SKELETAL MUSCLE FAT METABOLISM DURING POST-EXERCISE RECOVERY IN HUMANS

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CANDIDATE DECLARATION

I certify that the thesis entitled

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Full Name **Nicholas Eden Kimber**

Signed……..

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DECLARATION

This thesis reports original previously unpublished observations conducted in the Exercise, Muscle and Metabolism Unit, School of Exercise and Nutrition Sciences, Deakin University, Burwood, Australia, except where indicated otherwise, during the period 2000-2004. This thesis is the result of work performed solely by the author with the following exceptions. Subject recruitment and testing procedures for Study I were conducted in the Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada under the guidance of Dr. David Dyck, Professor Lawrence Spriet and Professor George Heigenhauser. Malonyl-CoA analysis for Study II was completed by Dr. Asish Saha and Professor Neil Ruderman at the Diabetes & Metabolism Research Unit, Boston Medical Centre, USA. The author performed intramuscular triglyceride analysis for studies II and III at the Royal Melbourne Institute of Technology University. Qualified medical practitioners performed the skeletal muscle biopsies.

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ABSTRACT

Recovery after prolonged or high-intensity exercise is characterised by a substantial increase in adipose tissue lipolysis, resulting in elevated rates of plasma-derived fat oxidation. Despite the large increase in circulating fatty acids (FAs) after exercise, only a small fraction of this is taken up by exercised muscle in the lower extremities. Indeed, the predominant fate of non-oxidised FAs derived from post-exercise lipolysis is reesterification in the liver. During recovery from endurance exercise, a number of changes also occur in skeletal muscle that allow for a high metabolic priority towards glycogen resynthesis. Reducing muscle glycogen during exercise potentiates these effects, however the cellular and molecular mechanisms regulating substrate oxidation following exercise remain poorly defined. The broad aim of this thesis was to examine the regulation of fat metabolism during recovery from glycogen-lowering exercise in the presence of altered fat and glucose availability. In study I, eight endurance-trained males completed a bout of exhaustive exercise followed by ingestion of carbohydrate (CHO)-rich meals (64-70% of energy from CHO) at 1, 4, and 7 h of recovery. Duplicate muscle biopsies were obtained at exhaustion and 3, 6 and 18 h of recovery. Despite the large intake of CHO during recovery (491 ± 28 g or 6.8 ± 0.3 g · kg^{-1}), respiratory exchange ratio values of 0.77 to 0.84 indicated a greater reliance on fat as an oxidative fuel. Intramuscular triacylglycerol (IMTG) content remained unchanged in the presence of elevated glucose and insulin levels during recovery, suggesting IMTG has a negligible role in contributing to the enhanced fat oxidation after exhaustive exercise. It appears that the partitioning of exogenous glucose towards glycogen resynthesis is of high metabolic priority during immediate post-exercise recovery, supported by the trend towards reduced pyruvate dehydrogenase (PDH) activity and increased fat oxidation. The effect of altering plasma FA availability during post-exercise recovery was examined in study II. Eight endurance-trained males performed three trials consisting of glycogen-lowering exercise, followed by infusion of either saline (CON), saline + nicotinic acid (NA) (LFA) or Intralipid and heparin (HFA). Muscle biopsies were obtained at the end of exercise (0 h) and at 3 and 6 h in recovery. Altering the availability of plasma FAs during recovery induced changes in whole-body fat oxidation that were unrelated to differences in skeletal muscle malonyl-CoA. Furthermore, fat oxidation and acetyl-CoA carboxylase (ACC) phosphorylation

appear to be dissociated after exercise, suggesting mechanisms other than phosphorylation-mediated changes in ACC activity have an important role in regulating malonyl-CoA and fat metabolism in human skeletal muscle after exercise. Alternative mechanisms include citrate and long-chain fatty acyl-CoA mediated changes in ACC activity, or differences in malonyl-CoA decarboxylase (MCD) activity. Reducing plasma FA concentrations with NA attenuated the post-exercise increase in MCD and pyruvate dehydrogenase kinase 4 (PDK4) gene expression, suggesting that FAs and/or other factors induced by NA are involved in the regulation of these genes. Despite marked changes in plasma FA availability, no significant changes in IMTG concentration were detected, providing further evidence that plasma-derived FAs are the preferential fuel source contributing to the enhanced fat oxidation post-exercise during recovery. To further examine the effect of substrate availability after exercise, Study III investigated the regulation of fat metabolism during a 6 h recovery period with or without glucose infusion. Enhanced glucose availability significantly increased CHO oxidation compared with the fasted state, although no differences in whole-body fat oxidation were apparent. Consistent with the similar rates of fat metabolism, no difference in AMPK or ACCβ phosphorylation were observed between trials. In addition, no significant treatment or time effects for IMTG concentration were detected during recovery. The large exercise-induced PDK4 gene expression was attenuated when plasma FAs were reduced during glucose infusion, supporting the hypothesis that PDK4 is responsive to sustained changes in lipid availability and/or changes in plasma insulin. Furthermore, the possibility exists that the suppression of PDK4 mRNA also reduced PDK activity and thus maintained PDH activity to account for the higher rates of CHO oxidation observed during glucose infusion compared with the control trial.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACCβ-P	acetyl-CoA carboxylase beta phosphorylation
AICAR	5'-aminoimidazole-4-carboxamide ribonucleoside
AMPK	AMP-activated protein kinase
AMPKα-P	AMP-activated protein kinase alpha phosphorylation
ATP	adenosine triphosphate
β-actin	beta actin
β-HAD	beta-hydroxyacyl-CoA dehydrogenase
cAMP	cyclic adenosine monophosphate
cDNA	copy deoxyribonucleic acid
Ca^{2+}	calcium
CHO	carbohydrate
CO_2	carbon dioxide
CoA	coenzyme A
CON	control
CPT I	carnitine palmitoyltransferase I
C_T	critical threshold
EDTA	ethylenediamine tetraactetic acid
EGTA	ethylene glycolbis (β-aminoethyl ether) N,N,N,N,N,-tetraacetic acid
EMTG	extramuscular triglyceride
ERK	extracellular signal-regulated kinase
FA	fatty acid
$FABP_C$	cytoplasmic fatty acid binding protein
$FABP_{PM}$	plasma membrane fatty acid binding protein
FAT/CD36	fatty acid translocase
FATP-1	fatty acid transport protein – 1
FFA	free fatty acid
FKHR	forkhead homolog in rhabdomyocarcoma
g	grams
G-6-P	glucose-6 phosphate
GLU	glucose infusion

GLUT4	glucose transporter 4
GI	glycemic index
HCL	hydrogen chloride
HFA	high fatty acid availability
HK	hexokinase
^{1}H-MRS	magnetic resonance spectroscopy
HRP	horse radish peroxidase
HSL	hormone sensitive lipase
kcal	calorie
kJ	kilojoule
kDa	kilodalton
KCl	potassium chloride
IMTG	intramuscular triacylglycerol
LCAD	long chain acyl-CoA dehydrogenase
LCFA	long chain fatty acid
LFA	low fatty acid availability
LPL	lipoprotein lipase
LXR	liver X receptor
M	mole
MAPK	mitogen-activated protein kinase
MCD	malonyl-CoA dehydrogenase
MGL	monoglyceride lipase
min	minute
ml	millilitre
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acid
NTC	no template control
O2	oxygen
pH	potential of hydrogen
PCA	perchloric acid
PCr	phosphocreatine
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase

PFK	phosphofructokinase
PGC-1α	PPARγ coactivator-1α
PKA	protein kinase A
PPAR	peroxisome proliferators-activated receptor
PUFA	polyunsaturated fatty acid
RER	respiratory exchange ratio
Rn	normalised reporter
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
RXR	retinoid X receptor
sec	second
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SREBP-1c	sterol regulatory element binding protein-1c
TG	triglyceride
V	volt
$\dot{V}CO_2$	carbon dioxide production
VLDL	very low density lipoprotein
$\dot{V}O_2$	oxygen consumption
$\dot{V}O_{2\,max}$	maximal oxygen consumption
W	Watt
°C	degrees celcius

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

The production of ATP (oxidative phosphorylation) for resting and contracting skeletal muscle is derived mainly from stores of fat and carbohydrate in well-fed humans. Skeletal muscle is the primary site for glucose and fatty acid (FA) disposal and therefore has a significant role in maintaining whole body metabolic homeostasis. Fat is available to skeletal muscle in the form of FAs, derived primarily from lipolysis within peripheral adipose tissue stores and possibly from adipocytes located between skeletal muscle fibers. FAs are also obtained in small amounts from circulating chylomicrons and very low-density lipoproteins (VLDL) as well as intramuscular triacylglycerols (IMTG). Carbohydrates however, are stored primarily as glycogen within muscle fibers. The liver also stores limited amounts of glycogen, which serves to maintain plasma glucose levels during rest and muscle contraction. An overview of fat and carbohydrate (CHO) energy stores is provided in Table 1.1.

As an energy source, fat has a higher energy yield per unit mass than CHO (37.5 *vs* 16.9 kJ/g) and is stored in relatively large quantities in the body (Table 1.1). Therefore, fat can provide a substantial amount of substrate for oxidative phosphorylation at rest and during submaximal endurance exercise. Although fat is an important metabolic fuel, the capacity to oxidise FAs from extra- and intramuscular fat stores is often limited and in many cases CHOs are the major substrate. The underlying mechanisms for this limitation are not completely understood and the interpretation of studies examining the regulation of fat metabolism remain equivocal. FAs are also known to function as precursors for lipid-signaling molecules and ligands for transcription factors that control gene expression. Thus, there is an increasing need to understand the role of FAs at the molecular level and how they regulate cellular metabolism.

Table 1.1. Energy stores of an 80 kg man

Substrate		Weight (g)	Energy (kJ)
Fat	Plasma fatty acids	0.4	15
	Plasma triacylglycerols	4.0	150
	Intramuscular triacylglycerols	300	11 250
	Adipose tissue	12 000	450 000
	Total (approximately)	12 304	461 400
Carbohydrate	Plasma glucose	20	338
	Liver glycogen	100	1790
	Muscle glycogen	350	6265
	Total (approximately)	470	7943

Based on estimates for a “normal”, non-obese male with a body mass of 80 kg.

1.2. OVERVIEW OF SKELETAL MUSCLE FAT METABOLISM

Skeletal muscle is the predominant tissue regulating whole body fat oxidation, accounting for up to 90% of energy requirements at rest (Dagenais *et al.*, 1976; van Loon *et al.*, 2001). In the post-absorptive state, plasma FAs derived from peripheral adipose tissue triacylglycerols (TG) contribute almost exclusively to oxidative energy production (van Loon *et al.*, 2001), most likely from a FA mediated reduction in glucose metabolism (Spriet *et al.*, 2004). The role of fat as an energy substrate during short and long-term exercise has been well reviewed in recent publications (Holloszy *et al.*, 1998; Jeukendrup *et al.*, 1998b, a; Wolfe, 1998b; Jeukendrup, 2002; Spriet, 2002), however much less is known about the mechanisms regulating FA metabolism during post-exercise recovery.

1.2.1. *Fat as a Fuel During Exercise*

In the transition from rest to low intensity exercise (25% of $\dot{V}O_{2\,max}$), FAs released from adipose tissue continue to provide a majority of the oxidative fuel for working muscle (Romijn *et al.*, 1993a). During the first 2 h of moderate intensity exercise (55–65% of $\dot{V}O_{2\,max}$), the relative contribution of plasma FA and TG sources (sum of IMTG and lipoprotein derived triglycerides) to total fat oxidation is approximately equal in endurance-trained individuals (Romijn *et al.*, 1993a; van Loon *et al.*, 2001). However, when moderate intensity exercise extends beyond 2 h the progressive increase in plasma FFA is associated with a greater reliance on blood-borne fat oxidation (Watt *et al.*, 2002a). At high exercise intensities (>80% of $\dot{V}O_{2\,max}$) circulating FAs are markedly reduced, although this does not entirely account for the decline in fat oxidation (Romijn *et al.*, 1993a; Romijn *et al.*, 1995). Evidence suggests that FA oxidation during high intensity exercise is also limited by direct inhibition of long-chain FAs (LCFAs) entry into the mitochondria (Sidossis *et al.*, 1997; Kiens *et al.*, 1999; van Loon *et al.*, 2001).

During moderate intensity exercise, fat oxidation from sources other than plasma FAs is thought to be derived primarily from IMTG since the contribution from circulating lipoproteins (VLDL) under normal dietary conditions is typically less than 10% of total fat metabolism (Helge *et al.*, 2001). On the basis of these findings, many investigators believe that IMTG is an important energy substrate during long-term endurance activities (Havel *et al.*, 1967; Essen *et al.*, 1977; Romijn *et al.*,

1993a; Klein *et al.*, 1994; Romijn *et al.*, 1995; Watt *et al.*, 2002b). Studies using tracer methodology have generally reported IMTG utilisation across a range of exercise intensities (30–80% $\dot{V}O_{2\,max}$) in both untrained (Martin *et al.*, 1993; Phillips *et al.*, 1996; Sidossis *et al.*, 1997; Friedlander *et al.*, 1999; Schrauwen *et al.*, 2000) and trained individuals (Romijn *et al.*, 1993a; Coyle *et al.*, 2001; van Loon *et al.*, 2001) during cycle exercise lasting 30-120 min. Using the non-invasive technique of magnetic resonance spectroscopy (^{1}H-MRS), a majority of studies also demonstrate a net utilisation (~20-50%) of IMTG during 90-120 min of exercise (Boesch *et al.*, 1997; Boesch *et al.*, 1999; Krssak *et al.*, 2000; Rico-Sanz *et al.*, 2000; Brechtel *et al.*, 2001b; Decombaz *et al.*, 2001; Larson-Meyer *et al.*, 2002), while direct measurement of IMTG content from skeletal muscle biopsies have been controversial (for review, see (Watt *et al.*, 2002b). The discrepancies reported in these studies may be explained by the methodological differences in measuring IMTG, limitations associated with the muscle biopsy technique, and the variability in IMTG content between trained and untrained individuals (Szczepaniak *et al.*, 1999). A major limitation when using the muscle biopsy procedure is the technical difficulty in separating intra- from extramuscular triglyceride (EMTG). Indeed, EMTG has been found to be highly variable in humans as measured by ^{1}H-MRS (Szczepaniak *et al.*, 1999). Training status may also be an important consideration for reducing the variability associated with IMTG measurement from muscle biopsies (Wendling *et al.*, 1996; Watt *et al.*, 2002a), as EMTG storage is lower in endurance trained compared to untrained individuals (Hoppeler, 1986; Szczepaniak *et al.*, 1999).

1.2.2. *Fat Metabolism During Post-Exercise Recovery*

In the fasted state, there is substantial FA mobilisation from adipose tissue following prolonged exercise (Mulla *et al.*, 2000), resulting in elevated rates of plasma-derived fat oxidation (Ahlborg & Felig, 1982; Krzentowski *et al.*, 1982; Bielinski *et al.*, 1985; Devlin *et al.*, 1989; Wolfe *et al.*, 1990; Tuominen *et al.*, 1996; Schrauwen *et al.*, 1997; Tuominen *et al.*, 1997; Kiens & Richter, 1998; Mulla *et al.*, 2000). Despite the large increase in circulating FAs after exercise, only a small fraction of this is taken up by exercised muscle in the lower extremities (Mulla *et al.*, 2000). Evidence suggests the predominant fate of the non-oxidised FAs derived from post-exercise lipolysis is reesterification in the liver, accounting for up to 90% of the FAs released during recovery (Wolfe *et al.*, 1990). Furthermore, exercise intensity is positively related to fat oxidation during recovery (Pritzlaff *et al.*, 2000), whilst FA uptake and

oxidation are enhanced during moderate-intensity exercise in a glycogen depleted state (Wojtaszewski *et al.*, 2003). These data suggest that the magnitude of muscle glycogen depletion during exercise influences the utilisation of LCFAs as an energy substrate (Blomstrand & Saltin, 1999).

During recovery from glycogen-depleting exercise in well-trained individuals, it has been demonstrated using the muscle biopsy technique that IMTG is also an important source of FAs for skeletal muscle oxidation (Kiens & Richter, 1998). Paradoxically, the reduction in IMTG concentration occurred during conditions of elevated glucose and insulin which are known to reduce hormone-sensitive lipase (HSL) activity (Watt *et al.*, 2004) and calculated IMTG oxidation during low intensity exercise (Coyle *et al.*, 1997). Studies using ^{1}H-MRS in endurance-trained subjects have failed to demonstrate a reduction in muscle triglyceride (TG) when a high-CHO diet is consumed during post-exercise recovery, reporting either unchanged (Decombaz *et al.*, 2000; Decombaz *et al.*, 2001) or increased (Larson-Meyer *et al.*, 2002) IMTG content. It should be noted that there are several differences between the biopsy and MRS studies which may result in different findings. First, IMTG is measured in soleus or tibialis anterior muscles (primarily Type I fibres) in the MRS studies, as opposed to the vastus lateralis (50% Type I / 50% Type II) in the biopsy studies. Second, the recruitment pattern is likely to be different in these muscles given the different modes of exercise (running, MRS; cycling, biopsy). Third, the timing of post-exercise sampling may be an issue, since an initial post-exercise IMTG measurement was not determined until 22 h of recovery using MRS (Larson-Meyer *et al.*, 2002) as opposed to a more detailed time course in the first 6 h of recovery using biopsies (Kiens & Richter, 1998). Therefore, further research is warranted to elucidate whether IMTG is a significant source of FAs contributing to post-exercise skeletal muscle fat metabolism.

The role of CHO ingestion on muscle TG resynthesis after prolonged exercise has also been examined. Following glycogen reducing exercise, IMTG concentration failed to return to baseline after 24 h of high CHO feeding (83% of total energy) (Starling *et al.*, 1997). Subsequent studies using ^{1}H-MRS in endurance-trained males (Van Loon *et al.*, 2003) and females (Larson-Meyer *et al.*, 2002) have also demonstrated that IMTG repletion is impaired when a typical CHO-rich athlete's diet consumed for 48 h (Van Loon *et al.*, 2003) and 70 h (Larson-Meyer *et al.*, 2002)

after prolonged exercise. These data suggest that a certain quantity of dietary fat is required to replenish IMTG after endurance exercise. Indeed, high-fat feeding (68% of total energy) was observed to increase IMTG stores and reduce muscle glycogen synthesis 24 h after prolonged cycling compared with a high-CHO diet (Starling *et al.*, 1997). More recent studies have also demonstrated that IMTG content increases in response to high-fat feeding after glycogen-lowering exercise (Decombaz *et al.*, 2000; Decombaz *et al.*, 2001; Larson-Meyer *et al.*, 2002) and that this effect is independent of training status (Decombaz *et al.*, 2001). In the absence of glycogen-lowering exercise, increases in IMTG stores are also observed during conditions of elevated plasma FAs with (Bachmann *et al.*, 2001; Brechtel *et al.*, 2001a) and without (Boden *et al.*, 2001) a hyperinsulinemic glucose clamp. Conversely, a 3 week low-fat diet (2% of total energy) significantly reduces IMTG concentration compared with an intake of 22% of energy from fat (Coyle *et al.*, 2001).

1.3. PATHWAY OF SKELETAL MUSCLE FAT OXIDATION

Fatty acid oxidation within skeletal muscle involves a complex sequence of events that include: 1) mobilisation from peripheral adipose tissue, 2) transport to the muscle, 3) uptake of plasma FAs by the muscle cell, 4) mobilisation from IMTG pools, 5) transport into the mitochondria, and 6) oxidation within the mitochondria. Some of these steps are also potential sites for the regulation of fat metabolism which will be discussed in section 1.4.

1.3.1. *Adipose Tissue Lipolysis and FFA Delivery to Muscle*

The mobilisation of FAs from adipose tissue is the first important step in the regulation of fat metabolism and overall energy homeostasis during rest and exercise. Lipolysis is mediated by the activation of the rate-limiting enzyme HSL in adipose tissue (Fredrikson *et al.*, 1981). Control of lipolysis is complex and occurs mainly by sympathetic nervous system activity, plasma catecholamine concentrations (norepinephrine and epinephrine) and plasma insulin levels. Stimulation of adrenergic receptors on the adipocyte membrane activates a cyclic AMP-dependent protein kinase A (PKA) and simulataneously phosphorylates HSL and the lipid-droplet associated protein, perilipin, resulting in increased HSL activity and the translocation of the enzyme from the cytosol to the surface of the lipid droplet. Recent evidence indicates that β-adrenergic stimulation may also activate HSL via extracellular signal-regulated kinases (ERK), one of the mitogen-activated protein

kinase (MAPK) pathways that transduce extracellular signals into intracellular responses (Greenberg *et al.*, 2001). Increased HSL activity alone catalyses the hydrolysis of triglycerides and diglycerides to yield two molecules of nonesterified FAs, whereas the participation of monoglyceride lipase (MGL) is required to liberate the remaining FA and a glycerol molecule (Figure 1.1). Insulin is the most important physiological inhibitor of catecholamine-induced lipolysis, inducing a decrease in cAMP levels and concomitant decrease in HSL activity. Indeed, ingestion of carbohydrate can be a powerful mediator of adipose tissue lipolysis at rest and during exercise. Small elevations in plasma insulin concentration (i.e., 10-30 μU/ml) dramatically reduce lipolysis at rest, although lipolytic rate still remains in excess of FA oxidation in basal conditions (Campbell *et al.*, 1992).

Studies using microdialysis in abdominal adipose tissue in man have shown antilipolytic α_2-adrenergic receptors to modulate lipolysis at rest (Arner *et al.*, 1990) and during exercise (Wolfe *et al.*, 1990), whereas β_1-adrenergic stimulatory effects are predominant only during exercise (Arner *et al.*, 1990). Following 1 h of exercise at 70% of $\dot{V}O_{2\,max}$, in vivo lipolytic sensitivity to epinephrine mediated β-adrenergic stimulation does not increase in endurance trained individuals (Klein *et al.*, 1995). However, triglyceride-FA cycling in the resting state is markedly increased in highly trained endurance athletes, providing FAs in excess of the rate of FA oxidation that may enhance the potential for increasing FA oxidation rapidly at the onset of exercise (Romijn *et al.*, 1993b).

