALCULATIONS

To calculate absorbance change during reaction for standards, subtract mean first read values from mean final read values. Zero value standard indicates any non-specific activity but is not subtracted from the other standards.

Plot absorbance change vs  $\beta$ -Hydroxybutyrate conc in mmol/l. Find  $y = mx + c$  for line of best fit.

To calculate absorbance change during reaction for samples, if the supernatant has been removed prior to freezing, same should be done as with standards (subtract first read).

But if the supernatant was not removed before freezing it will be tainted with grey from the blood precipitate, which then bleaches during incubation.

In this case use an extra sample well with no enzyme added as the blank (as described method)

Subtract this final absorbance from the mean of the duplicate sample reaction wells.

Calculate concentration in whole blood in mmol/l using  $y = mx + c$ .

The method is written for 150 $\mu$ l whole blood + 300 $\mu$ l 10% PCA, but for this study samples were 50 $\mu$ l WB + 50 $\mu$ l 10%PCA, from which 50 $\mu$ l of supernatant was used.

Standards were prepared as described but then 25 $\mu$ l of each concentration was added to 25 $\mu$ l 10% PCA to produce 50 $\mu$ l of solution equivalent to the samples.

Samples were then neutralized with 260 $\mu$ l 1.5% KOH and pH checked, this enabled sufficient supernatant for 2 wells of sample at 100 $\mu$ l/well.



Standards (being less acidic) were neutralized with 120µl 1.5% KOH and 140µl water to form equivalent volume supernatant as samples. pH checked and 100ul/well used. Standard curve in duplicate.

## **Appendix 7: Serum and Urine Urea Assay**

### **Principle**

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with  $\alpha$ - oxoglutarate and NADH in the presence of glutamate-dehydrogenase to yield glutamate and  $\text{NAD}^+$ .

### **Samples**

Heparinized plasma or urine samples. Dilute urine 1 to 20 with distilled water and multiply the result by 21.

### **Reagent Composition**

<b>Buffer</b>	<b>Initial Concentration of Solutions</b>
Tris Buffer	150 mmol/l, pH 7.6
<b>Enzyme Reagent</b>	
Urease	$\geq 10$ U/ml
GLDH	$\geq 2$ U/ml
NADH	0.26 mmol/l
ADP	3 mmol/l
$\alpha$ - oxoglutarate	14 mmol/l
<b>Standard</b>	13.3 mmol/l

### **Procedure**

Serial dilutions of the standards were made and a standard curve method was used to read off the concentrations in the samples. Volumes of reagent were reduced to 250  $\mu\text{l}$ , samples and standards were reduced to 2.5  $\mu\text{l}$ , to adapt for the plate reader. Pipette sample or standard, reagent and enzyme mixture in duplicate, incubate for 30 min at  $37^{\circ}\text{C}$  and read at 340 nm.

## **Appendix 8: Measurement of ATP, PCr and G-6P**

### **Principle**

Glu-6-P + NADP  $\xrightarrow{\text{G-6-PDH}}$  P-gluconolactate + NADPH

ATP + Glucose  $\xrightarrow{\text{HK}}$  ADP ADP + G-6-P

PCr + ADP  $\xrightarrow{\text{CPK}}$  Cr + ATP

### **Method**

Make up the following reagent mixture using these quantities per well:

D1	(TEA pH 7.5-7.6 buffer)	(fridge)	22.2µl
DTT (B 197 777)	(7.8mg/ml)	(fridge)	4.4µl
NADP (B 128 058)	(20.9mg/ml)	(fridge)	8.9µl
ADP (B 236 675)	(5.1mg/ml)	(-20 °C)	0.9µl
Glucose (Sigma)	(22.5mg/ml)	(shelf)	8.9µl
Dist H <sub>2</sub> O			154.7µl
			<hr/> 200µl

Vortex and keep on ice

Make 1 mM standards for G6P, ATP and PCR (see below). Combine standards into one well.