The delivery of FA to skeletal muscle is a function of adipose tissue blood flow, the plasma FA concentration and muscle blood flow. Upon release into the blood stream, FA are quickly bound to serum albumin and transported primarily to skeletal muscle and liver tissue where they are oxidised or reesterified into a TG molecule (extracellular reesterification). At rest, non-oxidative lipolysis can account for ~70% of all nonesterified FA released from adipose tissue, resulting primarily from extracellular reesterification (Wolfe *et al.*, 1990). Delivery of plasma FA are closely matched to uptake at rest and during low intensity exercise, while FA levels increase progressively at a moderate exercise intensity (65% of $\dot{V}O_{2\,max}$) to maintain high plasma FA uptake and oxidation rates (Watt *et al.*, 2002a). During high intensity

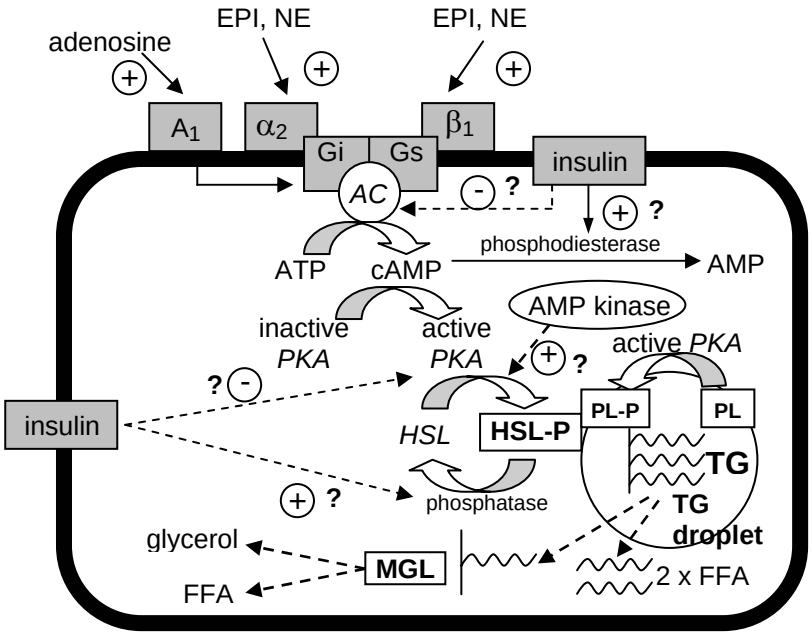

Figure 1.1. Schematic diagram outlining adipose tissue lipolysis. EPI, epinephrine; NE, norepinephrine; Gs and Gi, stimulatory and inhibitory G proteins; AC, adenylate cyclase; cAMP, cyclic AMP; PKA, protein kinase A; TG, triacylglycerol; HSL, hormone sensitive lipase; PL, perilipin; MGL, monoacylglycerol lipase; FFA, free fatty acid. Modified from Spriet (2002).

exercise (85% of $\dot{V}O_{2\,max}$), plasma FA concentration is markedly reduced despite the maintenance of lipolysis, suggesting that the decline in adipose tissue blood flow limits the availability of albumin for FA transport in the blood (Romijn *et al.*, 1993a).

1.3.2. *FA Transport Across the Muscle Cell Membrane*

Until recently, permeation of FAs across the plasma membrane was believed to occur via a simple process of passive diffusion down a concentration gradient because of the lipophilic nature of the FA molecule (Potter *et al.*, 1989). However, it is now recognised that at least part of the uptake of FAs may occur through a protein-mediated transport system that allows FA entry into the cell and movement within the cytosol for mitochondrial oxidation (Bonen *et al.*, 1998; Turcotte, 2000). Three proteins with potential roles in fat transport have been identified and cloned in skeletal muscle: the FA-binding protein in the plasma membrane ($FABP_{PM}$) (Calles-Escandon *et al.*, 1996), the FA translocase (FAT/CD36) and the FA transport protein (FATP-1) (Abumrad *et al.*, 1993). Consistent with the putative role of $FABP_{PM}$ and FAT/CD36 as FA transporters, palmitate transport rates into giant sarcolemmal vesicles appears to be a saturable process (Bonen *et al.*, 1998), and palmitate uptake into red and white muscles correlates well with the expression of $FABP_{PM}$ and FAT/CD36 (Luiken *et al.*, 1999). The role of FATP in fat transport is uncertain however as FATP content is inversely correlated with LCFA uptake (Luiken *et al.*, 1999).

Endurance training has been demonstrated to increase the content of $FABP_{PM}$ in human skeletal muscle (Kiens *et al.*, 1997), although equivocal findings have been observed in the rodent model (Turcotte *et al.*, 1999; Dyck *et al.*, 2000a). The expression of FAT/CD36 mRNA and protein is also enhanced when the oxidative capacity of skeletal muscle is increased by chronic electrical stimulation (Bonen *et al.*, 1999). Furthermore, muscle contraction enhances palmitate uptake by giant vesicles that occurs in parallel with increases in plasma membrane FAT/CD36 content (Bonen *et al.*, 2000). More interestingly however, the same study has demonstrated that the acute regulation of FA transport during muscle contractions is mediated by translocation of the FAT/CD36 protein from an intracellular pool to the muscle membrane (Bonen *et al.*, 2000), in a manner analogous to that reported for GLUT 4 stimulated glucose uptake. Therefore, exercise appears to be a powerful stimulus for promoting the intracellular redistribution of the FAT/CD36 transporter

and the rapid uptake of FAs from the plasma. Fat transporter activity and translocation is also under hormonal regulation. Insulin has recently been demonstrated to induce the translocation of FAT/CD36 to the plasma membrane (Luiken *et al.*, 2002), thus enhancing FA uptake into resting skeletal muscle (Dyck *et al.*, 2001; Luiken *et al.*, 2002). However, factors that regulate fat transporter activation/translocation remain unknown. One possible candidate includes AMP-activated protein kinase (AMPK) because it is associated with regulation of fat oxidation in resting and contracting skeletal muscle and has a role in controlling GLUT 4 translocation to the sarcolemma (Winder, 2001).

1.3.3. *Muscle Triacylglycerol Utilisation*

The IMTG content in human skeletal muscle varies greatly (Wendling *et al.*, 1996) and is relatively small compared with peripheral stores in adipose tissue. Normally skeletal muscle contains around 5–15 $mmol \cdot kg^{-1}$ wet weight of TG or a total store of 300–400g, located as small fat droplets within the cytoplasm of muscle cells in close proximity to the mitochondria (Boesch *et al.*, 1997; Hoppeler *et al.*, 1999). In contrast to the uniform distribution of glycogen in muscle fibers, IMTG stores are variable within individual muscle fibres, between different fibre types and between different muscle groups. Histochemical techniques using oil red O staining has revealed that type I fibers contain considerably more TG (2.8-fold) than type II fibers, and type IIa fibers contain more TG than type IIb fibers (Essen, 1977). Quantifying the volume density of fat droplets using morphometric analysis has also shown similar patterns of fat storage in muscle fibers (Howald *et al.*, 1985). Triglyceride stored between muscle fibers (EMTG) may also supply FFA for oxidation but little is known about the role of extra- versus intramuscular triacyglycerol stores. Recent ^{1}H-MRS technology has established that EMTG is a relatively stable TG pool in comparison with IMTG during exercise and post-exercise recovery (Larson-Meyer *et al.*, 2002).

Skeletal muscle contains three different types of triacylglycerol lipases (Oscai *et al.*, 1990). One is a lysosomal lipase with an *in vitro* pH optimum of 5. Another is a lipoprotein lipase (LPL) that was previously believed to control IMTG degradation because of the direct relationship between LPL activity and TG depletion in muscle. However, the presence of a neutral TG lipase, or the muscle version of HSL with a pH optimum of 7 has been identified in skeletal muscle (Holm *et al.*, 1987) that

appears to have an important role in muscle TG hydrolysis (Langfort *et al.*, 1999). HSL is covalently activated by kinase phosphorylation as previously described for adipose tissue (Figure 1.1). There are at least five known kinases that stimulate or inhibit HSL activity. Skeletal muscle HSL expression is correlated to fibre type, being higher in oxidative than in glycolytic fibres (Langfort *et al.*, 1999). By using neutralising antibodies it was further confirmed that HSL accounts for a significant fraction of the TG lipase activity in skeletal muscle fibres (Langfort *et al.*, 1999). HSL is also activated in parallel with glycogen phosphorylase in response to epinephrine, suggesting simultaneous activation of glycogen and TG breakdown (Langfort *et al.*, 1999). The mechanisms involved in the β-adrenergic–induced activation of HSL are likely to mimic those in adipose tissue as described above. Indeed, increased ERK phosphorylation has been demonstrated to coincide with HSL activity in response to epinephrine infusion in resting human skeletal muscle (Watt *et al.*, 2003c).

1.3.4. *FA Transport Across the Mitochondrial Membrane*

After being transported across the muscle cell membrane, FAs can either be esterified and incorporated into various lipid pools (Dyck & Bonen, 1998) or bound to a FA binding protein in the cytosol (FABPc) and shuttled to the mitochondrial membrane. Once LCFA have been activated to fatty acyl-CoA by acyl-CoA synthase, their transport into the mitochondria for subsequent oxidation is facilitated by the carnitine palmitoyltransferase (CPT) complex, consisting of CPT I, acylcarnitine translocase and CPT II. Fatty acyl-CoA is free to permeate the outer mitochondrial membrane, however a carrier is required for the passage through the inner membrane. Translocation of fatty acyl-CoA requires the addition of carnitine, achieved by the conversion of the acyl-CoA ester to acyl-carnitine by CPT I, located on the outer mitochondrial membrane. Upon translocation into the mitochondrial matrix, the enzyme CPT II facilitates the reconversion of acyl-carnitine into fatty acyl-CoA, which undergoes enzymatic cleavage in the process of β-oxidation. The release of carnitine from CPT II enables the exchange with acyl-carnitine across the inner mitochondrial membrane in a 1:1 ratio, a process controlled by the enzyme acyl carnitine translocase (Figure 1.2).

1.3.5. *Mitochondrial β-Oxidation*

Mitochondrial β-oxidation is initiated when acyl-CoA is oxidised to enoyl-CoA by a family of four closely related, chain length specific enzymes known as fatty acyl-CoA dehydrogenases (ACD). The next step involves hydration of enoyl-CoA to hydroxyacyl-CoA via enoyl-CoA hydratase, which is then oxidised to ketoacyl-CoA by the rate limiting enzyme beta-hydroxyacyl-CoA dehydrogenase (β-HAD). Studies have demonstrated that β-HAD is responsive to endurance training (Spina *et al.*, 1996) and to increases in FA availability induced by a fat-rich diet (Helge & Kiens, 1997). The third and final step in the β-oxidation pathway is the cleavage of ketoacyl-CoA by acetyl-CoA acyltransferase, yielding an acetyl-CoA and fatty acyl-CoA molecule. Acetyl-CoA immediately enters the tricarboxylic acid (TCA) cycle to ultimately generate ATP (Figure 1.2), whilst the fatty acyl-CoA repeats the β-oxidative pathway until the entire carbon chain has been cleaved.

1.4. REGULATION OF SKELETAL MUSCLE FAT AND CARBOHYDRATE METABOLISM

Many theories may account for the interaction between fat and CHO oxidation at rest and during exercise (Spriet & Watt, 2003), however substrate availability and the activity of key regulatory enzymes are believed to be the primary mechanisms. The reciprocal relationship between fat and CHO oxidation was first examined by (Randle *et al.*, 1963) using contracting rat heart and resting diaphragm muscles. The 'glucose-fatty acid cycle' was postulated to describe the down regulation of glucose oxidation due to the inhibitory effects of FAs on the enzymes phosphofructokinase (PFK), pyruvate dehydrogenase (PDH) and hexokinase (HK). The accelerated flux of FAs through β-oxidation has been proposed to increase the ratio of acetyl-CoA to CoA and inhibit PFK, whereas a TCA cycle mediated increase in citrate inhibits PDH. An increase in glucose-6-phosphate resulting from a reduction in PFK and PDH activities has been proposed to inhibit hexokinase activity and ultimately decrease the uptake of glucose presumably by decreasing the glucose gradient across the muscle cell membrane (Randle *et al.*, 1963).

Since the original *in vitro* glucose-fatty acid cycle was proposed, attempts at examining this theory in human skeletal muscle suggest that a reciprocal relationship between glucose and FA oxidation is evident but the mechanisms controlling this shift are clearly different. Several studies have utilised infusion of a triglyceride

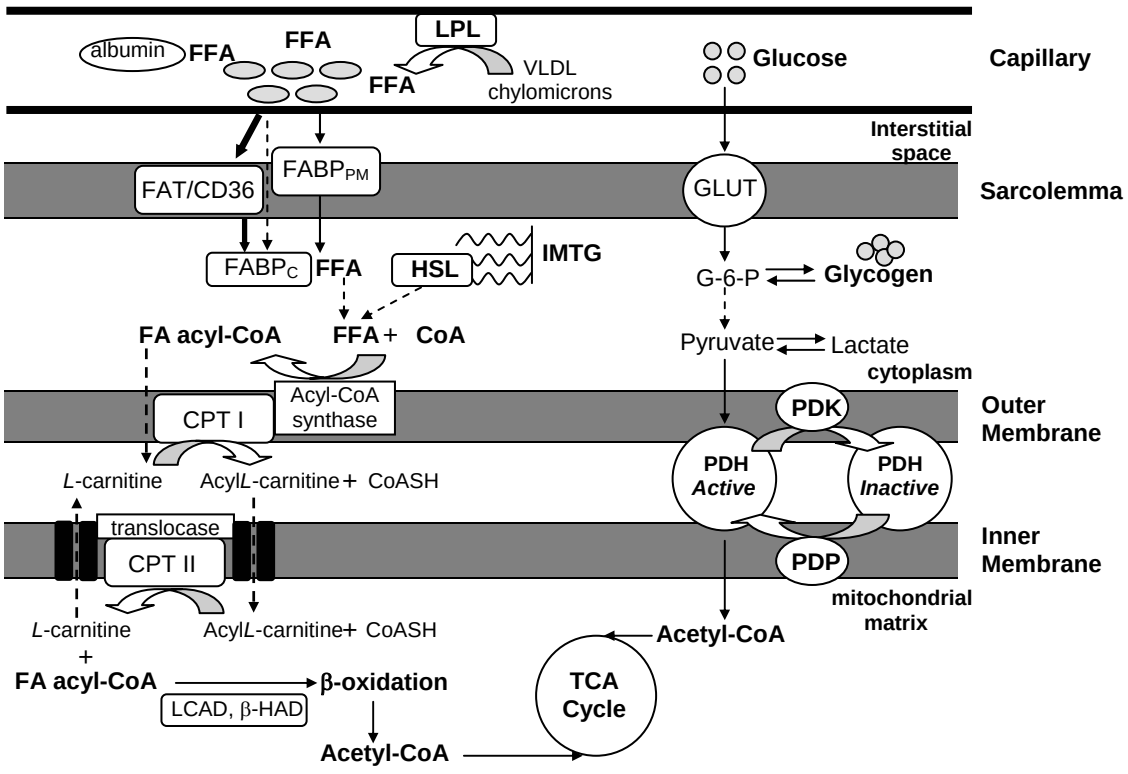

Figure 1.2. Schematic diagram of the fatty acid glucose metabolic pathways in skeletal muscle. FFA, free fatty acids; LPL, lipoprotein lipase; $FABP_{PM}$, plasma membrane fatty acid binding protein; FAT/CD36, fatty acid translocase; $FABP_C$, cytoplasmic fatty acid binding protein; FA acyl-CoA, fatty acid acyl-CoA; HSL, hormone sensitive lipase; IMTG, intramuscular triglyceride; G-6-P, glucose-6-phosphate; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; CPT I and II, carnitine palmitoyltransferase I and II; LCAD, long chain fatty acyl-CoA dehydrogenase; β-HAD, β-hydroxyacyl-CoA dehydrogenase.

emulsion (Intralipid) and heparin to acutely elevate arterial FA levels during exercise without additional substrate or hormonal changes that are associated with high-fat feeding. However these studies have yielded inconsistent results, demonstrating either no change in CHO or fat oxidation during prolonged low-intensity exercise (Ravussin *et al.*, 1986), or a significant increase in glycogen sparing and FA oxidation during prolonged moderate-intensity exercise (Vukovich *et al.*, 1993). Using moderate-intensity one-legged knee extensions, thigh glucose uptake is attenuated without a decrease in muscle glycogen utilisation, suggesting that FAs may directly inhibit glucose transport rather than the classic glucose-fatty acid cycle (Hargreaves *et al.*, 1991). During high-intensity exercise, Intralipid infusion increased total fat oxidation with a concomitant reduction in CHO oxidation due solely to a decrease in muscle glycogen utilisation (Romijn *et al.*, 1995). Furthermore, the significant sparing of muscle glycogen during high-intensity cycling with high circulating FAs appears to be regulated at the level of glycogen phosphorylase, as opposed to citrate and acetyl-CoA suggested in the original glucose-fatty acid cycle (Dyck *et al.*, 1993; Dyck *et al.*, 1996). In contrast, studies using glucose ingestion (Coyle *et al.*, 1997), a hyperinsulinemic, hyperglycaemic clamp (Sidossis *et al.*, 1996; Sidossis & Wolfe, 1996) and dextrose infusion (Sidossis *et al.*, 1999) provide evidence that glucose, rather than FA, controls the relative use of fat and CHO as energy substrates during rest and exercise. Indeed, the direct inhibition of FA oxidation by accelerated carbohydrate metabolism has led to the hypothesis that the glucose-fatty acid cycle is reversed in human skeletal muscle (Wolfe, 1998a). The regulation of skeletal muscle fat and CHO metabolism is clearly multifactorial, and different mechanisms may dominate during rest compared to exercising conditions. Most studies in resting humans support the concept that an increased availability of FAs suppresses the oxidation of intracellular or extracellular glucose or both (Baron *et al.*, 1989; Walker *et al.*, 1991; Yki-Jarvinen *et al.*, 1991). However, the underlying mechanisms are largely unknown and although there are indications that the Randle cycle is operative in resting human skeletal muscle, other plausible mechanistic links between FA availability and fat and CHO metabolism do exist.

The key sites of metabolic regulation for CHO and fat metabolism include the PDH complex and the CPT I system, respectively. The PDH reaction catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA, thus controlling the rate of

entry of pyruvate into the mitochondria (Figure 1.2). CPT I primarily governs fat oxidation by committing LCFA to mitochondrial β-oxidation. Competition exists between the two major substrates, glucose and FAs, and the choice substrate for oxidation is heavily dependent on the balance between the activities of the key enzymes PDH and CPT 1.

1.4.1. *Regulation of CPT I by Malonyl-CoA*

The CPT complex plays a pivotal role in the regulation of LCFA entry into the mitochondria for subsequent β-oxidation. Skeletal muscle CPT 1 is considered the rate limiting step in the oxidation of LCFA and is reversibly inhibited by malonyl-CoA, which is synthesised by the enzyme acetyl-CoA carboxylase (ACC) in the first committed step in FA biosynthesis (McGarry *et al.*, 1983). CPT I activity is very sensitive to malonyl-CoA concentration (McGarry *et al.*, 1983), and it is believed that malonyl-CoA could be the key regulator of fat oxidation in skeletal muscle during muscle contraction (Winder, 1998). Studies in rodent skeletal muscle show that malonyl-CoA levels are highest at rest to inhibit CPT I activity and maintain low rates of fat transport. During exercise, malonyl-CoA concentration decreases, relieving the inhibition of CPT I and allowing for enhanced LCFA oxidation (Winder *et al.*, 1989; Winder *et al.*, 1990). However, studies in human skeletal muscle have not observed a decrease in malonyl-CoA concentration in response to moderate-intensity exercise when there are large increases in the rate of fat oxidation (Odland *et al.*, 1996; Odland *et al.*, 1998; Dean *et al.*, 2000). Furthermore, a malonyl-CoA resistant CPT I subfraction has been detected in rodent skeletal muscle (Kim *et al.*, 2002), suggesting that malonyl-CoA is not the sole regulator of CPT I activity at rest or during exercise. Indeed, CPT I is also inhibited by *in vitro* decreases in pH that may explain the decrease in fat metabolism in the shift from moderate to high intensity exercise (Starritt *et al.*, 2000). Taken together, these findings suggest that the regulation of CPT I activity is more complex than simple changes in malonyl-CoA levels and/or that malonyl-CoA inhibition is overridden during exercise (Bezaire *et al.*, 2004)

Acutely increasing plasma FFA provision during moderate-intensity exercise does reduce malonyl-CoA levels in human skeletal muscle however, and along with the decline in PDH activity, may explain the enhanced lipid oxidation in the presence of elevated FFA availability (Odland *et al.*, 2000). Significant increases in malonyl-

CoA concentration have also been observed following a resting euglycemic (Bavenholm *et al.*, 2000) and hyperglycaemic (Rasmussen *et al.*, 2002) clamp with hyperinsulinemia in human skeletal muscle. These data suggest that in the presence of high glucose and insulin, elevated malonyl-CoA appears to decrease muscle FA oxidation, inhibit functional CPT I activity and shunt LCFA away from oxidation and towards storage (Rasmussen *et al.*, 2002). Furthermore, malonyl-CoA is reduced in response to Intralipid infusion at rest (Odland *et al.*, 2000) and remains depressed for up to 90 min after a single bout of exercise in rats which has been postulated to allow for increased fat utilisation and a partitioning of glucose towards muscle glycogen resynthesis (Rasmussen *et al.*, 1998). Therefore, while it appears that factors other than changes in malonyl-CoA concentration are involved in regulating the shift in fuel utilisation during exercise, evidence suggests that malonyl-CoA has an important role in regulating FA oxidation in resting skeletal muscle (Dean *et al.*, 2000).

1.4.2. *AMP Activated Protein Kinase and Regulation of Malonyl-CoA Concentration*

Regulation of malonyl-CoA concentration and potentially skeletal muscle fat metabolism is likely to be mediated by the metabolic fuel sensor or "fuel gauge" 5'AMP-activated protein kinase (AMPK) (Hardie & Hawley, 2001). AMPK is a heterotrimeric protein kinase, consisting of one catalytic subunit (α) and two regulatory subunits (β and γ) (Hardie *et al.*, 1998; Kemp *et al.*, 1999). Two catalytic subunits have been identified (α1 and α2), of which α1 is the most abundant and widely distributed, whilst α2 is predominant in skeletal muscle, heart and liver tissue (Stapleton *et al.*, 1996). AMPK activity is upregulated in part by increases in the allosteric activator AMP and decreases in the allosteric inhibitor phosphocreatine. The primary regulator of AMPK is an upstream kinase, AMP-activated protein kinase kinase (AMPKK), that phosphorylates a threonine residue (Thr-172) of the α-subunit resulting in activation (Hardie & Hawley, 2001). An increase in AMP that occurs during cellular stress such as exercise also promotes AMPKK phosphorylation both by binding to dephospho-AMPK and enhancing it as a substrate, and by activating AMPKK itself (Hawley *et al.*, 1995).

An increasing body of evidence suggests that activation of the catalytic subunit (α) of AMPK (AMPKα) increases FA oxidation by lowering the concentration of

malonyl-CoA (Ruderman *et al.*, 2003). AMPK is postulated to decrease malonyl-CoA levels through two mechanisms: 1) phosphorylating and therefore inactivating ACC on serine residue 221 (ser^{221}) (Park *et al.*, 2002b), 2) phosphorylating and activating malonyl-CoA decarboxylase (MCD), an enzyme involved in the degradation of malonyl-CoA (Park *et al.*, 2002a). ACC catalyses the carboxylation of acetyl-CoA to form malonyl-CoA in the first committed step in FA biosynthesis. There are two major isoforms of ACC (ACCα and ACCβ) that are encoded by separate genes and expressed in mammalian tissues. ACCα is highly expressed in non-oxidative tissues and generates malonyl-CoA primarily for FA synthesis, whereas ACCβ is the predominant isoform expressed in the heart, skeletal muscle and liver that produces malonyl-CoA primarily for the regulation of CPT I activity (McGarry & Brown, 1997). Consistent with its role in the regulation of mitochondrial LCFA transport and oxidation, ACCβ is located in close proximity to CPT I on the outer mitochondrial membrane in skeletal muscle (Abu-Elheiga *et al.*, 2000). In contrast, MCD is an enzyme that decreases the concentration of malonyl-CoA by catalysing the conversion of malonyl-CoA to acetyl-CoA in a wide variety of rodent tissues (Voilley *et al.*, 1999). Although the metabolic role of this reaction has not been fully defined in mammalian cells, two isoforms of MCD have been identified from a single gene in the goose uropygial gland (Courchesne-Smith *et al.*, 1992). The longer of the two proteins is ubiquitously expressed and targets the mitochondrial matrix, whereas the shorter form has a more restricted pattern of expression as a cytosolic enzyme. In rat cardiac muscle, a 50-kDa MCD isoform is expressed that has a putative role in regulating fatty acid oxidation rates (Dyck *et al.*, 1998), while increased MCD activity appears to account for the disposal of malonyl-CoA in oxidative skeletal muscle (Alam & Saggerson, 1998).

The dual control of skeletal muscle malonyl-CoA by MCD and ACC, via AMPK, is increasingly being recognised as having a major role in regulating fat metabolism (Saha *et al.*, 2000; Park *et al.*, 2002a). AMPK activation by 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR) decreases ACC activity (Merrill *et al.*, 1997) and activates MCD in skeletal muscle (Saha *et al.*, 2000), suggesting that MCD and ACC may be jointly regulated by AMPK (Figure 1.3). Indeed, an increase in MCD and decrease in ACC activities are coordinately regulated by AMPK activation in response to exercise and AICAR treatment (Park *et al.*, 2002a). Since AICAR induced activation of AMPK decreases malonyl-CoA in skeletal muscle (Merrill *et*

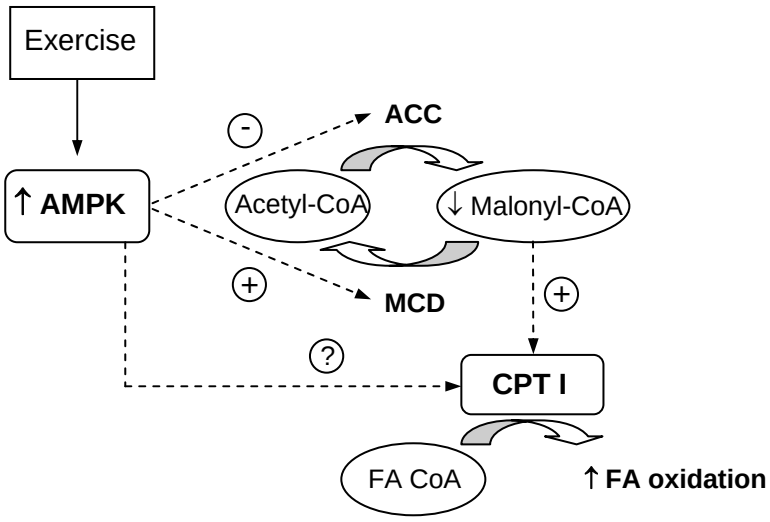

Figure 1.3. A proposed model of how AMPK activation causes changes in ACC and MCD activity that lower the concentration of malonyl-CoA in skeletal muscle. AMPK, 5'AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase, CPT I, carnitine palmitoyltransferase I; FA CoA, fatty acid CoA. Modified from Park et al. (2002).

al., 1997; Kaushik et al., 2001), such coordinated changes in MCD and ACC attributable to AMPK are suggested to lower malonyl-CoA concentration and result in an increased oxidation and decreased synthesis of FAs (Park *et al.,* 2002; Ruderman *et al.,* 2003). Not all studies demonstrate that MCD is a substrate for AMPK however, suggesting instead that the principal mechanism by AMPK regulates malonyl-CoA content is through its regulation of ACC (Habinowski *et al.*, 2001). Furthermore, activation of MCD may also be regulated by increased protein content (Dyck *et al.*, 2000b; Sakamoto *et al.*, 2000), or by the existence of a separate protein or allosteric regulator (Dyck *et al.*, 2000b; Young *et al.*, 2001), although the exact nature of these regulatory mechanisms remain unknown. Gene expression may also be an important determinant of MCD activity, since MCD mRNA is most abundant in human cardiac and skeletal muscles, tissues in which cytoplasmic malonyl-CoA is a potent inhibitor of CPT I and, thus, of mitochondrial LCFA oxidation (Sacksteder *et al.*, 1999).

1.4.3. *Activity of the Pyruvate Dehydrogenase (PDH) Complex*

Carbohydrate oxidation is primarily regulated by PDH, a multi-enzyme complex which catalyses the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA. This reaction controls the flux of pyruvate across the mitochondrial membrane and through the tricarboxylic acid (TCA) cycle. The complex consists of three subunits: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) and is tightly controlled by two specific regulatory enzymes, PDH kinase (PDK) and PDH phosphatase (PDP). PDK phosphorylates and inactivates the E1α-subunit of the PDH complex, whereas dephosphorylation by PDP restores the enzyme activity (Figure 1.2). PDK activity is highly regulated by the intramitochondrial effectors ATP, acetyl-CoA and NADH, and is inhibited by elevated pyruvate concentrations, whereas PDP activity is Mg^{2+} dependent and is stimulated by Ca^{2+} and increased pyruvate (Cooper *et al.*, 1975; Howlett *et al.*, 1998).

Four PDK isoforms (1-4) have been identified in human tissues that phosphorylate and inactivate the PDH complex. Each isoenzyme has unique regulatory properties that vary in their level of expression in different cell types. PDK1 is expressed predominately in heart tissue whilst PDK2 is ubiquitously expressed and most abundant in heart and skeletal muscle (Gudi *et al.*, 1995). In contrast, PDK3 is found

almost exclusively in heart and skeletal muscle in low amounts (Gudi *et al.*, 1995). PDK4 is primarily expressed in heart and skeletal muscle (Rowles *et al.*, 1996) and is fibre type dependent, as a higher content has been observed in oxidative muscle (type I and IIa) compared to fast twitch glycolytic muscle (type IIb) (Peters *et al.*, 2001a). PDK expression and activity in several oxidative tissues is increased in response to nutritional and endocrine manipulations that increase fat availability and oxidation (Sugden & Holness, 2003). In particular, PDK4 is modified when there is a sustained change in tissue FA delivery and/or handling and is hypothesised to be a "lipid status"-responsive PDK isoform (Sugden *et al.*, 2001). During conditions of reduced glycolytic flux and increased FA oxidation, PDK activity is elevated in resting human skeletal muscle (Peters *et al.*, 1998; Peters *et al.*, 2001b). PDK4 expression is induced in skeletal muscle during fasting (Wu *et al.*, 1999; Holness *et al.*, 2002; Spriet *et al.*, 2004), high fat feeding (Peters *et al.*, 1998; Holness *et al.*, 2000) and streptozotocin-induced diabetes in rats (Wu *et al.*, 1999) and correlates with IMTG concentration in obese patients before and after weight loss (Rosa *et al.*, 2003). Furthermore, the progressive increase in PDK4 mRNA during prolonged fasting in humans has been associated with a decline in PDH activity (PDHa) (Spriet *et al.*, 2004). Hence, one long-term mechanism of PDK regulation in response to altered fat delivery and oxidation involves changes in the relative expression of PDK4 and this may relate to changes in PDHa.

1.4.4. *Regulation of IMTG Hydrolysis*

Evidence from the dual-label "pulse-chase" technique in isolated rodent skeletal muscle has demonstrated that the IMTG pool is a dynamic substrate, undergoing simultaneous esterification and hydrolysis at rest and during tetanic contractions (Dyck & Bonen, 1998). In isolated rat skeletal muscle, the activity of the neutral TG lipase HSL appears to be under dual control by epinephrine (Langfort *et al.*, 1999) and contraction-related mechanisms (Langfort *et al.*, 2000). Epinephrine has also been demonstrated to induce IMTG hydrolysis in exercising human skeletal muscle (Cleroux *et al.*, 1989; Kjaer *et al.*, 2000). During prolonged exercise to exhaustion, nonselective β-adrenergic blockade completely inhibited IMTG degradation compared to placebo treatment (Cleroux *et al.*, 1989). In addition, HSL activation was undetected in adrenalectomised individuals during moderate and high intensity cycling, however when epinephrine was infused to mimic control levels, HSL activity increased at both moderate and high power outputs (Kjaer *et al.*, 2000).

Furthermore, epinephrine infusion and contraction appear to have an additive effect on HSL activation during exercise (Watt *et al.*, 2003c). However, at the onset of exercise, HSL activity is independent of epinephrine levels at power outputs corresponding to 30, 60 and 90% of $\dot{V}O_{2\,peak}$, suggesting that HSL activation is regulated by factors related to the onset of exercise such as intracellular Ca^{2+} levels (Watt *et al.*, 2003b) and increased ERK phosphorylation (Watt *et al.*, 2003c). Moreover, given the apparent mismatch between activation of HSL early in exercise and the negligible IMTG and fat oxidation, it is postulated that HSL activation may not be the only rate-limiting factor regulating IMTG hydrolysis and that other postactivational factors (i.e. HSL translocation and perilipin phosphorylation) may also mediate IMTG degradation in skeletal muscle (Watt *et al.*, 2003b).

HSL activity is also thought to be allosterically inhibited by long chain fatty acyl CoA (LCFA CoA) (Jepson & Yeaman, 1992). One inviting hypothesis is that LCFA-CoA inhibits HSL activity after binding to a specific site on the enzyme, thus preventing further mobilisation of FFAs from the IMTG pool. In support of this theory, LCFA-CoA accumulation has been observed during 2 h of moderate-intensity exercise which may account for the decrease in HSL activity (Watt *et al.*, 2003a) and the reduction in IMTG utilisation previously reported late in prolonged exercise (Watt *et al.*, 2002a). Clearly, the regulation of skeletal muscle HSL during exercise is complex and is thought to involve both β-adrenergic and perhaps more importantly AMPK-mediated mechanisms (Watt *et al.*, 2004c). In circumstances where AMPK is elevated, net IMTG degradation is measurable despite reduced HSL activity. Therefore, the physiological importance of activating HSL for triacylglycerol hydrolysis is uncertain (Watt *et al.*, 2004c) and requires further investigation during exercise and the immediate recovery period.

1.5. METABOLIC GENE EXPRESSION IN SKELETAL MUSCLE

Control of substrate metabolism in response to sustained alterations in substrate availability and exercise training is influenced by changes in gene expression that encode for key enzymatic, regulatory and myofibrillar proteins. Alterations in gene expression are responsible for both morphologic and phenotypic changes and importantly are indicative of cellular responses to environmental stimuli and perturbations such as stress and growth factors, energy availability and contractile activity (Lockhart & Winzeler, 2000).

Metabolic protein synthesis is initiated by the transcription of genomic DNA resulting in the transient increase of messenger ribonucleic acid (mRNA). Increases in muscle mRNA levels reflect the balance between rates of production and the decay of mRNA or its chemical half life, and thus are most likely to result from transcriptional activation and/or increased mRNA stability (Hargreaves & Cameron-Smith, 2002). The half-life of many mRNAs can fluctuate in response to environmental stimuli such as hormones, temperature shifts and substrate availability (Day & Tuite, 1998). Similarly, the rate of translation and the rate of degradation will determine the amount of functional protein at any one time, although the half-life of a given protein is typically longer than the half-life of its corresponding mRNA. Therefore, transcriptional rather than translational control of mRNA may result in measurable changes in protein synthesis and content (Williams & Neufer, 1996).

The rate of gene transcription into mRNA is controlled by multiple regulatory pathways that precede the initiation of transcription by RNA polymerase. Genes consist of coding regions for the synthesis of proteins as well as non-coding or promoter regions that contain binding sites for transcription factors which are critical to the regulation of gene expression. Transcription factors are proteins which upon activation, move from the cytosol to the nucleus and bind to enhancer and repressor DNA sites, resulting in increased and decreased transcriptional activity, respectively (Baar *et al.*, 1999). Activation of appropriate transcription factors is the culmination of a signaling pathway that can be initiated by changes in nutrient availability (Young *et al.*, 2001; Muoio *et al.*, 2002b) and exercise (Pilegaard *et al.*, 2003). Once transcription factors are bound to form a stable transcriptional complex, RNA polymerase initiates the continuous polymerization of ribonucleotide precursors into RNA (Jiang *et al.*, 1996). In addition to the regulation of gene transcription rates, post-transcriptional control mechanisms also influence the change in protein content. These regulatory factors include nuclear export of mRNA, localisation and stability within the cytosol and translational regulation and degradation. Post-translational modifications or proteolytic clevage may also alter protein function and activity. Although these pre-translational, translational and post-translational events are all involved in the molecular regulation of skeletal muscle metabolism, activation of gene expression is still an integral part of the adaptive process.

1.5.1. *Metabolic Gene Expression During Post-Exercise Recovery*

The cellular and molecular mechanisms facilitating the increase in skeletal muscle fat oxidation following prolonged exercise remain poorly defined. Increases in metabolic gene transcription relevant to carbohydrate and fat oxidation are well documented during exercise and appear to increase further during the post-exercise recovery period (Kraniou *et al.*, 2000; Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). For many genes, transcriptional activity peaks during the initial recovery phase after endurance exercise and returns to basal levels within a ~24 h period (Pilegaard *et al.*, 2000). Indeed, transcriptional activation of key regulatory proteins increase by three- to sevenfold after 1–2 h of post-exercise recovery, followed by increases in mRNA that peak at 2–4 h after exercise (Pilegaard *et al.*, 2000). These findings suggest that the cumulative effect of transient increases in the transcriptional activity of metabolic genes may be important in the cellular adaptation to repeated bouts of exercise (Williams & Neufer, 1996).

The role of gene expression in regulating fat metabolism in the post-exercise recovery period has received little attention. Gene expression most integral to FFA uptake include $FABP_{PM}$ and FAT/CD36, while those relevant to oxidation are CPT I and the key enzyme of β-oxidation, β-hydroxyacyl-CoA dehydrogenase (β-HAD) (Tunstall *et al.*, 2002). In response to a single bout of exercise following 9 days of aerobic training, the expression of genes involved in FFA uptake and oxidation ($FABP_{PM}$, FAT/CD36, CPT 1, β-HAD) were unchanged at the cessation of exercise and at 3h post-exercise (Tunstall *et al.*, 2002). However, the increased capacity for lipid oxidation after exercise training was accompanied by an increased mRNA abundance of FAT/CD36 and CPT 1 and FAT/CD36 protein expression. Therefore, it appears that increases in total lipid oxidation in response to short-term endurance training are associated with the genes involved in regulating FFA uptake across the plasma (FAT/CD36) and mitochondrial membrane (CPT 1) (Tunstall *et al.*, 2002). Similarly, 5 days of knee extensor training also increases CPT I mRNA abundance at rest, whilst more dramatic increases in LPL and CPT I gene transcription and expression were observed during a 4 h post-exercise recovery period (Pilegaard *et al.*, 2000).

During recovery from endurance exercise a number of changes occur in skeletal muscle that allow for a high metabolic priority towards glycogen resynthesis (Richter

et al., 2001). Insulin stimulated glucose uptake and glycogen synthase activity are increased during post-exercise recovery in human skeletal muscle (Mikines *et al.*, 1988; Wojtaszewski *et al.*, 2000) and this appears to be influenced by the extent of muscle glycogen depletion (Nielsen *et al.*, 2001). Furthermore, skeletal muscle insulin sensitivity is increased at least 48 h after a bout of exercise (Mikines *et al.*, 1988), leading to a rapid rate of glycogen resynthesis when high-carbohydrate foods are consumed following glycogen-depleting exercise (Kiens & Richter, 1998). Consistent with these findings, reduced muscle glycogen is associated with greater PDK4 transcription and mRNA levels after prolonged exercise (Pilegaard *et al.*, 2002). PDK4 is a highly exercise-responsive gene in human skeletal muscle (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). Therefore, the possibility exists that signaling mechanisms sensitive to muscle glycogen content may be linked to the transcriptional control of exercise-responsive genes (Pilegaard *et al.*, 2002). Further studies are required to delineate the role of exercise in mediating the expression of genes pertinent to the regulation of fat metabolism.

1.5.2. *Effect of Substrate Availability on Metabolic Gene Expression*

Sustained utilisation of FAs as a primary energy substrate for skeletal muscle can be induced by lipid infusion, dietary manipulation and fasting. Increasing circulating FAs *in vivo* using Intralipid infusion has frequently been utilised to investigate the effect of FAs on hepatic and adipose tissue gene expression, however relatively few have examined this effect in skeletal muscle. Intralipid infusion is reported to increase the gene expression of FAT/CD36 in rat skeletal muscle (Fabris *et al.*, 2001; Vettor *et al.*, 2002) and in human adipose tissue (Nisoli *et al.*, 2000). Furthermore, skeletal muscle mRNA abundance of GLUT4 is decreased in the rat, whilst PDK4 mRNA is markedly increased (~10-fold) following a continuous infusion of triglycerides and heparin in human skeletal muscle (Tunstall, 2003, unpublished observations).

Increases in the mRNA abundance of selected fat and CHO metabolic genes are also observed after consuming high fat in comparison to high CHO diets (Young *et al.*, 2001; Cameron-Smith *et al.*, 2003). Following 5 days of high-fat feeding (>65% of energy as lipids), the gene expression of FAT/CD36, β-HAD and PDK4 and the protein concentration of FAT/CD36 were markedly higher in comparison to 5 days of a high-CHO intake (70-75% of energy as CHO) in endurance cyclists (Cameron-

Smith *et al.*, 2003). In response to a similar dietary intervention of shorter duration (2 days), the mRNA abundance of selected genes regulating CHO metabolism were increased (GLUT4 and glycogenin) and decreased (PDK4) in response to a high-CHO intake, whereas only FAT/CD36 mRNA increased after high-fat feeding (Arkinstall *et al.*, 2004). Thus, it appears that CHO-related genes are more responsive to short-term dietary manipulation than those involved in the oxidation of FAs (Arkinstall *et al.*, 2004). These findings support previous studies utilising high fat feeding (Peters *et al.*, 2001b), fasting (Spriet *et al.*, 2004) and lipid infusion (Tunstall, 2003, unpublished observations) in humans that PDK4 gene expression is highly sensitive to changes in plasma FA availability. The increase in PDK4 gene expression during prolonged fasting also reduces PDHa, thus PDK4 maybe an important factor mediating the starvation induced decrease in CHO oxidation (Spriet *et al.*, 2004). Furthermore, elevated plasma FA levels in response to fasting, diabetes and high-fat feeding are associated with increased MCD mRNA in rat skeletal muscle, suggesting that FAs are involved in the regulation of MCD gene expression and possibly MCD activity, although posttranscriptional mechanisms regulating MCD activity appear to exist (Young *et al.*, 2001). However, it remains unknown whether FA availability also influences MCD mRNA abundance and MCD activity during post-exercise recovery in human skeletal muscle.

1.6. FATTY ACID REGULATION OF SKELETAL MUSCLE GENE EXPRESSION

In addition to providing an energy source and its effects on membrane lipid composition, FAs can regulate gene expression leading to changes in metabolism, growth and cell differentiation (Jump & Clarke, 1999). Once in the cell, FAs can undergo β-oxidation within the mitochondria or be elongated, desaturated, peroxidised, exchanged with membrane phospholipids and participate or interfere with eicosanoid synthesis (Duplus *et al.*, 2000). Therefore, it is not only FAs *per se* but also products of FA metabolism that can act as signaling molecules in regulating gene expression. The majority of genes targeted by FAs encode proteins with roles in FA transport or metabolism. It is well known that polyunsaturated FAs (PUFAs), in particular n-3 unsaturated FAs, rapidly suppress hepatic *de novo* lipogenesis and triglyceride synthesis/secretion while inducing peroxisomal and microsomal FA oxidation (Jump & Clarke, 1999). These effects on fat metabolism are mediated by changes in gene expression leading to induction or suppression of mRNAs encoding for key metabolic enzymes (Jump & Clarke, 1999).