Dilute the following enzymes:

Glucose-6-Phosphate-Dehydrogenase\*(Sigma G-5885). Dilute 1 part enzyme to 1 part Dist H<sub>2</sub>O. Use 3µl per well.

Hexokinase (HK) (B 1426 362). Dilute 1 part enzyme to 1 part Dist H<sub>2</sub>O. Use 5µl per well.

Creatine phosphokinase (CPK) (Sigma C-3755) (-20°C). Dissolve 15mg/ml in 0.5% NaHCO<sub>3</sub> + 0.05% BSA (D5). Dilute 2 parts enzyme to 1 part Dist H<sub>2</sub>O. Use 3µl per well. Vortex and keep on ice.

Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)

Set the plate reader to read samples at 340nm.

Pipette 200µl of the reagent mix into each well.

Add 20µl of water (blank)/sample. Add 21µl (3x7 µl of each standard) to a single well. Read absorbance (A1).

Add 3µl of the G6PDH. Incubate for 10-15 min. Read absorbance (A2).

Add 5µl of the HK. Incubate for 15-20 min. Read absorbance (A3).

Add 3µl of the CPK. Incubate for 30-35 min. Read absorbance (A4).

### **To make up 1 mM standards**

10 mM ATP (B 127 523): 6.052 mg/ml then dilute 10 times

10 mM PCr (B 127 574): 3.272 mg/ml then dilute 10 times

10 mM G-6P (B 127 027): 3.042 mg/ml then dilute 10 times

### **Calculations** (units = mmol/kg dm weight)

$$\text{Conc.} = \frac{((\text{final vol} * (\text{Ab}_n - \text{blank}_n)) - (\text{vol before enzyme} * (\text{Ab}_{n-1} - \text{blank}_{n-1}))) * \text{ext factor} * 1.25 * \text{dil factor}}{6.22 * \text{volume of sample}}$$

### **Notes**

Remember that volumes increase as each subsequent enzyme is added.

Ext factor is extraction factor; Dil factor is dilution factor and 6.22 is extinction factor

\*G6PDH from Boeh. 127 035 (dilution 1 part enzyme to 2 parts Dist H2O) also works

## **Appendix 9: Measurement of Glucose (Insoluble and soluble Glycogen)**

### **Method**

Make up the following reagent mixture using these quantities per well:

G1	TEA	7.00g/100ml	64µl
	KOH	0.80g/100ml	
	Mg (AC) <sub>2</sub> .4H <sub>2</sub> O	2.40g/100ml	
	EDTA Na <sub>2</sub> .2H <sub>2</sub> O	0.14g/100ml	
	pH=8.2 (KOH)		
ATP	Sigma A-2383 (-20°C)	27.72mg/ml	4µl
DTT	Boeh 197 777 (fridge)	9.36mg/ml	4µl
NAD	Boeh 127 965 (fridge)	19.92mg/ml	8µl
H <sub>2</sub> O			120µl
			<b>Σ 200 µl</b>

Vortex and keep on ice

Make glucose standard (1.5mmol/l): 2.70 mg/10 ml of water)

Dilute the following enzymes:

G-6-PDH	Sigma G-5885, 200 units/mg	Reconstitute with 200µl H <sub>2</sub> O
HK	Sigma H-4502, 256 units/mg	Reconstitute with 256µl H <sub>2</sub> O
	Or Sigma 1131 1500 units	

Mix equal parts of G-6-PDH and HK (ie 50µl + 50µl)

Vortex and keep on ice.

Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)

Set the plate reader to read samples at 340nm

Pipette 200µl of the reagent mix into each well.

Add 20µl of water/standard/sample.

Read absorbance (A1).

Add 2µl of the mixed enzyme and incubate for 15-20 min.

Read final absorbance (A2).