A number of postulated mechanisms can account for the FA regulation of gene transcription, as summarised in Figure 1.4. FAs, FA-CoAs or FA metabolites can initiate a signal transduction cascade to induce covalent modification of a transcription factor, thereby modifying its transcriptional activity. Alternatively, FAs or their derivatives may act as ligands for a transcription factor, which can then bind directly to DNA at a FA response element and activate or repress transcription. FAs can also act indirectly by modifying transcription factor mRNA stability or gene transcription, resulting in variations of *de novo* transcription factor synthesis that impacts on the transcription rate of proteins involved in FA transport or metabolism. Furthermore, transcription factors can bind to the cognate response element in isolation as a monomer or together with the same (homodimer) or different (heterodimer) transcription factor (Duplus *et al.*, 2000).

1.6.1. *Fatty Acid–Responsive Transcription Factors*

Specific FA-regulated transcription factors that have been identified in mammalian cells include peroxisome-proliferator-activated receptor (PPAR) family, nuclear factor κB (NFκB), sterol regulatory element-binding protein-1c (SCREBP1-c), retinoid X receptors (RXRα, β and γ), liver X receptors (LXRα and β) (Duplus *et al.*, 2000) and more recently in skeletal muscle forkhead homolog in rhabdomyosarcoma (FKHR) (Kamei *et al.*, 2003). Since the initial identification of PPARα (Issemann & Green, 1990), several PPAR family members have been cloned. These include PPAR gamma (PPARγ1 and γ2) and PPAR delta (PPARδ) (Clarke *et al.*, 1999). Each isoform is encoded by a separate gene and display unique tissue distribution patterns. PPARα is expressed most abundantly in highly oxidative tissues where it plays a key role in activating pathways of β-oxidation (Braissant *et al.*, 1996). Conversely, PPARγ is expressed primarily in adipose tissue and thus promotes adipocyte differentiation and activates transcription of genes involved in lipid storage (Braissant *et al.*, 1996). PPARδ is ubiquitiously expressed and its function is unclear, however it appears to have a role in regulating cholesterol metabolism (Chawla *et al.*, 2003).

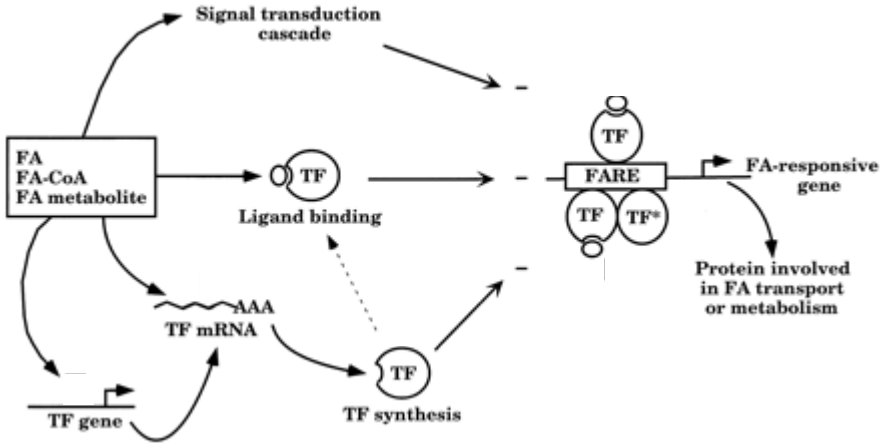

Figure 1.4. Postulated mechanisms for fatty acid control of gene transcription. TF, transcription factor; FARE, fatty acid response element; TF*, different transcription factor. Modified from Duplus et al. (2000).

PPARα is considered the major subtype that mediates FA-induced activation of fat oxidative genes, since PPARα knockout mice exhibit decreased fat oxidative gene expression together with low rates of β-oxidation and FA accumulation in both cardiac and hepatic tissues during fasting (Leone *et al.*, 1999). In primary cultured human skeletal muscle cells, specific activation of PPARα increases the gene expression of CPT I, MCD and PDK4 along with enhancing FA oxidation (Muoio *et al.*, 2002b). PDK4 mRNA abundance, PDK4 protein and PDK4 activity are also increased with PPARα activation in rodent skeletal muscle (Wu *et al.*, 1999). Furthermore, treatment with a PPARα agonist increases skeletal and cardiac muscle MCD mRNA and activity, while specific PPAR gamma (PPARγ) activation decreases muscle MCD gene expression (Young *et al.*, 2001). The same study demonstrated that FAs increased the expression of MCD mRNA, suggesting that FA-induced MCD expression is mediated through PPARα in rodent skeletal and cardiac muscle (Young *et al.*, 2001; Campbell *et al.*, 2002). Whether the same FA-induced regulation of MCD gene expression exists in human skeletal muscle is unknown. However, other studies demonstrate that the role of PPARs in regulating FA-induced gene expression is more complex as there may be no obligatory participation of PPARα in the upregulation of PDK4 gene expression during fasting (Holness *et al.*, 2002; Muoio *et al.*, 2002a; Spriet *et al.*, 2004) and a PPARδ compensatory mechanism may exist in PPARα knockout mice (Muoio *et al.*, 2002a).

1.7. SUMMARY

Skeletal muscle is the predominant tissue regulating whole body fat oxidation at rest and in the post-exercise recovery period. After prolonged or glycogen-lowering exercise plasma FAs increase in response to the substantial FFA mobilisation from adipose tissue to support the elevated rate of fat oxidation. Some evidence also suggests that IMTGs are an important fuel source during post-exercise recovery in the presence of elevated glucose and insulin, however the use of IMTG as an energy substrate after exercise requires further investigation. Many studies also suggest that glucose uptake after strenuous exercise is partitioned away from oxidation and towards storage in skeletal muscle, reflecting the high metabolic priority of glycogen resynthesis during post-exercise recovery. However, the underlying mechanisms accounting for the interaction between fat and CHO oxidation after exercise remain poorly defined. An increasing body of evidence suggests that AMPK activation

increases post-exercise skeletal muscle FA oxidation by lowering the concentration of malonyl-CoA and thus allowing for enhanced CPT I mediated LCFA transport into the mitochondria. AMPK is postulated to decrease malonyl-CoA levels through inactivating ACC and increasing MCD activity in rodent skeletal muscle, although it is unknown whether this mechanism is also operative in human skeletal muscle after exercise. Exercise increases transient changes in metabolic gene expression during the post-exercise recovery period which may be important in the cellular adaptation to repeated bouts of exercise. Numerous studies in rodent skeletal muscle as well as investigations conducted in human adipose and hepatic tissue indicate substrate availability may also be an important mediator of oxidative gene expression at rest. However it remains to be determined if altering FFA and glucose availability during post-exercise recovery can induce the same effect.

The broad aim of this thesis was to examine the regulation of fat and CHO metabolism during recovery from glycogen-lowering exercise in the presence of altered fat and glucose availability. This series of studies examine alterations in metabolic protein activity, content and gene expression in human skeletal muscle in response to a strenuous exercise stimulus followed by manipulation of substrate availability.

1.8. AIMS

Specifically the purpose of this thesis was to:

1. Examine the utilisation of IMTG as an energy substrate and provide a more in-depth understanding of the metabolic changes which occur in skeletal muscle after exhaustive exercise when combined with high-CHO feeding to replenish muscle glycogen.

2. Determine the effect of acute alterations in circulating plasma FFA availability on malonyl-CoA concentration and whole-body fat and CHO oxidation during post-exercise recovery.

3. Determine whether changes in whole body fat and CHO oxidation are related to post-translational modifications in AMPKα and ACCβ phosphorylation during post-exercise recovery.

4. Determine the effect of acute alterations in circulating plasma FFA and glucose availability on IMTG concentration during post-exercise recovery.

5. Determine whether acute changes in circulating plasma FFA and glucose availability alters the mRNA expression of key proteins involved in the regulation of fat and CHO oxidation during post-exercise recovery.

CHAPTER TWO
GENERAL METHODS

This chapter provides a detailed description of the methods and procedures that were used in the studies presented in this thesis. Experimental protocols specific to a study are described in the methods section of the relevant chapter. Throughout this thesis, each study is referred to numerically as outlined below:

Study I *Skeletal muscle fat and carbohydrate metabolism during recovery from glycogen-depleting exercise in humans*

Study II *Regulation of skeletal muscle fat metabolism after exercise in humans: influence of fat availability*

Study III *Effect of glucose availability on the regulation of fat metabolism in human skeletal muscle during post-exercise recovery*

Study I was conducted in the Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada under the guidance of Dr. David Dyck, Professor Lawrence Spriet and Professor George Heigenhauser. Studies II and III were conducted at the School of Exercise and Nutrition Sciences, Deakin University, Burwood, Australia. Malonyl-CoA analysis for Study II was completed by Dr. Asish Saha and Professor Neil Ruderman at the Diabetes & Metabolism Research Unit, Boston Medical Centre, USA.

2.1. SUBJECTS

Individuals who volunteered as subjects for the above studies were endurance trained males aged between 18 and 36 years. Volunteers were eligible to participate if they achieved a $\dot{V}O_{2\,max}$ greater than 55 $ml \cdot kg^{-1} \cdot min^{-1}$ and had a history of endurance training and/or racing for at least the previous 3 months. A total of twenty subjects were recruited for all studies from a variety of competitive backgrounds including road cycling (4), mountain biking (4), swimming (3), triathlon (2), running (2) canoeing (1), rowing (1), whilst three were recreationally active. All subjects were

considered to be healthy, did not smoke or take any medications, and had no evidence of cardiovascular or metabolic diseases. The experimental procedures, possible risks and benefits were fully explained to each subject before their written, informed consent was obtained. All experiments were approved by the ethics committees of each institution (Study I: University of Guelph; Studies II and III: Deakin University), and were performed according to the Declaration of Helsinki.

2.2. PROCEDURES

2.2.1. *Dietary Analysis*

Daily energy intake and composition of each subject's habital food and fluid intake was assessed using a 4-day food record (3 weekdays and 1 weekend day). Dietary data were analysed for energy intake and macronutrient composition using dietary analysis software (Food Processor Nutrition Analysis Software, version 7, ESHA Research, Salem OH, USA). For Study I, total energy intake provided during recovery was matched closely to the average energy intake reported from individual 4-day food records.

2.2.2. *Maximal Oxygen Uptake ($\dot{V}O_{2\,max}$) and Familiarization*

Prior to each experimental trial the maximal oxygen uptake of all participants was assessed using an incremental cycle ergometer test on an electronically-braked cycle ergometer (Study I: Lode Excalibur, Quinton Instruments, Seattle, WA, USA; Studies II and III: Excalibur II, Lode Groningen, The Netherlands). The protocol started at a workload of 150 W and increased by either 30, 40 or 50 W every 2 min for 5 work intervals, after which the workload increased either 15, 20 or 25 $W \cdot min^{-1}$ until volitional exhaustion. Expired air from subjects was collected through a mouthpiece and plastic hosing and was analysed by an on-line metabolic cart (Study I: Quinton Q-plex 1, Quinton Instruments; Studies II and III: Applied Electrochemical Instrument, Pittsburgh). The analysers were calibrated prior to each test using commercial gases of known composition. The duration of each test was ~15 min and $\dot{V}O_{2\,max}$ was determined from a plateau in $\dot{V}O_{2\,max}$ (increase <2 $ml \cdot kg^{-1} \cdot min^{-1}$) with a further increase in power output. Heart rate was measured continuously via a heart rate monitor (Polar Sports Tester, Polar Electro, Finland). From the $\dot{V}O_{2\,max}$ test, power outputs corresponding to submaximal $\dot{V}O_2$ values to be used during the experimental trial were determined using linear regression analysis

for each subject. A familiarization trial was conducted approximately one week before the first trial to verify the experimental power outputs determined from the $\dot{V}O_{2\,max}$ test. In addition, all subjects were familiarized with the intensity of the glycogen-lowering protocol and the approximate number of intervals to be completed during each high-intensity phase.

2.2.3. *Muscle Biopsies*

In all studies muscle samples were obtained from the vastus lateralis muscle using the percutaneous needle biopsy technique (Bergstrom & Hultman, 1967). For studies II and III, the biopsy technique was modified to include suction (Evans *et al.*, 1982). Incisions were made after a local anaesthetic (Study I: 2% lidocaine; Studies II and III: 1% xylocaine) injection in the mid-thigh region and spaced approximately 2-3cm apart. Muscle biopsies were always performed through a new incision on a previously intact part of the thigh. Duplicate muscle biopsies at each time point were performed in study I for the measurement of muscle triacylglyercol. Excised muscle tissue from the biopsy was immediately frozen in liquid nitrogen (N_2), removed from the needle while frozen and stored in liquid N_2 until subsequent analysis.

2.2.4. *Venous Catheterisation*

Venous catheters were inserted into an antecubital vein for blood sampling and infusion of Intralipid, glucose or saline solutions. Approximately 1 ml of blood waste was drawn and discarded prior to each 5 ml blood draw for sampling. The catheter was flushed with 0.5 ml of saline immediately after each blood sample to prevent blood coagulation within the sampling site.

2.2.5. *Respiratory Exchange Ratio (RER) and Substrate Oxidation*

$\dot{V}O_2$, $\dot{V}CO_2$, and RER were determined at rest and during recovery using a Medical Graphics Corporation metabolic cart (Cardio2 and CPX/D system, St Paul, MN). Expired air samples were analysed breath by breath (reported as 30 sec average) and RER calculated by averaging for the final 5 min of a 10-min collection period. The metabolic cart was manually calibrated prior to each test using 0.01% alpha rated gases. Assuming a non-protein RER value, whole body CHO and fat oxidation (g) were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ measurements during the last 5 min of each collection period according to the following equations (Peronnet & Massicotte, 1991):

$$\text{CHO oxidation} = 4.585\ \dot{V}CO_2 - 3.226\ \dot{V}O_2$$

$$\text{Fat oxidation} = 1.695\ \dot{V}O_2 - 1.701\ \dot{V}CO_2$$

2.3. ANALYTICAL TECHNIQUES

2.3.1. *Blood Analysis*

Plasma Free Fatty Acids

For Study I, a portion of whole blood (1.5 ml) from a heparinised collection tube was centrifuged and 800 μl of plasma added to 200 μl of 5 M NaCl at 56°C for 30 min to inactivate lipoprotein lipase. The treated plasma was stored at –20°C for subsequent analysis. For Studies II and III, a portion of whole blood (1 ml) from a non-heparinised syringe was added to 20 μl of EGTA and reduced glutathione. In addition, 10 μl of tetrahydrolipstatin (120 $mg \cdot l^{-1}$) from Xenical (Orlistat) capsules (Roche, Nutley, NJ) was added to prevent in vitro lipolysis (Krebs *et al.*, 2000). The samples were centrifuged at 13000 r.p.m. for 2 min and the treated plasma stored at –20°C prior to analysis for free fatty acids (FFAs). All plasma samples were analysed for FFAs using a Wako NEFA C test kit (Wako Chemicals, Richmond, VA, USA) and the concentration of FFAs determined in 10 μl of sample (Study I) and 5 μl of sample (Studies II and III) by extrapolation from a standard curve. The mean intra-assay coefficient of variation (CV) from two samples with low and high FFA concentrations was 4.8%, obtained from a total of sixteen FFA assays performed in studies II and III.

Glycerol

A 200 μl aliquot of whole blood was deproteinised in 1 ml of 0.6 M $HClO_4$ (perchloric acid, PCA) and spun at 13000 r.p.m. for 2 min. The supernatant was stored at –80°C for subsequent fluorometric determination of whole blood glycerol (Bergmeyer, 1974). The mean intra-assay CV from two samples with low and high glycerol concentrations was 2.9%, obtained from a total of four assays performed in studies II and III.

Glucose and Lactate

In Study I, PCA treated whole blood (as described for glycerol) was used for the fluorometric determination of whole blood glucose and lactate (Bergmeyer, 1974). For studies II and III, blood was placed into heparinised vials, mixed, and

centrifuged at 13000 r.p.m. at 4°C for 2 min. The plasma was subsequently decanted and stored at –20°C until time of analysis. Plasma glucose and lactate were measured using an automated glucose/lactate analyser (EML 105, Radiometer, Copenhagen, Denmark). The mean intra-assay CV for the glucose and lactate assays were 1.0% and 4.8%, respectively.

Plasma Insulin

Blood for insulin analysis was placed in a heparinised tube, mixed, and centrifuged at 13000 r.p.m. at 4°C for 2 min. The plasma was subsequently decanted and stored at –20°C until time of analysis. Plasma insulin was assayed in duplicate using an insulin radioimmunoassay kit (Study I: LINCO Research, St Charles, MO, USA; Studies II and III: Phadeseph, Pharmacia & Upjohn, Sweden), which utilises ^{125}I-labelled insulin and insulin antiserum in a double antibody technique. The mean intra-assay CV from three samples was 8.6% for studies II and III.

2.3.2. *Muscle Glycogen*

Briefly, freeze-dried muscle was dissected free of blood, connective tissue and visible fat and powdered. In studies II and III, a small aliquot of the powdered muscle (2-3 mg) was suspended in 1 M HCl and incubated for 2 h at 95-100°C. After incubation, the extract was neutralised with 750 μl of 0.667 M NaOH and stored at –80°C until analysis. Muscle glycogen was measured using an enzymatic flurometric technique as previously described (Harris *et al.*, 1974) and expressed in mmol·kg^{-1} dry weight. The mean intra-assay CV from three samples was 6.4% for studies II and III.

2.3.3. *Muscle Triacylglycerol*

Total muscle triacylglycerol (IMTG) was determined by extracting a 4-8 mg sample of powdered muscle in chloroform-methanol (2:1) (Frayn & Maycock, 1980). After phospholipid removal using silicic acid (study I only), the reconstituted extracts were saponified with ethanolic KOH at 60°C for 1 h, and neutralised with $MgSO_4$. Extracts were stored at –80°C until IMTG concentration was determined by assaying the liberated glycerol fluorometrically (Froberg & Mossfeldt, 1971). For study I, IMTG content was calculated from the mean of the paired biopsies at each time point. For studies II and III, a CV of 7.1% for IMTG concentration was obtained

when two aliquots from the same muscle were analysed separately using four muscle samples. The mean intra-assay CV was 2.8% for glycerol from five samples.

2.3.4. *Muscle Pyruvate Dehydrogenase Activity*

PDH activation (PDHa) was determined from a small (10-20 mg) sample of wet muscle in Study I using a previously described radioisotopic method (Constantin-Teodosiu *et al.*, 1991) with some modifications. Briefly, the assay buffer for PDHa was slightly modified as previously reported (Putman *et al.*, 1993). During the assay, 200 μl aliquots were sampled at 1, 2 and 3 min intervals. The reaction was stopped by addition of each aliquot to 40 μl of 0.5 M PCA. After 5 min each aliquot was neutralised with 1.0 M K_2CO_3, centrifuged for 3 min at 13000 r.p.m. and the resulting supernatant stored at –80°C until analysed for acetyl-CoA by the method of Cederblad et al. (1990). Plots of acetyl-CoA as a function of time were used to determine the reaction rates.

2.3.5. *Malonyl-CoA*

Malonyl-CoA concentration was determined from a small sample of wet muscle tissue (~10 mg) using the previously described radioisotopic method (McGarry *et al.*, 1978) with modifications (Saha *et al.*, 1995; Chien *et al.*, 2000). Briefly, this method is based on the malonyl-CoA-dependent incorporation of labelled acetyl-CoA into palmitic acid catalyzed by fatty acid synthetase in the presence of NADPH. An internal malonyl-CoA standard was utilised with each sample to correct for differing levels of acetyl-CoA.

2.3.6. *Muscle Metabolites*

An aliquot of powdered muscle was extracted in a solution of 0.5 M PCA and 1 M Ethylenediaminetetraacetric acid (EDTA) and was neutralised with 2.2 M $KHCO_3$. For study I, the neutralised PCA extracts were assayed for ATP, phosphocreatine (PCr) and creatine by spectrophotometry as previously described (Bergmeyer, 1974; Harris *et al.*, 1974). Pyruvate was determined flurometrically (Passoneau & Lowry, 1993) and acetyl-CoA and acetylcarnitine were determined by radiometric assays (Cederblad *et al.*, 1990). In studies II and III, extracts were assayed for ATP, PCr and creatine using an enzymatic, fluorometric technique (Bergmeyer, 1974).

2.3.7. *RNA Isolation*

RNA Extraction

Total RNA was isolated from 5–10 mg skeletal muscle using a modification of the acid guanidium thiocyanate-phenol-chloroform extraction technique (FastRNA™ Kit-Green; Bio101, Carlsbad). Muscle tissue was added to 550 μl of RNA-bee solution (BioTex Laboratories, USA) and processed using the FastPrep™ Instrument (BIO 101, Vista CA; Savant Farmingdale, NY), twice for 20 sec at a rate of 5.5, separated by 5 min on ice. Following a series of centrifugation and separation steps, a pellet of RNA was obtained and suspended in 5 μl of EDTA-treated water.

To determine the RNA concentration, 1 μl of the suspension was diluted with 400 μl of sterile water to measure the OD 260 and OD280 using UV spectrometry (Heλios, Unicam, Cambridge, United Kingdom). Total RNA concentration was determined using the equation OD260/0.0625 $\mu g \cdot \mu l^{-1}$ (Sambrook *et al.*, 1989). RNA purity was determined by the equation OD260/OD280, with values between 1.7-2.0 indicative of the sample being free of genomic and protein contaminants. RNA was stored at –80^{o}C until subsequent reverse transcription (RT).

cDNA Synthesis

RNA was diluted to 0.1 $\mu g \cdot \mu l^{-1}$ in a total volume of 5 μl and heated to 65^{o}C for 10 min immediately prior to first-strand cDNA being generated using AMV reverse transcriptase (Promega, Madison, WI). RNA was combined with oligo $(dT)_{15}$ primers (1 $\mu g \cdot \mu l^{-1}$ of RNA), in the presence of 5 mM MgCl, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1% Triton® X-100, 1 mM of each dNTP and 20 U of recombinant RNasin ribonuclease inhibitor (40 $U \cdot \mu l^{-1}$). A RT negative was obtained by not adding any RNA to an aliquot of the RT mix. After the addition of 15 U AMV Reverse Transcriptase (20 $U \cdot \mu l^{-1}$), the mixture was incubated at 42^{o}C for 60 min, before the reaction was terminated by incubation at 99^{o}C for 5 min followed by 5 min at 4^{o}C using the PCR*express* Thermal Cycler (Hybaid, Middlesex, United Kingdom). All samples being compared underwent RT together and cDNA was stored at –20^{o}C until subsequent analysis.

2.3.8. *Real Time RT-PCR*

Real time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA). A real time RT-PCR mix of 0.5 X SYBR Green 1 PCR Master Mix, forward and reverse primers (3 μM) and 12ng cDNA was run for 40 cycles of PCR in a volume of 20 μl. Samples for each gene were run on two plates for study II (with a control sample) and a single plate for study III using a no template control and in duplicate to control for amplification efficiency. To compensate for variations in input RNA amounts and efficiency of RT, the mRNA expression of the housekeeping gene cyclophilin (CYC) was quantitated, and results normalised to these values. Recent work in this laboratory has identified CYC as an appropriate housekeeping gene for quantification of skeletal muscle mRNA abundance during both dietary and exercise interventions (Murphy *et al.*, 2003). Fluorescent emission data were captured, and mRNA levels were quantified for each gene by use of the critical threshold (C_T) value (Schmittgen *et al.*, 2000). The ΔC_T was calculated by subtracting the C_T for CYC from the C_T for the gene of interest. The relative expression of the gene of interest is calculated using the expression $2^{-\Delta CT}$ and reported as arbitrary units.

2.3.8.1. *Primer Design and Optimisation*

Forward and reverse primers (Table 2.1) were designed using Primer Express software package version 1.0 (Applied Biosystems, Foster City, CA) from gene sequences obtained from GenBank. A BLAST search for each primer and probe confirmed homologous binding to the desired mRNA of human skeletal muscle (Altschul *et al.*, 1990). Primers were purchased from Geneworks (Adelaide, SA, Australia).

Primers were optimised for real time RT-PCR to determine the minimum primer concentrations giving the highest fluorescence at the lowest C_T value. A PCR mix was prepared to run triplicates of each of the four conditions as shown in Table 2.2. The results demonstrated that optimal C_T values were obtained using 3 μM of both forward and reverse primer for each gene except for GLUT4 where 9 μM was optimal (Table 2.2).

Table 2.1.

Gene primer sequences

Gene		Primer Sequence (5' → 3')	GenBank Accession (Amplicon Size, bp)	Conc, μM
Cyclophilin	Forward:	CCC ACC CTC TTC TTC GAC AT	NM_021130.1 (80)	3
	Reverse:	CCA GTG CTC AGA GCA CGA AA		3
GLUT4	Forward:	CCA ACA GAT AGG CTC CGA AGA	NM_001042 (2128)	3
	Reverse:	CGC AGA GAA CAC AGC AAG GA		9
LPL	Forward:	TTG TGA AAT GCC ATG ACA AGT CT	NM_000237 (3549)	3
	Reverse:	CAT GCC GTT CTT TGT TCT CTA GA		3
MCD	Forward:	TCA TAA AGC GAG TCG TCA AGG A	NM_012213 (2136)	3
	Reverse:	CAG GTA TAG GTG ACA GAC TG AAA ACA		3
PDK4	Forward:	CCC GAG AGG TGG AGC ATT T	NM_002612 (82)	3
	Reverse:	GCA TTT TCT GAA CCA AAG TC AGT A		3

See text for definitions.

Table 2.2.

Concentration combinations for forward and reverse primers for primer optimisation.

	Forward Primer Conc. (μM)	
Reverse Primer Conc. (μM)	**3**	**9**
3	3 / 3	3 / 9
9	9 / 3	9 / 9

2.3.9. *Protein Analysis*

For detection of ACCβ and total AMPKα (α_1 and α_2) phosphorylation (ACCβ-P and AMPK-P), approximately 10–15 mg of muscle were homogenised in 20 volumes of homogenisation buffer (50 mM Tris pH 7.5, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 1mM DTT, 50mM NaF, 5 mM Na pyrophosphate, 10% glycerol) for approximately 30 sec on ice. The homogenate was then centrifuged (5 min, 1000 *g* at 4°C) and stored at –80°C for subsequent analysis. Protein concentration was determined using the BCA method.

Proteins were separated and identified using SDS-PAGE. 35μg of protein from each sample were loaded onto 1.5mm 7% acrylamide gels before undergoing electrophoresis for 75 min at 150V. Proteins were wet transferred to a nitrocellulose membrane for 120 min at 100V. Membranes were then cut at ~100 kDa and the top portion (ACCβ-P) blocked for 1 h in blocking buffer (7% skim milk powder in Tris buffered saline and 0.25% Tween (TBST)) and exposed overnight at 4°C to the anti-phospho-ACCβ-221 polyclonal antibody (1:500) (Chen *et al.*, 2000). The lower membrane (AMPKα-P) was blocked overnight at 4°C in 7% blocking buffer and exposed for 60 min at room temperature to the phospho-specific AMPKα thr^{172} antibody (1:1000). Membranes were exposed to anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibodies (diluted 1:10,000) in blocking buffer for 45 min at room temperature. Antibody binding was viewed by incubating in enhanced chemiluminescence substrate (Pierce SuperSignal Chemiluminescent, Rockford) and exposing to a Kodak Image Station 440CF (NEN Life Science Products, Boston). Bands were identified and quantified using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester).

CHAPTER THREE

SKELETAL MUSCLE FAT AND CARBOHYDRATE METABOLISM DURING RECOVERY FROM GLYCOGEN-DEPLETING EXERCISE IN HUMANS

3.1. INTRODUCTION

Fat is an important source of energy for resting and contracting muscle, and is available exogenously (FAs, chylomicrons, very low density lipoproteins (VLDL)), as well as within the muscle fibre. Intramuscular triacylglycerol (IMTG) is found in the form of lipid droplets adjacent to mitochondria (Hoppeler *et al.*, 1999) and are believed to be an energy substrate during long-term endurance activities (Havel *et al.*, 1967; Essen, 1977; Staron *et al.*, 1989; Romijn *et al.*, 1993; Klein *et al.*, 1994; Romijn *et al.*, 1995; Watt *et al.*, 2002a). However, the specific contribution of the IMTG pool as a metabolic substrate during prolonged submaximal exercise is debatable. The majority of evidence from isotopic tracer and ^{1}H-magnetic resonance spectroscopy (MRS) studies demonstrate a net utilization of IMTG during 90-120 min of exercise, while the findings of studies measuring IMTG content directly from skeletal muscle biopsies have been controversial (for review, see (Watt *et al.*, 2002b)).

Relatively little attention has been given to lipid utilisation during the recovery period following exhaustive, glycogen-depleting exercise in human beings. The data from several studies indicate an increase in whole body fat oxidation after exercise, as demonstrated by a decrease in the respiratory exchange ratio (RER) (Krzentowski *et al.*, 1982; Bielinski *et al.*, 1985; Kiens & Richter, 1998). Recently, it was also demonstrated that IMTG content decreased significantly during the initial 18 h of recovery in well-trained individuals (Kiens & Richter, 1998). These authors suggested that following glycogen-depleting exercise, muscle glycogen resynthesis is of high metabolic priority, resulting in the preferential utilization of IMTG and exogenous fat by the recovering skeletal muscle. Paradoxically, the reduction in IMTG content occurred during a period of high-CHO feeding when circulating insulin, an antilipolytic hormone, was elevated. However, more recent ^{1}H-MRS studies in endurance-trained subjects have failed to demonstrate a reduction in IMTG content during recovery, with reports of either unchanged (Decombaz *et al.*, 2000;

Decombaz *et al.*, 2001) or increased (Larson-Meyer *et al.*, 2002) IMTG content. It should be noted that there are several differences between the biopsy and MRS studies which may result in different findings. First, IMTG is measured in soleus or tibialis anterior muscles (primarily Type I fibres) in the MRS studies, as opposed to the vastus lateralis (50% Type I / 50% Type II) in the biopsy studies. Second, the recruitment pattern is likely to be different in these muscles given the different modes of exercise (running, MRS; cycling, biopsy). Third, differences in the timing of post-exercise sampling may be an issue i.e. post-exercise IMTG content was not determined until 22 hr of recovery in the study by Larson-Meyer (Larson-Meyer *et al.*, 2002) as opposed to a more detail time course in the first 6 hr of recovery in the study by Kiens and Richter (Kiens & Richter, 1998).

Since the existing MRS data suggests that IMTG are not utilised as an energy source in the post-exercise recovery period, verifying these findings with direct measurements in human biopsy samples is important. Furthermore, potential mechanisms underlying altered substrate utilisation following glycogen-depleting exercise, when combined with the standard practice of high-CHO feedings to replenish muscle glycogen stores, remain unknown. Accordingly, the objectives of this study were to: 1) examine the role of IMTG as an energy substrate after exhaustive exercise, and 2) provide a more thorough examination of the metabolic changes which occur in skeletal muscle during the recovery period following exhaustive, glycogen-depleting exercise. In order to facilitate comparison to the only previous study to measure IMTG content in muscle biopsies during recovery, the experimental protocol of Kiens & Richter (1998) was essentially replicated. There were two major differences in design between this study and the previous one (Kiens & Richter, 1998). First, the sampling of muscle biopsies was in duplicate at each time point, which should permit a more accurate determination of IMTG content (our coefficient of variation between duplicate biopsies in trained individuals is 12% (Watt *et al.*, 2002a) compared to 24% in untrained individuals (Wendling *et al.*, 1996). Second, additional metabolic measurements were included, namely pyruvate dehydrogenase activation (PDHa), and changes in muscle pyruvate, acetyl CoA and acetylcarnitine contents during recovery. Based on 1) the premise that circulating lipids could likely support the increased FA oxidation in resting muscle during the recovery period, 2) that elevated insulin levels would be expected to inhibit IMTG lipolysis, and 3) recent MRS data which does not support the use of IMTG following

exercise, it was hypothesised that IMTG content would remain unchanged during the recovery phase. Furthermore, despite the elevation of insulin subsequent to the feeding of high-CHO meals, it was hypothesised that PDHa would decrease during recovery to facilitate the partitioning of glucose towards glycogen resynthesis.

3.2. METHODS

Subjects

Eight endurance-trained males, age, 25 ± 3 years; body mass, 72.7 ± 2.1 kg; and $\dot{V}O_{2\,max}$, 63.1 ± 2.6 ml·kg^{-1}·min^{-1} (mean ± SEM) volunteered to participate in this study. Six of the subjects were competitive athletes (3 swimmers, 2 runners, 1 canoeist) and two were highly active. All subjects were considered to be healthy, did not smoke or take any medications, and had no evidence of cardiovascular or metabolic diseases. The experimental procedures and possible risks of the study were explained verbally and in writing to all participants before obtaining their informed, written consent to participate. The study protocol (Figure 3.1) was approved by the ethics committees of both institutions, and was performed according to the Declaration of Helsinki.

Pre-Experimental Protocol

Daily energy intake and composition of each subject's habitual diet was assessed using a 4-day food record (3 weekdays and 1 weekend day). Dietary data (Table 3.1) was analysed for energy intake and macronutrient composition (FoodPro Version 7.11). Subjects' maximal oxygen uptake ($\dot{V}O_{2\,max}$) was determined on an electromagnetically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA, USA). $\dot{V}O_{2\,max}$ was determined from a plateau in $\dot{V}O_2$ (increase <2 ml·kg^{-1}·min^{-1}) with a further increase in power output. From the $\dot{V}O_{2\,max}$ test, power outputs corresponding to submaximal $\dot{V}O_2$ values to be used during the experimental trial (see below) were determined using linear regression analysis for each subject. All subjects were familiarised with the intensity of the glycogen-depleting protocol and the experimental power outputs verified ~1 week before the experiments. For two days prior to the experimental trials, all subjects consumed a high-CHO diet to maximise glycogen stores (Table 3.1) and abstained from exercise, and consumption of caffeine and alcohol.

Experimental Protocol

Subjects reported to the laboratory at 1000 on the experiment day after a 10-12 hr overnight fast. After voiding, body mass was recorded and a teflon catheter inserted into an antecubital vein for blood sampling. Following 30 min of rest in a supine position, resting $\dot{V}O_2$ and $\dot{V}CO_2$ were measured (for determination of the respiratory

exchange ratio (RER)), and a 5 mL venous blood sample was drawn prior to exercise. Subjects then consumed a light breakfast consisting of CHO-rich, high glycemic index (GI) foods (Table 3.1). During a subsequent 2 h rest period, two incisions were made over the vastus lateralis muscle under local anesthesia (2% licodaine without adrenaline (epinephrine)). Respiratory and blood samples were then obtained and exercise initiated on a Lode Excalibur cycle ergometer. The glycogen-depleting protocol was performed as previously described (Kuipers *et al.*, 1987; Kiens & Richter, 1998). This consisted of cycling at 75% $\dot{V}O_{2\,max}$ for 20 min, followed by alternating 2-min bouts of 90% and 50% of VO_{2max} for 4-5 intervals, then decreasing intensity to 80% and 50% 2-min bouts for another 4-5 intervals, and finishing with 2-min 70% and 50% bouts until exhaustion. The mean exercise duration was 85.8 ± 3.2 min. During exercise, subjects ingested water ad libitum and a blood sample was taken just prior to exhaustion. After cessation of exercise (exhaustion), subjects were moved from the cycle ergometer to an examination table, at which point two percutaneous needle muscle biopsies were obtained from the same thigh. Approximately 3-4 minutes lapsed between the termination of the exercise, and the procurement of the muscle samples. During the recovery period, duplicate muscle biopsies were obtained at 3, 6 and 18 h by alternating between left and right thighs through different incisions spaced 2-3 cm apart. Blood and breath samples were obtained after 30 min, and every hour during the initial 6 h of recovery, and again after 18 h the following morning. $\dot{V}O_2$, $\dot{V}CO_2$, and RER were determined at rest and during exercise using a metabolic cart (Quinton Q-plex 1, Quinton). RER was calculated by averaging expired air samples for the final 5 min of a 10-min collection period.

Recovery Diet

Energy and macronutrient intake for the 3 meals consumed during the recovery period (Table 3.1) were calculated for each subject, and were similar to that used previously (Kiens & Richter, 1998). All subjects ate the same type of common food items and each meal was carefully prepared and weighed to the nearest gram prior to each trial. The first meal was ingested after 1 h of recovery and provided sufficient energy to replace that used during the exercise bout (~1450 kcal). A much lighter second meal was eaten after 4 h of recovery, followed by a third meal 7 h into the recovery period. After the final meal, subjects were only permitted to drink water until their final two muscle biopsies the next morning at 18 h of recovery. Total

energy intake during recovery was matched closely to the average energy intake reported from individual 4-day food records (Table 3.1). The composition of the post-exercise recovery diet was calculated to provide 65-70% of energy from CHO, 20 % from fat, and 10-15 % from protein, with an average GI of 60-65 using glucose as a reference (Table 3.1).

Analyses

Blood samples were analysed for whole blood glycerol and plasma FFA, insulin, glucose and lactate according to procedures outlined in chapter 2. Muscle PDHa, glycogen, IMTG, ATP, PCr, creatine, acetyl-CoA and acetylcarnitine were analysed as previously described in chapter 2.

Statistics

Results were analysed using a one-way ANOVA with repeated measures for the time factor to test for changes in each variable measured during the recovery period. A Newman–Keuls *post hoc* test was used if differences between time points were detected. Significance was accepted at $P \leq 0.05$. Values are presented as mean ± SEM.

3.3. RESULTS

Diet Analysis

Total energy intake (Table 3.1) during trial day (breakfast + recovery meals) averaged 13212 ± 687 kJ and was closely matched to daily habitual energy intake (12505 ± 961 kJ). Dietary composition during recovery consisted of an average of 67 ± 0.9 % of energy from CHO, 21 ± 0.8 % from fat and 13 ± 0.3 % from protein, and an average GI of 64.5 ± 0.6. Total CHO intake during the recovery period was 491 ± 28 g or 6.8 ± 0.3 $g \cdot kg^{-1}$. Total fat intake was 73 ± 3 g during the recovery period. The first meal consumed during recovery provided an average of 46 ± 0.9% and 52 ± 0.5%, respectively, of the total energy and CHO intake for trial day.

Respiratory and Blood Responses

Resting RER was 0.77 ± 0.02 at 2 h prior to exercise, and 0.80 ± 0.02 immediately prior to exercise. These were not statistically significant (Table 3.2). During the hour following exhaustive exercise, RER was significantly lower than baseline (P < 0.001), and remained unchanged for the remaining 17 h of recovery.

Resting whole blood glucose was 4.1 ± 0.10 $mmol.l^{-1}$ (Table 3.2) prior to the commencement of exercise. After 2 h of recovery (1 h after the first high-CHO meal), blood glucose concentrations rose significantly above baseline and remained elevated for another 3 h (P < 0.001). Plasma insulin concentrations increased markedly above baseline after meal 1 to reach a peak of 68.3 ± 2.0 μU/mL (P < 0.001) at 2 h of recovery (Table 3.2). For the following 4 h, plasma insulin concentration remained significantly elevated and returned to baseline levels by the following morning at 18 h after exercise. Plasma FFA levels were 0.22 ± 0.03 $mmol.l^{-1}$ prior to exercise and increased significantly (P < 0.001) during the first hour of recovery to 1.54 ± 0.17 mM (Table 3.2). After ingestion of the first meal, FFA concentration rapidly declined at 2 h (0.40 ± 0.07 mM) and remained near baseline throughout the recovery period. Plasma glycerol concentrations were highest near exhaustion (0.23 ± 0.02 $mmol.l^{-1}$), and similar to FFA responses, showed a marked reduction at 2h and throughout recovery (Table 3.2). The exercise protocol produced a significant elevation in plasma lactate concentration (5.2 ± 0.6 mM, P < 0.001), although levels quickly returned to baseline after 1 h and remained unchanged during the recovery (Table 3.2).

IMTG

At the termination of exercise, IMTG content was 23.5 ± 3.5 mmol·kg^{-1} dry wt (Figure 3.2), and remained constant at 24.6 ± 2.6, 25.7 ± 2.8 and 28.4 ± 3.0 mmol·kg^{-1} dry wt after 3, 6 and 18 h of recovery, respectively.

Muscle Glycogen and Metabolites

Muscle glycogen content was 37 ± 11 mmol·kg^{-1} dry wt upon completion of the exercise bout and increased significantly throughout the recovery period to reach a maximal content of 424 ± 22 mmol·kg^{-1} dry wt ($P < 0.001$) after 18 h of recovery (Figure 3.3). Muscle ATP, PCr and creatine were generally unchanged throughout the recovery period (Table 3.3). Acetyl CoA and acetylcarnitine content decreased significantly during the first 3 h of recovery ($P < 0.001$) and remained significantly below post-exercise concentrations throughout the recovery period (Table 3.3). Pyruvate ($P < 0.01$) was also significantly reduced compared to levels at exhaustion and remained low throughout the post-exercise period (Table 3.3).

PDHa

PDHa was 1.37 ± 0.17 mmol·min^{-1}·kg^{-1} w.w. after exhaustive exercise, and decreased during recovery, being significantly different from exhaustion at 6 (0.66 ± 0.10 mmol·min^{-1}·kg^{-1} w.w., $P < 0.01$) and 18 h (0.71 ± 0.15 mmol.min^{-1}·kg^{-1} w.w., $P < 0.05$) of recovery (Figure 3.4).

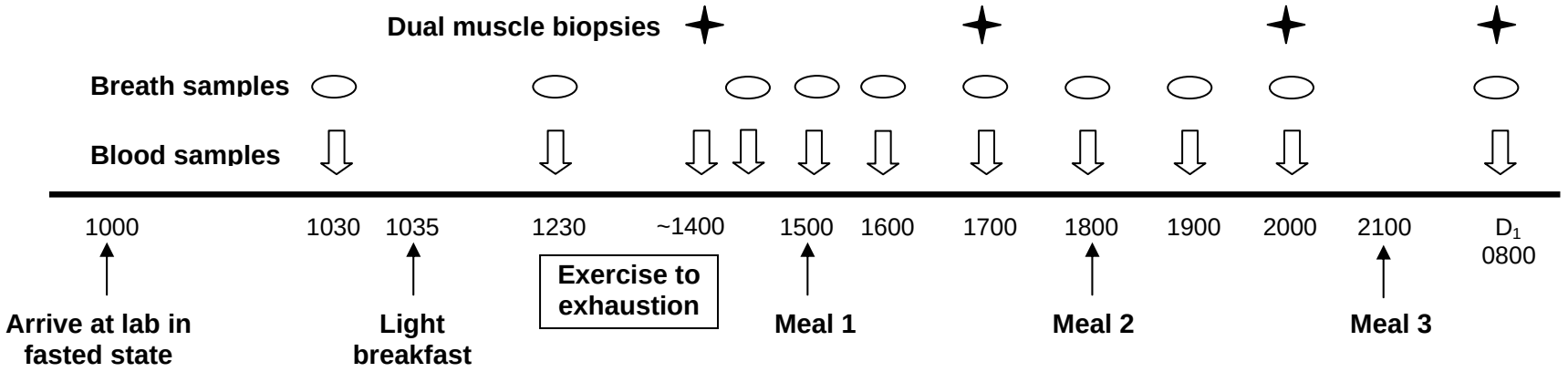

Figure 3.1. Schematic representation of protocol.

Table 3.1. Daily energy intake, dietary composition and glycemic index before and during post-exercise recovery.