**Calculations:** (units = mmol glucosyl units/kg dm weight)

$$\text{Conc.} = \frac{[(A2 \times V2) - (A1 \times V1)] - [(AB2 \times V2) - (AB1 \times V1)]}{\text{Standard Volume} \times 6.22} \times \text{Extraction Factor}$$

[6.22 is Extinction factor]

## **Appendix 10: Measurement of Creatine**

### **Principle**

Pyruvate + NADH  $\xrightarrow{\text{LDH}}$  Lactate + NAD

ADP + PEP  $\xrightarrow{\text{PK}}$  ATP + Pyruvate

Cr + ATP  $\xrightarrow{\text{CPK}}$  PCr + ADP

### **Method:**

Make up the following reagent mixture using these quantities per well:

D4	(Glycine buffer)				75µl
D3	(KCl 15g/100ml)				3.75µl
ATP	(15.4mg/ml)	Sigma 2383	A- (-20°C)		15µl
PEP	(11.6mg/ml)	Sigma 7252	P- (-20°C)		11.25µl
NADH	(9mg/ml)	Boeh. 727 or 107 735	107 (fridge)		3.75µl
LDH		Boeh. 069 (pig heart)	107 (fridge)		0.375µl
PK		Boeh. 155	128 (fridge)		0.375µl
Dist H <sub>2</sub> O					115.5µl
					<hr/> 225µl

Vortex and keep on ice

Make standard (see below).

Prepare the enzyme:

CPK (Sigma C-3755 (-20°C)). Dissolve 15mg/ml in 0.5% NaHCO<sub>3</sub> + 0.05% BSA

(D5). Use 5µl per well. Vortex and keep on ice.

Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)

Set the plate reader to read samples at 340nm.

Pipette 225µl of the reagent mix into each well.

Add 15µl of water/standard/sample into each well. Read absorbance (A1).

Add 5µl of the CPK. Incubate for 30min. Read absorbance (A2).

**To make up 1mM standard (0.1965 mg/ml):**

Creatine (anhydrous, Sigma C-0780, kept at room temperature) MW 131.1

Weigh out an amount (~2mg) and add to 1ml H<sub>2</sub>O. Divide MW by the measured weight, divide this by 1000. Divide 1 by this answer – this will give you the mM of the new solution. Then calculate the dilution factor to make up a 1.5mM solution. (ie divide new mM by 1.5 then ratio *1:answer from this division*)

**Calculations:** (units = mmol/kg dm weight)

$$\text{Conc.} = \frac{((\text{final vol} * (\text{Ab}_n - \text{blank}_n)) - (\text{vol before enzyme} * (\text{Ab}_{n-1} - \text{blank}_{n-1}))) * \text{ext factor} * 1.25 * \text{dil factor}}{6.22 * \text{volume of sample}}$$

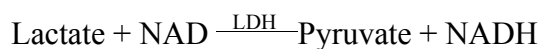
**Notes**

Remember that volumes increase as each subsequent enzyme is added.

Ext factor is extraction factor, Dil factor is dilution factor, 6.22 is extinction factor.

## **Appendix 11: Measurement of Lactate**

### **Principle:**



### **Method:**

1. Make up the following reagent mixture with these amounts per cuvette:

D2	(glycine buffer)	(F)	75ul
NAD (B 127 965)	(16.6 mg/ ml)	(F)	25ul
H <sub>2</sub> O			100ul

Vortex and keep on ice

2. Set plate reader to read at 340nm
3. Pipette 200ul of reagent into each well
4. Defrost the samples quickly in hot water, vortex and spin for 3 min at 14000rpm.
5. Pipette 20ul of water/sample/blank into each well. Mix and read absorbance (A1).
6. Add 3ul of LDH (B 107 069) to each well and mix.
7. After 20-30 min read final absorbance (A2).

### **Calculation:**

$$\text{Conc. Lactate} = \frac{223 * (A2-B12) - 220 * (A1-B11)}{20*6.22} * \text{Ext Fac} * 1.25$$

Units = mmol/Kg dm weight