	Pre-exercise			Recovery			
	Habitual	2 Days Before	Breakfast	Meal 1 1 h	Meal 2 4 h	Meal 3 7 h	Average
Energy, kJ	12505 ± 961	12578 ± 1254	858 ± 0	6051 ± 393	2890 ± 139	3413 ± 239	13212 ± 687
CHO, %	54 ± 1.6	60 ± 1.8*	84 ± 0	70 ± 0.5	64 ± 0.8	65 ± 1.7	66 ± 0.9
Fat, %	32 ± 1.2	26 ± 1.6*	12 ± 0	19 ± 0.3	22 ± 0.4	22 ± 1.9	21 ± 0.8
Protein, %	14 ± 0.7	14 ± 0.9	4 ± 0	11 ± 0.2	14 ± 0.4	13 ± 0.3	13 ± 0.3
CHO, $g \cdot kg^{-1}$	ND	ND	0.63 ± 0.02	3.5 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	2.3 ± 0.2
Glycemic Index (GI)	ND	ND	68.7 ± 0.0	64.1 ± 0.3	62.3 ± 0.3	55.8 ± 0.5	64.5 ± 0.6

Values are means ± S.E.M., $n = 8$. CHO, carbohydrate; ND, not determined. *Significantly different ($P < 0.05$) from habitual.

Table 3.2. Blood glucose, glycerol and lactate, plasma insulin and FFA, and RER before exercise, near exhaustion and during an 18 hour post-exercise recovery period.

Time (h)	RER	Glucose (mmol l^{-1})	Insulin (μU ml^{-1})	FFA (mmol l^{-1})	Glycerol (mmol l^{-1})	Lactate (mmol l^{-1})
Rest	0.77 ± 0.02	4.2 ± 0.12	8.9 ± 0.9	0.26 ± 0.04	0.08 ± 0.01	0.3 ± 0.05
Pre-exercise	0.80 ± 0.02	4.1 ± 0.10	14.3 ± 2.0	0.22 ± 0.03	0.09 ± 0.003	0.5 ± 0.04
End-exercise	ND	3.6 ± 0.15	4.5 ± 0.6	0.40 ± 0.06	0.23 ± 0.02‡	5.2 ± 0.6‡
0.5	0.62 ± 0.03‡	3.6 ± 0.10	5.5 ± 0.4	1.27 ± 0.16‡	0.20 ± 0.02‡	1.7 ± 0.3†
1	0.66 ± 0.03‡	3.7 ± 0.18	5.5 ± 1.3	1.54 ± 0.17‡	0.19 ± 0.02‡	0.8 ± 0.15
2	0.77 ± 0.02	6.3 ± 0.30‡	68.3 ±13.3‡	0.40 ± 0.07	0.11 ± 0.01	1.2 ± 0.2
3	0.77 ± 0.02	5.7 ± 0.31‡	65.7 ± 1.6‡	0.19 ± 0.03	0.09 ± 0.01	1.2 ± 0.1
4	0.80 ± 0.02	5.3 ± 0.30‡	61.9 ± 9.4‡	0.18 ± 0.03	0.09 ± 0.01	0.9 ± 0.08
5	0.84 ± 0.02	4.8 ± 0.25‡	66.6 ± 8.3‡	0.18 ± 0.02	0.09 ± 0.01	0.9 ± 0.10
6	0.79 ± 0.02	4.2 ± 0.21	41.7 ± 3.1*	0.22 ± 0.07	0.08 ± 0.01	0.8 ± 0.09
18	0.76 ± 0.02	4.3 ± 0.11	10.2 ± 1.0	0.44 ± 0.06	0.10 ± 0.04	0.4 ± 0.05

Values are means ± S.E.M., n = 8. FFA, free fatty acids; RER, respiratory exchange ratio; ND, not determined. Significantly different from before exercise, * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Table 3.3. Muscle metabolite concentrations immediately after exercise and during an 18 hour post-exercise recovery period.

Metabolite	0 h	3 h	6 h	18 h
ATP	23.5 ± 0.9	25.0 ± 1.0	26.2 ± 1.1	27.0 ± 1.6†
PCr	64.4 ± 3.0	68.0 ± 3.0	69.2 ± 3.1	69.1 ± 4.8
Creatine	48.4 ± 3.1	44.8 ± 2.5	43.7 ± 2.0	43.8 ± 2.6
Acetyl CoA	22.0 ± 1.8	3.7 ± 1.0‡	9.5 ± 2.0†	7.3 ± 0.8‡
Acetyl carnitine	17.5 ± 2.7	1.9 ± 0.4‡	2.3 ± 0.6‡	3.0 ± 0.5‡
Pyruvate	0.34 ± 0.07	0.15 ± 0.03†	0.14 ± 0.05†	0.10 ± 0.01†

Values are means ± S.E.M., $n = 8$. Significantly different from 0 hours, † $P < 0.01$, ‡ $P < 0.001$

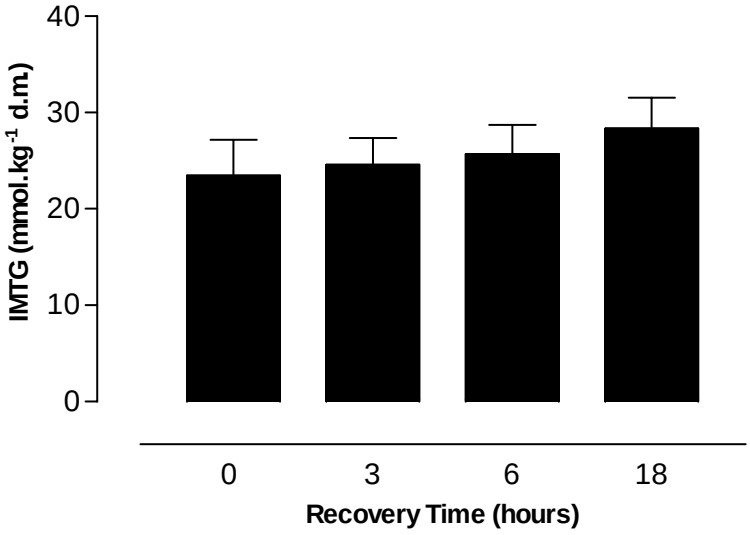

Figure 3.2. Muscle triacylglycerol (IMTG) contents in the vastus lateralis muscle immediately after exercise and during an 18 hour recovery period. Values are means ± SEM for 8 subjects.

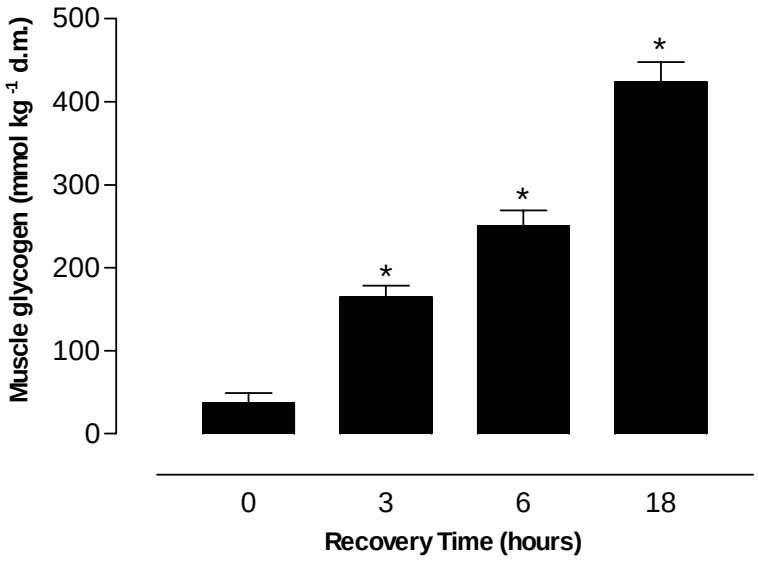

Figure 3.3. Muscle glycogen contents in the vastus lateralis muscle immediately after exercise and during an 18 hour recovery period. Values are means ± SEM for 8 subjects. *Significantly different (P < 0.001) from each previous time point.

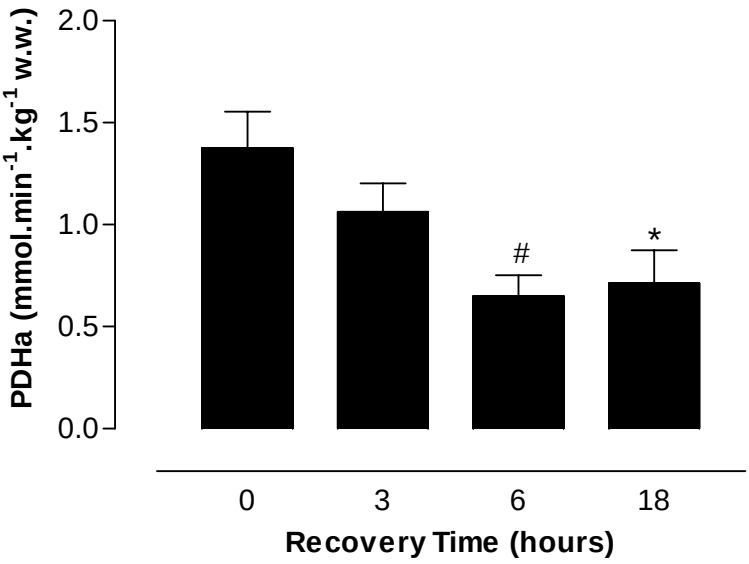

Figure 3.4. Muscle pyruvate dehydrogenase activation (PDHa) in the vastus lateralis muscle immediately after exercise and during an 18 hour recovery period. Values are means ± SEM for 8 subjects. #Significantly different (P < 0.01) from 0 hours; *significantly different (P < 0.05) from 0 hours.

3.4. DISCUSSION

Consistent with previous studies (Krzentowski *et al.*, 1982; Bielinski *et al.*, 1985; Kiens & Richter, 1998), these results indicate a greater contribution from fat oxidation during recovery in the presence of reduced muscle glycogen. This is in support of the hypothesis that a metabolic priority of recovering muscle is to resynthesise glycogen stores, resulting in a relative shift from CHO to fat oxidation. Indeed, activation of glycogen synthase in exercised human muscle has been recently demonstrated (Wojtazsewski *et al.*, 2001), supporting the contention that glycogen resynthesis is of high priority early in recovery. However, the present study does not support the previous findings of (Kiens & Richter, 1998) that IMTG are a significant source of lipid to be utilised during recovery. Rather, this study supports the more recent findings of ^{1}H-MRS studies that IMTG content does not decrease during recovery from exhaustive exercise in well-trained individuals even when low fat meals were ingested after exercise (Decombaz *et al.*, 2000; Decombaz *et al.*, 2001; Larson-Meyer *et al.*, 2002). This suggests that the primary lipid source during recovery may be exogenously derived (i.e. plasma FAs and TG), which is in agreement with the observation of a rapid reduction of plasma FAs during the first 2 hours of recovery. However, this reduction may also be due in part to reduced lipolysis, as indicated by the low glycerol. Furthermore, the large reduction in muscle acetylcarnitine stores early in recovery may have provided additional acetyl units for oxidation, and therefore have contributed to energy provision during this period. Despite the significant increase of insulin in response to the high-CHO feedings during recovery, muscle PDHa decreased. This reduction in activity is likely due, at least in part, to reduced substrate (pyruvate) availability as a result of the preferential partitioning of glucose towards glycogen resynthesis.

Responses During the First Hour of Recovery

During the first hour of recovery, we observed very low RER values (i.e. below 0.70). The explanation for this is uncertain, but is likely due to a shift towards predominantly fat oxidation, as well as the replenishment of the body's CO_2 stores within the bicarbonate pool (Jones & Heigenhauser, 1996). This would lead to a reduction in CO_2 production and consequently, an underestimation of the RER. The rapid increase in plasma FAs post-exercise, subsequent to the release of fatty acid trapped within adipose tissue (Hodgetts *et al.*, 1991; Romijn *et al.*, 1993) would have provided a large source of energy for the recovering muscle. In addition, the reduced

insulin and glucose levels, i.e. factors that are anti-lipolytic and inhibit fat oxidation, are likely to contribute to the increase in fat utilisation during the immediate recovery period. Muscle acetyl-CoA and acetylcarnitine accumulation was also evident post-exercise as previously reported (Howlett *et al.*, 1998; Watt *et al.*, 2002a).

Responses During 1 – 6 Hours of Recovery

After ingestion of the first high carbohydrate meal at 1 h, insulin and glucose levels were increased markedly while FAs and glycerol concentrations were rapidly reduced. Despite these responses, RER values remained low (0.77 to 0.80) until the following meal at 4 h, indicating predominantly fat oxidation, as previously observed during the early phase of recovery from prolonged (Krzentowski *et al.*, 1982; Bielinski *et al.*, 1985; Horton *et al.*, 1998) or exhaustive exercise (Kiens & Richter, 1998). The source of increased fat oxidation after glycogen depleting exercise has previously been attributed to the utilisation of IMTG (Kiens & Richter, 1998). However, in this study, IMTG content remained unchanged during the first 6 hours of recovery, suggesting that exogenous sources such as plasma FAs, and possibly plasma TG, are major sources of fat oxidation during recovery. Indeed, previous studies have found an increase in the activity of the muscle lipoprotein lipase (LPL) activity after exhaustive exercise (Lithell *et al.*, 1981; Kiens & Richter, 1998); (Kiens *et al.*, 1989), as well as increased muscle LPL mRNA at 4 h and protein content at 8 h post-exercise (Seip *et al.*, 1997). Glycogen content increased rapidly after 3 and 6 h of recovery (4.5 and ~7 fold respectively) in the presence of high circulating insulin and glucose. However, in spite of the elevated insulin, PDHa declined after 3 h and showed a further downward trend at 6 h to a value significantly ($P < 0.01$) below that observed immediately post-exercise (Figure 3.4). Coincident with this reduction in PDHa was a reduction in muscle acetylcarnitine stores, indicating that this pool may have been a significant source of acetyl units for oxidation early in recovery. Taken together, these data indicate a relative partitioning of glucose uptake during recovery towards muscle glycogen resynthesis and an increased reliance on FAs as an oxidative fuel. Approximately 365 g of glucose were consumed during the first 6 hours of recovery. During this period, muscle glycogen content increased by ~200 $mmol \cdot kg^{-1}$ d.w., which corresponds to a total of ~100 g of glucosyl units, assuming that ~12 kg of muscle mass were utilised during the exhausting exercise and actively resynthesising glycogen during recovery. Thus, assuming complete absorption, ~40% of the ingested glucose was directed towards muscle glycogen resynthesis

during the first 6 hours of recovery. Of the remaining glucose absorbed (365 – 100 = 265 g), ~210 g can be accounted for by 1) whole-body glucose oxidation (~30 g) based on an average RER and VO_2 of 0.78 and 0.3 $L \cdot min^{-1}$, respectively, and 2) liver glycogen resynthesis (~180 g), assuming a liver mass of ~2 kg, and near repletion of its glycogen stores. The remaining unaccounted glucose (55 g) may be due to either incomplete glucose absorption, or an underestimation of the working muscle mass.

Responses During 6 – 18 Hours of Recovery

The main finding during this period of recovery was that there was no change in IMTG content. Glycogen content continued to significantly increase during this period, increasing from 250.2 ± 17.7 to 423.8 ± 22.2 $mmol \cdot kg^{-1}$ d.w. The RER remained low, as expected, following the overnight fast (0.76 ± 0.02), indicating a continued reliance on fat. In addition, plasma FA levels were slightly elevated in response to low concentrations` of circulating insulin. In further support of a shift in metabolic priority towards glycogen resynthesis and away from CHO oxidation during recovery, PDHa activity, muscle pyruvate and acetyl-CoA concentrations were similar to levels found at 6 h of recovery.

Mechanisms for Increased Lipid Metabolism During Recovery

The mechanism for increased fat oxidation during recovery, in the presence of elevated insulin and glucose, is not readily apparent. IMTG contentration was unchanged during recovery, suggesting muscle lipid stores are not an appreciable source of FAs for increasing fat oxidation. Therefore, it is likely that elevated plasma FAs during the first hour of recovery resulted in an increased uptake and oxidation of FAs within glycogen depleted muscle. The rapid decline in plasma FA levels after 1 hour recovery can be attributed to both the rapid clearance by peripheral tissues, including skeletal muscle, as well as an insulin-mediated reduction in peripheral lipolysis (Horowitz *et al.*, 1997; Mittendorfer *et al.*, 2002) following the first high-CHO meal during recovery. It is paradoxical, though, that fat oxidation remained elevated in the presence of elevated insulin levels. It should be noted, however, that resistance to insulin's inhibition of FA oxidation during recovery from a marathon has previously been reported (Tuominen *et al.*, 1996).

Regulation of IMTG Content During Post-exercise Recovery

Enzymatic regulation of muscle TG esterification and hydrolysis during resting and contracting conditions is poorly understood. Recently, a neutral hormone-sensitive lipase (HSL) was identified in skeletal muscle (Langfort *et al.*, 1998) and appears to be under dual control by adrenaline (Langfort *et al.*, 1999) and contraction-related mechanisms (Langfort *et al.*, 2000). Plasma adrenaline concentrations were not determined in the present study, but would be expected to decrease rapidly (i.e. within 30 min) during recovery. Regulation of HSL activity may also result from allosteric feedback inhibition by long chain fatty acyl CoA (Jepson & Yeaman, 1992). Thus, the large increase in plasma FAs observed early in recovery may have elevated cytosolic LCFA-CoA concentrations and reduced IMTG hydrolysis by inhibiting HSL. For example, during a resting hyperinsulinemic glucose clamp with elevated FA levels, a rapid increase in the IMTG pool was found during a 5 h time period (Brechtel *et al.*, 2001). It is therefore probably not surprising that IMTG content did not decrease during the early stages of recovery given the marked elevation of insulin, and the possible accumulation of cytosolic LCFA-CoA in the recovering muscle. However, in fairness, it should be pointed out that during the first hour of recovery in the study by Kiens & Richter (1998), plasma FAs were also significantly elevated (~ 2 mM), at a time when IMTG stores were being utilised.

In spite of conclusions regarding IMTG use, the possibility of simply being unable to detect a small, but physiologically meaningful contribution from IMTG hydrolysis/oxidation must be acknowledged. Estimation of IMTG utilization using biochemical analysis or other established techniques such as isotope tracer kinetics and ^{1}H-MRS is limited by the inability to account for TG/FFA cycling. Using the dual label pulse-chase technique in isolated rat soleus muscle, it has been demonstrated that the IMTG pool undergoes simultaneous esterification and hydrolysis during both rest and contraction (Dyck & Bonen, 1998). This raises the possibility that TG resynthesis during recovery may have masked the utilisation of IMTG as an oxidative fuel during recovery from exhaustive exercise. A recent pulse-chase study in exercising humans (Guo *et al.*, 2000) indicated that FA incorporation into IMTG was relatively small compared to the rates of IMTG oxidation i.e. ~8%, making this possibility unlikely. However, it is possible that the incorporation of FAs into TG might be greater during recovery. Furthermore, determining the specific contribution of IMTG as an oxidative fuel during post-exercise recovery is

complicated by the methodological limitations associated with the muscle biopsy technique. While the use of trained subjects reduces the coefficient of variation between biopsies to 12.3 ± 9.4% (Watt *et al.*, 2002a), compared to 23.5 ± 14.6% in untrained subjects (Wendling *et al.*, 1996), this does not explain the discrepancy between the present findings, and those of Kiens and Richter (Kiens & Richter, 1998), who used equally well-trained subjects in their study.

Regulation of PDHa During Post-exercise Recovery

While the regulation of PDH activity during acute and prolonged exercise has been widely examined, to our knowledge this study is the first to report PDH activation in human skeletal muscle during CHO feeding after exhaustive exercise. PDH activity is controlled by the relative activities of PDH kinase (PDK), which phosphorylates and deactivates PDH, and PDH phosphatase (PDP), which dephosphorylates and activates PDH. PDK is stimulated by a high ATP/ADP, acetyl-CoA/CoA-SH and $NADH/NAD^+$ ratios and is inhibited by elevated pyruvate concentrations, whereas PDP is stimulated by Ca^{2+} and increased pyruvate. Muscle ATP, acetyl-CoA and pyruvate concentrations were unchanged during recovery, while Ca^{2+} and the $NADH/NAD^+$ ratio were not measured in this study. However, since pyruvate is a substrate for PDH and an inhibitor of PDK, the possibility exists that a decline in pyruvate availability contributed to the trend in PDHa reduction between 3 and 6 h of recovery. In addition, it is well documented that PDK4 gene expression is increased during recovery from prolonged, exhaustive exercise (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002), which may also account for the observed decrease in PDHa.

Conclusions

In summary, we report that IMTG content remains unchanged during recovery from glycogen-depleting exercise in the presence of elevated glucose and insulin levels. It appears that the partitioning of exogenous glucose towards glycogen resynthesis is of high metabolic priority during immediate post-exercise recovery, and is supported by the trend towards reduced PDH activity and increased fat oxidation. Plasma FAs (and possibly plasma TG), as well as intramuscular acetylcarnitine stores are likely to be important fuels for muscle metabolism in the immediate recovery period. However, IMTG appears to have a negligible role in contributing to the enhanced fat oxidation during recovery from exhaustive exercise.

CHAPTER FOUR

REGULATION OF SKELETAL MUSCLE FAT METABOLISM AFTER EXERCISE IN HUMANS: INFLUENCE OF FAT AVAILABILITY

4.1. INTRODUCTION

After a bout of endurance exercise the recovering skeletal muscle relies primarily on plasma-derived fatty acids (FAs) as an oxidative energy source. In the fasted state, post-exercise lipolysis in peripheral adipose tissue is substantial (Mulla *et al.*, 2000), resulting in elevated rates of plasma-derived fat oxidation (Bielinski *et al.*, 1985; Devlin *et al.*, 1989; Wolfe *et al.*, 1990; Schrauwen *et al.*, 1997; Tuominen *et al.*, 1997; Mulla *et al.*, 2000). A major factor regulating skeletal muscle FA oxidation is malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase I (CPT I), the enzyme that regulates the transfer of long-chain fatty acids (LCFAs) into the mitochondria for β-oxidation (McGarry & Brown, 1997). Malonyl-CoA concentration in the cytoplasm is under the dual control of synthesis by acetyl-CoA carboxylase (ACC) and degradation by the enzyme malonyl-CoA decarboxylase (MCD) (Alam & Saggerson, 1998).

Regulation of malonyl-CoA concentration and potentially skeletal muscle fat metabolism during post-exercise recovery is likely to be mediated by the metabolic fuel sensor 5'AMP-activated protein kinase (AMPK) (Ruderman *et al.*, 2003). Exercise-induced activation of AMPK appears to co-ordinately regulate a decrease in ACC and increase in MCD activity during post-exercise recovery (Park *et al.*, 2002a). Such coordinated changes in MCD and ACC attributable to AMPK are suggested to lower malonyl-CoA concentration and result in an increased oxidation and decreased synthesis of FAs (Park *et al.*, 2002a; Ruderman *et al.*, 2003). While an increasing body of evidence suggests that AMPK, ACCβ and MCD have key roles in regulating malonyl-CoA and LCFA oxidation following exercise in rodent muscle (Ruderman *et al.*, 2003), it is unknown whether these mechanisms exist in human skeletal muscle. Furthermore, it also remains to be determined whether altering circulating free fatty acid (FFA) levels can induce changes in skeletal muscle fat oxidation during post-exercise recovery through modification of AMPK, ACCβ, and malonyl-CoA.

In addition to plasma FAs, intramuscular triglyceride (IMTG) may also be an important source of FAs for skeletal muscle oxidation after glycogen-depleting exercise (Kiens & Richter, 1998). Paradoxically, the reduction in IMTG concentration occurred during conditions of elevated glucose and insulin which are known to reduce hormone-sensitive lipase activity (Watt *et al.*, 2004a) and calculated IMTG oxidation during low intensity exercise (Coyle *et al.*, 1997). However, Chapter 3 which essentially replicated the experimental protocol of Kiens & Richter (1998), demonstrated that IMTG has a negligible role in contributing to the enhanced fat oxidation during recovery from exhaustive exercise. Thus, the available evidence is equivocal regarding the importance of IMTG is an energy substrate for post-exercise skeletal muscle metabolism.

Recovery from prolonged, endurance exercise is characterised by a transient increase in metabolic gene transcription (Baar *et al.*, 1999; Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). For many genes, transcriptional activation and increases in mRNA abundance peak after 1-4 of recovery and return to basal levels within a ~24 h period (Pilegaard *et al.*, 2000). Reduced muscle glycogen after prolonged exercise also appears to enhance the transcriptional activation and mRNA of pyruvate dehydrogenase kinase 4 (PDK4), raising the possibility that signalling mechanisms sensitive to muscle glycogen content may be linked to the transcriptional control of exercise-responsive genes (Pilegaard *et al.*, 2002). Fatty acids can also act as signals involved in regulating gene expression. Elevated plasma FA levels in response to fasting, diabetes and high-fat feeding are associated with increased MCD mRNA in rat skeletal muscle (Young *et al.*, 2001). This suggests that FAs are involved in the regulation of MCD gene expression and possibly MCD activity, although posttranscriptional mechanisms regulating MCD activity appear to exist (Young *et al.*, 2001). However, it remains unknown whether FA availability also influences MCD mRNA abundance and MCD activity during post-exercise recovery in human skeletal muscle. Furthermore, human skeletal muscle PDK4 gene expression is highly sensitive to changes in plasma FA availability during high fat feeding (Peters *et al.*, 2001), fasting (Spriet *et al.*, 2004) and lipid infusion (Tunstall, 2003, unpublished observations).

The objectives of this study were to examine the effect of plasma FFA availability during recovery from glycogen lowering exercise on: (1) malonyl-CoA

concentration, (2) ACCβ and AMPKα phosphorylation, (3) the importance of IMTG as an energy substrate, and (4) the mRNA abundance of proteins regulating fat (MCD, LPL) and carbohydrate (PDK4) oxidation. In the first of three trials, FFA levels were elevated by a combined infusion of Intralipid and heparin, a physiological intervention that is known to decrease malonyl-CoA concentrations at rest (Odland *et al.*, 2000) and increase fat oxidation (Nisoli *et al.*, 2000). During a second trial plasma FFA availability was lowered with oral administration of the lipolytic inhibitor nicotinic acid (NA), a drug previously described to increase CHO oxidation (Howlett *et al.*, 2001; Stellingwerff *et al.*, 2003) and PDH activity (Stellingwerff *et al.*, 2003) during exercise. The third trial involved saline infusion during recovery to serve as a control.

4.2. METHODS

Subjects

Eight endurance-trained males, age, 32 ± 1 years; body mass, 79.8 ± 5.5 kg; peak power output, 5.0 ± 0.04 watts·kg^{-1} and $\dot{V}O_{2\,max}$, 61.1 ± 3.0 ml·kg^{-1}·min^{-1} (mean ± SEM) volunteered to participate in this study. All subjects were considered to be healthy, did not smoke or take any medications, and had no evidence of cardiovascular or metabolic diseases. The experimental procedures and possible risks of the study were explained verbally and in writing to all participants before obtaining their informed and written consent to participate. The study protocol was approved by the Deakin University Human Research Ethics Committee and was performed according to the Declaration of Helsinki.

Pre-experimental protocol

Daily energy intake and composition of each subject's habitual diet was assessed using a 4-day food record (3 weekdays and 1 weekend day). Dietary data (Table 4.1) was analysed for energy intake and macronutrient composition (Food Processor Nutrition Analysis Software, version 7, ESHA Research, Salem OH, USA). One to two weeks prior to testing, maximal oxygen uptake ($\dot{V}O_{2\,max}$) was determined on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Expired air was analysed by O_2 and CO_2 analysers (AEI Technologies, Pittsburgh, PA, USA) and $\dot{V}O_{2\,max}$ determined from a plateau in $\dot{V}O_2$ (increase <2 ml·kg^{-1}·min^{-1}) with a further increase in power output. From the $\dot{V}O_{2\,max}$ test, power outputs corresponding to submaximal $\dot{V}O_2$ values to be used during the experimental trial were determined using linear regression analysis for each subject. All subjects were familiarised with the intensity of the glycogen-lowering protocol and the experimental power outputs verified ~1 week before the experiments. For 2 days prior to experimental trials, subjects abstained from exercise and the consumption of caffeine and alcohol. In addition, on the day before each experimental trial subjects were provided with a high-CHO diet to maximise glycogen stores (Table 4.1).

Experimental protocol

Subjects participated in three randomised, counterbalanced experimental trials, each separated by at least a 1 week period. On each occasion, subjects reported to the laboratory at 0715 after a 10-12 hr overnight fast. After voiding, body mass was

recorded and a teflon catheter inserted into an antecubital vein for blood sampling. Following 30 min of rest in a supine position, resting $\dot{V}O_2$ and $\dot{V}CO_2$ were measured (for determination of the respiratory exchange ratio – RER), and a 5 ml venous blood sample was drawn. Subjects then consumed a light, high-CHO breakfast (Table 4.1). During a subsequent 90 min rest period, three incisions were made over the vastus lateralis muscle under local anesthesia (1% xylocaine solution). Expired gas and blood samples were then obtained and exercise initiated on a Lode Excalibur cycle ergometer. The glycogen-lowering protocol was performed as previously described (Kuipers *et al.*, 1987). This consisted of cycling at 75% $\dot{V}O_{2\,max}$ for 20 min, followed by alternating 2 min bouts of 90% and 50% of $\dot{V}O_{2\,max}$ for 5-10 intervals, then decreasing intensity to 80% and 50% 2-min bouts for another 4-5 intervals, and finishing with 2 min 70% and 50% bouts until 90-min of exercise had been completed. During exercise, subjects ingested water *ad libitum* and after 60 min were administered either a capsule of nicotinic acid (8 mg·kg^{-1}) or placebo. A blood sample was also taken ~5 min prior to exhaustion. After cessation of exercise, subjects moved from the cycle ergometer to a bench where a percutaneous needle muscle biopsy was obtained from the thigh. Approximately 30 sec lapsed between the termination of the exercise, and the procurement of the muscle sample using the percutaneous needle biopsy technique with suction. Additional muscle biopsies were taken from separate incisions on the same thigh at 3 h and at completion of the 6 h recovery period.

Immediately after the initial muscle sample, a second teflon catheter was inserted into the opposite antecubital vein. Infusion of either an Intralipid 20% solution at 1.5 ml·min^{-1} (Baxter Healthcare, Deerfield, IL) and a 200 U bolus of heparin followed by 0.2 U·kg^{-1}·min^{-1} (Faulding, Parkville, Australia) (HFA) or 0.9% saline (Viaflex Baxter Healthcare, NSW, Australia) at 1.5 ml·min^{-1} (CON) was then commenced for 6 h via an IMED Gemini PC-1 volumetric infusion pump (Alaris Medical Systems, San Diego, CA). During recovery, NA (LFA) or placebo capsules were administered at 30 min and 1 h (3.7 mg·kg^{-1}), 2 and 3 (5 mg·kg^{-1}), and 4 and 5 h (3.7 mg·kg^{-1}). Blood samples were also obtained at 30 min intervals and breath samples collected every hour according to procedures outlined in Chapter 2.

Due to difficulties in collecting respiratory measurements for two subjects, RER and substrate oxidation data are presented for six subjects. Water ingestion was allowed *ad libitum* during the post-exercise recovery. An overview of the experimental protocol is provided in Figure 4.1.

Analyses

Blood samples were analysed for whole blood glycerol and plasma FFA, insulin, glucose and lactate according to procedures outlined in Chapter 2. Muscle glycogen, IMTG, malonyl-CoA, ATP, PCr, creatine, gene expression and AMPKα and ACCβ phosphorylation were analysed as previously described in Chapter 2.

Statistics

Results were analysed using a two-way ANOVA with repeated measures (time x trial) and specific differences were located using a Tukey *post hoc* test (SigmaStat, version 3). Statistical significance was accepted at $P \leq 0.05$. Values are presented as the mean ± SEM.

4.3. RESULTS

Diet Analysis

Total energy intake in the 2 days immediately prior to each trial was not different from habitual (Table 4.1). During the day prior to each trial, CHO intake was higher ($P < 0.001$) and fat intake lower ($P < 0.001$) to elevate levels of muscle glycogen.

RER and substrate oxidation

Resting and pre-exercise RER were not different between trials (Table 4.2). During the initial hour of recovery, RER was significantly lower ($P < 0.01$) than rest for CON and HFA trials only. Between 4 and 6 h of recovery during the LFA trial, RER was significantly higher than the CON ($P < 0.01$, 4 & 5 h) and HFA ($P < 0.01$, 5 & 6 h). Consistent with the RER data, CHO oxidation during the entire recovery period was significantly higher than fat oxidation for the LFA trial ($P < 0.001$) and greater for LFA ($P < 0.01$) compared with the HFA and CON trials (Figure 4.2). Fat oxidation increased ~2-fold compared with CHO oxidation during HFA ($P < 0.001$), and was significantly greater ($P < 0.05$) during HFA compared with LFA.

Blood responses

Plasma FFA levels were similar at rest, prior to and after exercise for all three trials (Figure 4.3B). During recovery, fat infusion significantly elevated FFA concentrations above rest ($P < 0.001$) and above the CON trial after 1.5 h ($P < 0.01$). NA ingestion lowered FFA levels to baseline values that were significantly below CON ($P < 0.01$) throughout recovery except at 4h. Plasma glycerol responses at the end of exercise were significantly higher than rest for CON ($P < 0.001$) and LFA ($P < 0.05$) trials only (Figure 4.3A). NA during recovery reduced plasma glycerol compared to HFA ($P < 0.01$) and CON ($P < 0.01$) except at 1 and 4 h. Plasma glucose did not change during all three trials, except for a lower ($P < 0.05$) post-exercise value during CON and higher ($P < 0.05$) 1 h value during LFA (Table 4.3). Pre-exercise plasma insulin was significantly higher ($P < 0.01$) than rest for CON and HFA trials as a result of the high-CHO breakfast (Table 4.3). During recovery, elevated FAs increased plasma insulin above levels observed during CON ($P < 0.05$, 2-6 h) and LFA ($P < 0.001$, 1-5 h). Plasma lactate increased significantly ($P < 0.01$) above rest in response to the high intensity exercise and returned to baseline levels throughout recovery for all trials (Table 4.3).

Muscle metabolites

No difference in muscle glycogen content between all three trials was observed upon completion of the exercise bout (Table 4.4). During the LFA trial, muscle glycogen increased significantly ($P < 0.001$) after 3 h with no further change at 6 h. A significant increase ($P < 0.01$) in glycogen was observed after 6 h for the HFA trial. No change in IMTG concentration was detected during recovery for all three trials (Table 4.4). Muscle ATP, PCr and creatine also remained unchanged throughout the recovery period (Table 4.4).

Malonyl-CoA

After glycogen depleting exercise (0 h), no difference in malonyl-CoA concentration was observed between trials (Figure 4.4). During recovery, a significant increase in malonyl-CoA occurred at 3 and 6 h for the CON trial only ($P < 0.001$). In comparison with CON, malonyl-CoA was significantly lower at 6 h during LFA ($P < 0.01$) and at 3 h ($P < 0.05$) and 6 h ($P < 0.01$) during the HFA trial.

ACCβ and AMPK phosphorylation

Immediately after exercise (0 h), ACCβ-P was ~4-fold higher than 3 or 6 h during CON ($P < 0.01$) and LFA and HFA ($P < 0.001$) (Figure 4.5A). No treatment effect for ACCβ-P was observed during recovery. An exercise or treatment effect was not observed for AMPK-P throughout the recovery period (Figure 4.5B).

Gene expression

Gene expression of the three genes measured during post-exercise recovery relative to the housekeeping gene CYC are presented in Figure 4.6. mRNA abundance of MCD increased by ~3-fold during CON and ~4-fold during HFA ($P < 0.001$), however this response was blunted during LFA resulting in a significantly lower expression ($P < 0.05$) after 6 h compared with the HFA trial (Figure 4.6A). A strong trend towards a reduction in MCD mRNA during LFA compared with CON ($P = 0.055$) was also observed. Intralipid infusion induced a ~2.5-fold increase ($P < 0.01$) in LPL mRNA after 6 h and a similar trend occurred during CON ($P = 0.074$) (Figure 4.6B). NA ingestion prevented an increase in LPL mRNA during the recovery period, although mRNA abundance was similar to the other trials. In response to the exercise bout and elevated FA levels during CON and HFA trials, PDK4 gene expression was markedly increased after 3 and 6 h of recovery during

CON (~19-fold, $P < 0.001$) and HFA (~15-fold, $P < 0.05$) (Figure 4.6C). Lowering FAs transiently abolished the substantial increase in PDK4 mRNA, resulting in significantly lower levels ($P < 0.05$) during LFA after 3 h compared to CON or HFA. However, no further suppression of PDK4 mRNA was evident as levels were significantly ($P < 0.05$) higher after 6 h than immediately post-exercise.

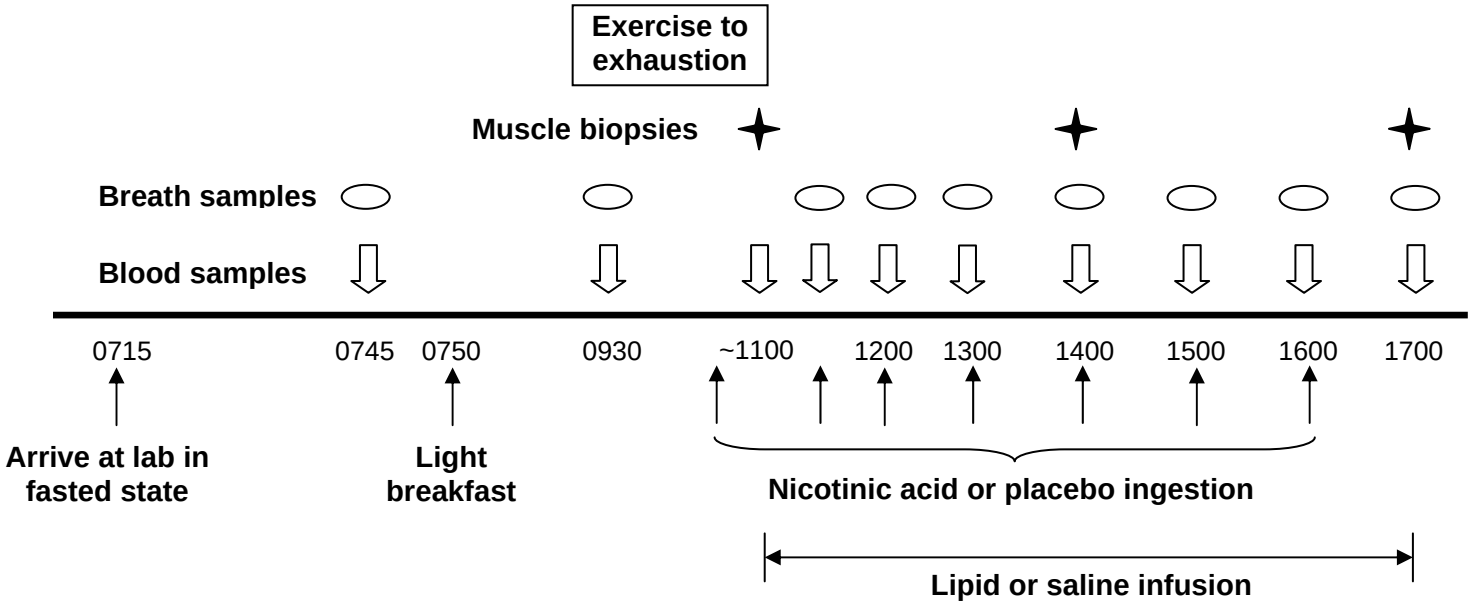

Figure 4.1. Schematic representation of protocol.

Table 4.1. Daily energy and macronutrient intake before post-exercise recovery.

	Habitual	2 days before	1 day before	Trial breakfast
Energy, kJ	14486 ± 1411	12814 ± 865	13768 ± 588	1281 ± 0
CHO, %	57 ± 2.9	59 ± 1.6	76 ± 1.3*	82 ± 0
Fat, %	25 ± 2.1	25 ± 2.2	11 ± 0.5*	13 ± 0
Protein, %	16 ± 1.9	16 ± 1.4	13 ± 0.9	5 ± 0

Values are means ± S.E.M., $n = 8$. CHO, carbohydrate. * Significantly different from 2 days before and habitual, $P < 0.001$.

Table 4.2. Respiratory exchange ratio values before exercise and during a 6 hour post-exercise recovery period.

Trial	Rest	Pre-exercise	1 h	2 h	3 h	4 h	5 h	6 h
CON	0.81 ± 0.02	0.84 ± 0.02	0.69 ± 0.04†	0.79 ± 0.02	0.81 ± 0.02	0.77 ± 0.01	0.75 ± 0.01	0.78 ± 0.02
LFA	0.79 ± 0.02	0.82 ± 0.03	0.77 ± 0.03	0.85 ± 0.02	0.82 ± 0.02	0.80 ± 0.02*	0.85 ± 0.02*‡	0.79 ± 0.01‡
HFA	0.79 ± 0.01	0.80 ± 0.03	0.69 ± 0.02†	0.78 ± 0.03	0.79 ± 0.02	0.73 ± 0.01	0.73 ± 0.01	0.74 ± 0.01

Values are means ± S.E.M., $n = 6$. † Significantly different from the resting value of the same trial, $P < 0.01$; * significantly different from CON trial, $P < 0.01$; ‡ significantly different from HFA trial, $P < 0.05$.

Table 4.3. Plasma glucose, insulin and lactate at rest, before exercise, end of exercise and during a 6 hour post-exercise recovery period.

Time (h)	CON			LFA			HFA		
	Glucose (mmol l^{-1})	Insulin (μU ml^{-1})	Lactate (mmol l^{-1})	Glucose (mmol l^{-1})	Insulin (μU ml^{-1})	Lactate (mmol l^{-1})	Glucose (mmol l^{-1})	Insulin (μU ml^{-1})	Lactate (mmol l^{-1})
Rest	4.9 ± 0.2	5.8 ± 0.8	1.4 ± 0.1	4.9 ± 0.2	5.3 ± 1.2	1.4 ± 0.1	4.9 ± 0.1	5.8 ± 0.6	1.4 ± 0.1
Pre-exercise	4.4 ± 0.3	8.8 ± 1.1†	1.7 ± 0.2	4.4 ± 0.3	10.1 ± 3.2	1.8 ± 0.2	4.6 ± 0.3	10.4 ± 1.4†	1.7 ± 0.1
End-exercise	4.3 ± 0.1#	4.3 ± 0.3	5.9 ± 0.9†	4.5 ± 0.4	3.8 ± 0.8	5.2 ± 0.6†	4.6 ± 0.4	4.6 ± 0.5	5.6 ± 0.6†
1	4.5 ± 0.1	3.8 ± 0.3	1.9 ± 0.1	5.0 ± 0.1*	2.8 ± 0.3‡	1.6 ± 0.1	4.7 ± 0.1	5.0 ± 0.4	2.1 ± 0.3
2	4.6 ± 0.1	3.7 ± 0.4	1.3 ± 0.1	4.7 ± 0.1	3.0 ± 0.5‡	1.4 ± 0.1	4.8 ± 0.1	5.4 ± 0.4*	1.6 ± 0.2
3	4.6 ± 0.2	4.0 ± 0.5	1.2 ± 0.1	4.8 ± 0.1	2.9 ± 0.5‡	1.3 ± 0.2	4.8 ± 0.1	6.3 ± 0.5*	1.6 ± 0.1
4	4.8 ± 0.1	3.8 ± 0.6	1.2 ± 0.1	4.9 ± 0.1	2.9 ± 0.5‡	1.3 ± 0.2	4.8 ± 0.1	6.4 ± 0.4*	1.4 ± 0.1
5	4.8 ± 0.1	3.9 ± 0.3	1.2 ± 0.2	4.8 ± 0.1	2.8 ± 0.6‡	1.3 ± 0.2	4.8 ± 0.1	7.5 ± 1.0*	1.3 ± 0.1
6	4.7 ± 0.1	3.7 ± 0.4	1.2 ± 0.1	4.8 ± 0.2	3.8 ± 0.9	1.4 ± 0.2	4.7 ± 0.1	6.1 ± 0.5*	1.4 ± 0.1

Values are means ± S.E.M., $n = 8$. Significantly different from the resting value of the same trial, # $P < 0.05$, † $P < 0.01$; * significantly different from CON trial, $P < 0.05$; ‡ significantly different from HFA trial, $P < 0.001$.

Table 4.4. Muscle metabolite concentrations immediately after exercise and during a 6 hour post-exercise recovery period.

	CON			LFA			HFA		
Metabolite	0 h	3 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h
ATP	23.1 ± 0.8	22.9 ± 0.8	23.3 ± 1.1	22.8 ± 0.7	22.2 ± 0.6	22.7 ± 0.6	23.3 ± 0.8	22.8 ± 0.7	23.2 ± 0.8
PCr	86.6 ± 3.2	86 ± 3.2	83.8 ± 2.7	86.1 ± 1.4	78.6 ± 3.2	81.0 ± 3.0	88.0 ± 2.6	83.8 ± 2.9	83.2 ± 2.2
Creatine	47.0 ± 4.9	47.6 ± 3.0	49.8 ± 3.1	47.5 ± 4.8	55.0 ± 3.2	52.6 ± 3.9	47.2 ± 4.7	49.8 ± 3.2	50.3 ± 3.7
Glycogen	138 ± 24	159 ± 19	163 ± 19	101 ± 24	155 ± 30†	153 ± 24†	127 ± 27	146 ± 24	170 ± 34*
IMTG	42.2 ± 5.8	35.3 ± 5.4	39.7 ± 4.6	30.7 ± 1.8	36.1 ± 6.8	38.0 ± 6.8	33.8 ± 5.4	36.0 ± 7.5	45.6 ± 10

Values are means ± S.E.M., $n = 8$. All values are expressed as mmol kg^{-1} d.m. PCr, phosphocreatine; IMTG, intramuscular triglyceride. Significantly different from 0 h, * $P < 0.01$, † $P < 0.001$.

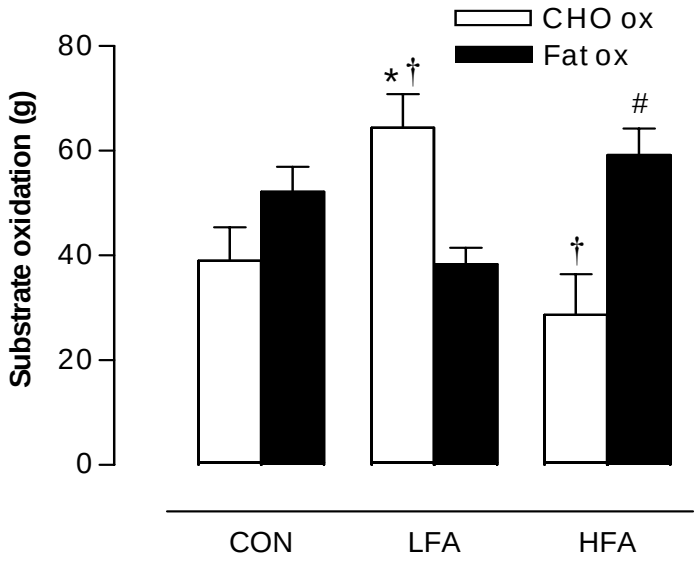

Figure 4.2. Total carbohydrate (CHO) and fat oxidation during a 6 h recovery period with either saline (CON) and Intralipid infusion (HFA) or nicotinic acid ingestion (LFA). Values are means ± S.E.M. for 6 subjects. * Significantly different from CON and HFA trials, $P < 0.01$; # significantly different from LFA trial, $P < 0.05$; † significantly different from fat oxidation, $P < 0.001$.

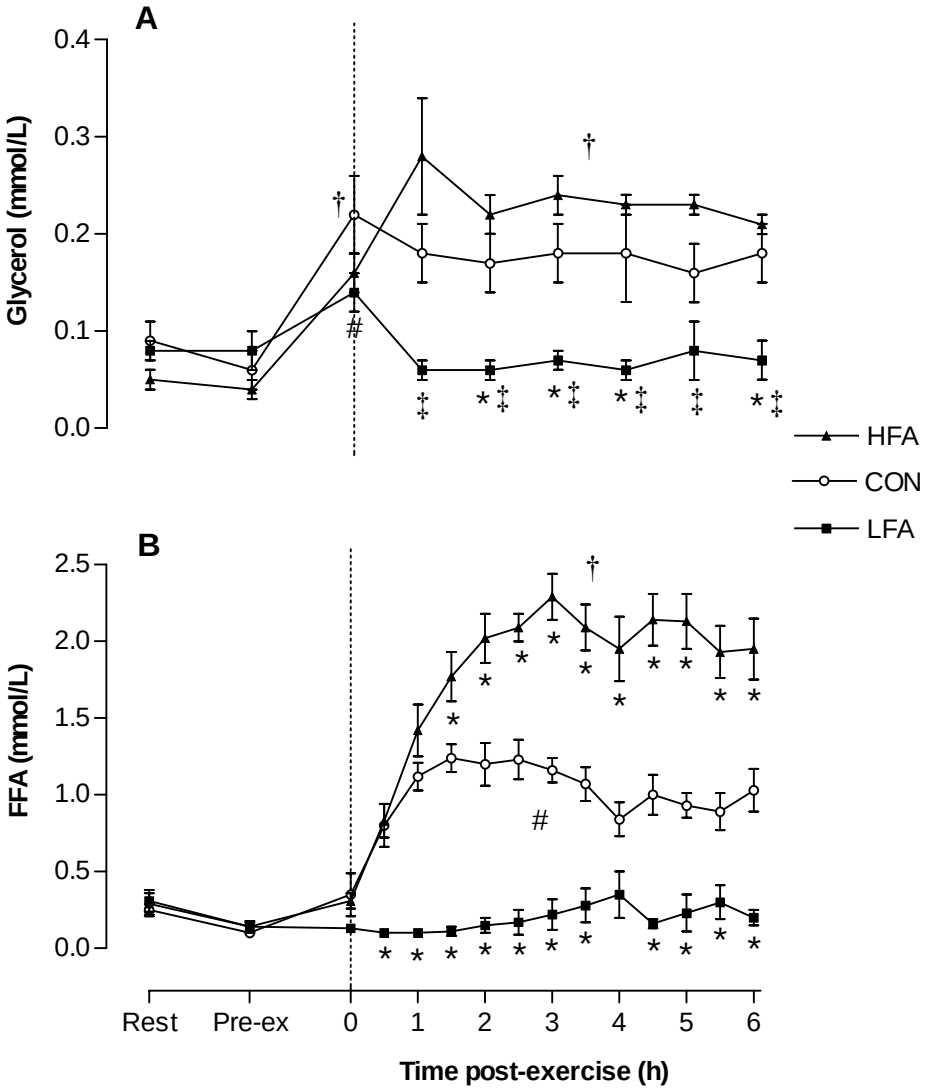

Figure 4.3. Plasma glycerol (A) and free fatty acid (FFA) (B) concentrations at rest, before and after exercise and during a 6 h recovery period with either saline (CON) and Intralipid infusion (HFA) or nicotinic acid ingestion (LFA). Values are means ± S.E.M. for 8 subjects. Significantly different from the resting value of the same trial, # $P < 0.05$, † $P < 0.001$; * significantly different from CON trial, $P < 0.01$; ‡ significantly different from HFA trial, $P < 0.01$.

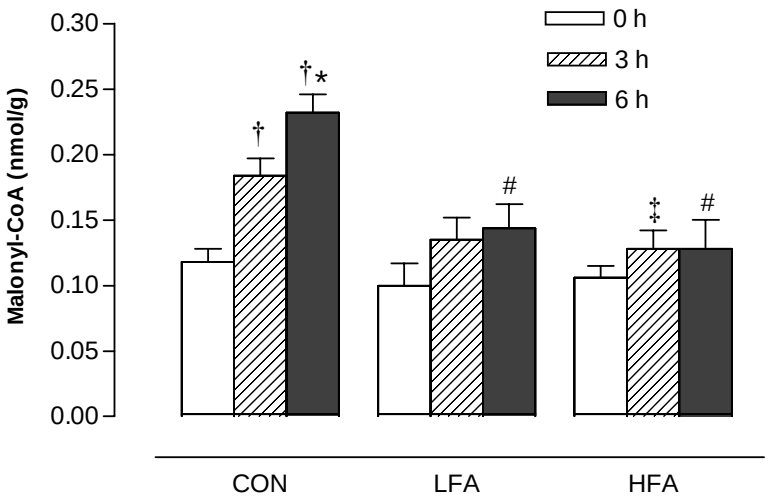

Figure 4.4. Malonyl-CoA concentration immediately after exercise (0 h) and during a 6 h recovery period with either saline (CON) and Intralipid infusion (HFA) or nicotinic acid ingestion (LFA). Values are means ± S.E.M. for 8 subjects. † Significantly different from 0 h, $P < 0.001$; * significantly different from 3 h, $P < 0.001$; significantly different from CON, ‡ $P < 0.05$, # $P < 0.01$.

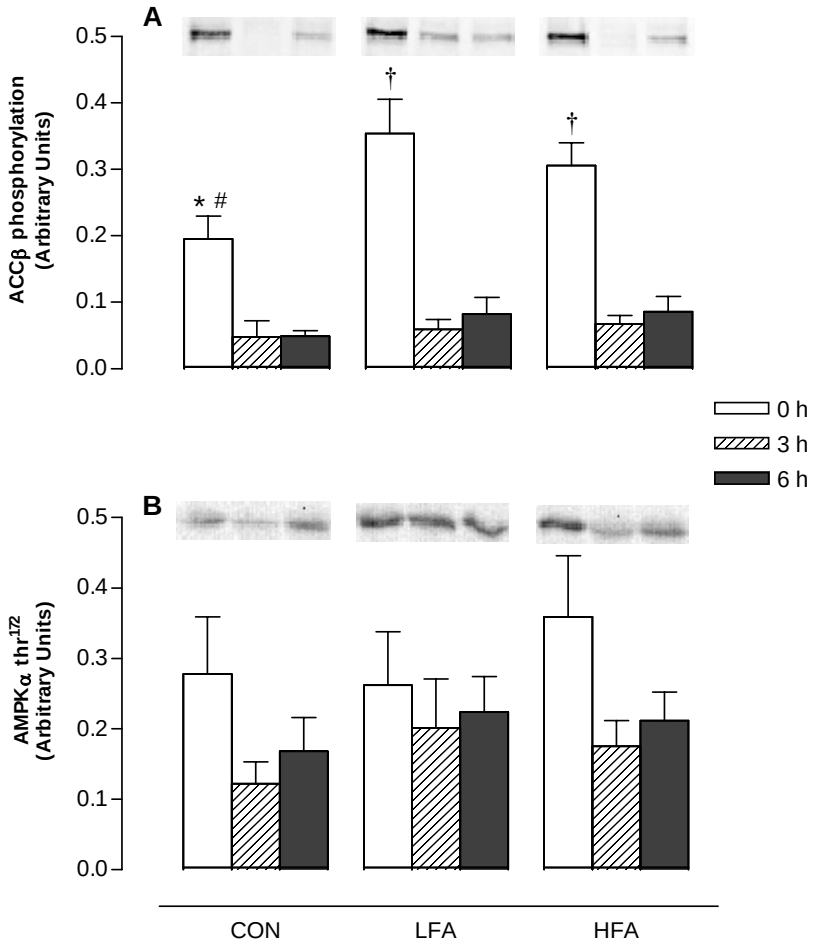

Figure 4.5. Acetyl-CoA carboxylase-β (ACCβ) (A) and AMP-activated protein kinaseα threonine 172 phosphorlyation (AMPK) using a phospho-specific antibody that recognises both the α_1 and α_2 AMPK subunits (B) immediately after exercise (0 h) and during a 6 h recovery period with either saline (CON) and Intralipid infusion (HFA) or nicotinic acid ingestion (LFA). Values are means ± S.E.M. for 8 subjects. Significantly different from 3 and 6 h, * $P < 0.01$, † $P < 0.001$; # significantly different from LFA trial, $P < 0.05$.

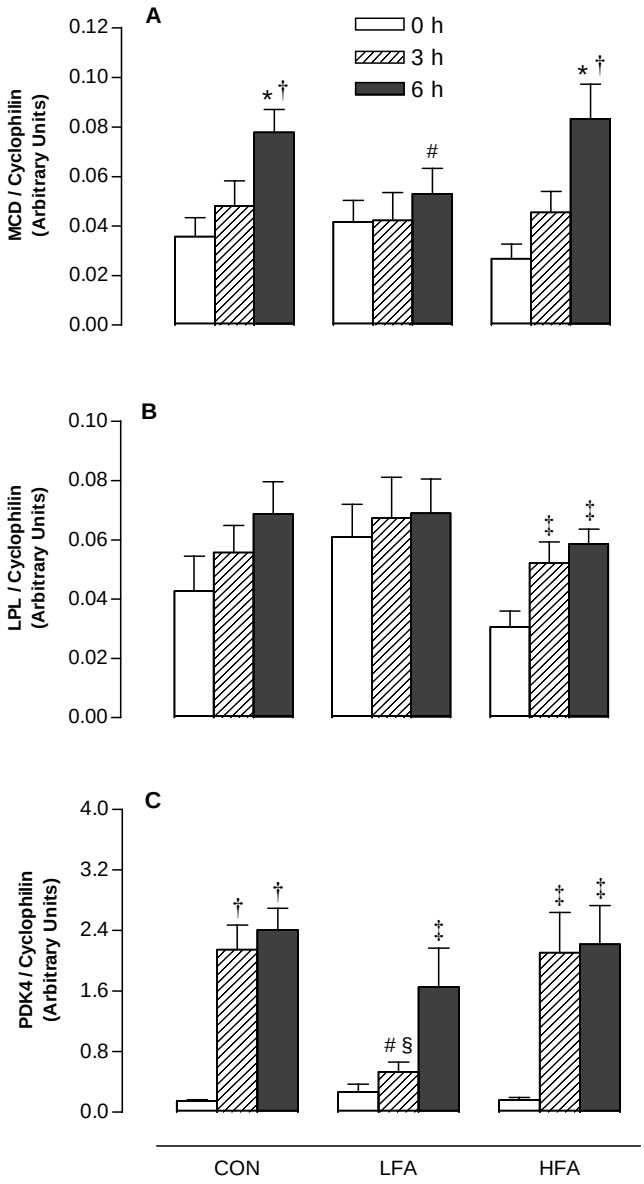

Figure 4.6. mRNA abundance of MCD (A), LPL (B) and PDK4 (C) immediately after exercise (0 h) and during a 6 h recovery period with either saline (CON) and Intralipid infusion (HFA) or nicotinic acid ingestion (LFA). Values are means ± S.E.M. for 8 subjects. Significantly different from 0 h, ‡ $P < 0.05$, † $P < 0.001$; * significantly different from 3 h, $P < 0.01$; § significantly different from CON trial, $P < 0.05$; # significantly different from HFA trial, $P < 0.05$.

4.4. DISCUSSION

This study examined the effect of fat availability on whole body substrate utilisation, factors regulating skeletal muscle fat oxidation (malonyl-CoA content, ACCβ and AMPKα phosphorylation), IMTG concentration and gene expression during post-exercise recovery. Contrary to results obtained from rodent skeletal muscle, changes in whole body fat metabolism after exercise are unrelated to levels of malonyl-CoA in human skeletal muscle. In particular, the significant increase in fat oxidation observed during 6 h of Intralipid infusion compared with NA ingestion was not associated with a difference in malonyl-CoA concentration. The following novel observations have also been made, (1) the significant increase in fat oxidation with elevated FAs were independent of an increase in ACCβ phosphorylation, (2) IMTG remained unchanged during recovery despite the dramatic changes in FFA levels, (3) administering NA attenuated the post-exercise increase in skeletal muscle MCD after 6 h and PDK4 gene expression after 3 h of recovery.

The CPT complex plays a pivotal role in the regulation of LCFA entry into the mitochondria for subsequent β-oxidation. Malonyl-CoA is a well known inhibitor of CPT I activity in a variety of tissues (McGarry & Brown, 1997) and it is believed that malonyl-CoA could be the key regulator of fat oxidation in rodent skeletal muscle during muscle contraction (Winder, 1998). However, malonyl-CoA is unchanged in human skeletal muscle during moderate-intensity exercise when there are large increases in the rate of fat oxidation (Odland *et al.*, 1996; Odland *et al.*, 1998; Dean *et al.*, 2000), suggesting that the regulation of CPT I activity is more complex than simple changes in malonyl-CoA levels and/or that malonyl-CoA inhibition is overridden during exercise (Bezaire *et al.*, 2004). Malonyl-CoA is decreased in response to Intralipid infusion at rest (Odland *et al.*, 2000) and remains depressed for up to 90 min after a single bout of exercise in rats which has been postulated to allow for increased fat utilisation and a partitioning of glucose towards muscle glycogen resynthesis (Rasmussen *et al.*, 1998). However, this study suggests that in the immediate post-exercise recovery period, human skeletal muscle malonyl-CoA is not a primary regulator of fat metabolism, since malonyl-CoA content was not correlated with changes in whole-body fat oxidation. Indeed, these data appear to be the first to indicate an increase in skeletal muscle malonyl-CoA during post-exercise recovery. It should also be noted that immediately after glycogen-lowering exercise, malonyl-CoA concentration was low in comparison with values reported

after high-intensity knee-extensor exercise to exhaustion (Dean *et al.*, 2000), suggesting a decline in malonyl-CoA content occurred during the exercise bout. Therefore, the observed increase in malonyl-CoA during CON may reflect a shift towards resting values that was blunted during the LFA and HFA trials. The reason for an increase in malonyl-CoA during recovery with saline infusion only remains unknown.

During post-exercise recovery, decreased malonyl-CoA content in rodent skeletal muscle is associated with a coordinate reduction in ACC and increase in MCD activity, mediated by the activation of AMPK (Park *et al.*, 2002a). In this study, ACCβ-P, which is inversely related to ACC activity in rat muscle (Park *et al.*, 2002b), rapidly declined after 3 h and remained unchanged after 6 h of recovery despite significant change in fat oxidation between the HFA and LFA trials. A substantial increase in ACCβ-P followed by a rapid decline after 1 h of moderate intensity exercise in well-trained individuals has also been reported, indicating that ACCβ-P is particularly sensitive to exercise when muscle glycogen is reduced (Wojtaszewski *et al.*, 2002b). Furthermore, AMPKα-P, which is correlated with total AMPK activity in rodent (Park *et al.*, 2002b) and human (Wojtaszewski *et al.*, 2002a; Clark *et al.*, 2004) skeletal muscle, did not decline throughout the 6 h recovery period in any trial. It should be noted however that AMPKα-P data in this study represent phosphorylation of both α_1 and α_2 subunits, raising the possibility that isoform-specific changes at the end of exercise and during recovery were not detected. Indeed, a higher degree of AMPKα_2 compared with AMPKα_1 activation in endurance-trained human skeletal muscle is reported in some (Wojtaszewski *et al.*, 2000b; Wojtaszewski *et al.*, 2003; Yu *et al.*, 2003) but not all (Clark *et al.*, 2004) studies in response to prolonged or high-intensity exercise. During immediate (1-3 hours) post-exercise recovery, isoform-specific changes are also observed. These include a rapid decrease in AMPKα_2 activity to near resting levels together with unchanged AMPKα_1 activation (Wojtaszewski *et al.*, 2000b; Wojtaszewski *et al.*, 2002a; Wojtaszewski *et al.*, 2003), whilst total AMPKα phosphorylation also decreases (Wojtaszewski *et al.*, 2002a). Although a pre-exercise biopsy was not obtained in this study, it can be inferred from previous studies that AMPKα-P was elevated immediately after exercise (Wojtaszewski *et al.*, 2000b; Wojtaszewski *et al.*, 2003; Yu *et al.*, 2003; Clark *et al.*, 2004). Therefore, despite the absence of a

significant decrease in AMPKα-P throughout recovery in all trials, AMPK activation is unlikely to have remained above resting levels during recovery. On the basis of this postulate, increased FA availability was unable induce AMPKα activity after exercise and thus may account for the lack of increase in ACCβ-P during the CON and HFA trials. In support of this hypothesis, 5 h of Intralipid infusion has no effect on AMPKα_1 or α_2 activity at rest (Tunstall *et al.*, unpublished observations). Furthermore, the low AMP/ATP ratio and stable ATP demand under resting conditions during recovery would be expected to keep AMPK in its inactive (dephosphorylated) form.

Fat oxidation was significantly higher during fat infusion compared with NA ingestion during recovery, however no difference in ACCβ-P was observed. These data suggest there is dissociation between skeletal muscle ACCβ-P and fat oxidation in humans during post-exercise recovery. A similar dissociation between ACCβ-P and fat oxidation has been reported in human skeletal muscle during exercise (Wojtaszewski *et al.*, 2002a; Clark *et al.*, 2004). Furthermore, the lack of association between ACCβ-P and malonyl-CoA in all trials suggests that factors other than the phosphorylation state of ACCβ are regulating ACC activity and thus malonyl-CoA in human skeletal muscle. Although not determined in this study, these factors could include the cytosolic concentration of citrate, an allosteric activator of ACC (Saha *et al.*, 1997; Saha *et al.*, 1999; Bavenholm *et al.*, 2000) or LCFA-CoA, an allosteric inhibitor of ACC (Saha *et al.*, 1999). Alternatively, differences in MCD activity could account for the differing malonyl-CoA values in the three trials.

Activation of AMPK during post-exercise recovery is also postulated to mediate an increase in MCD activity and further reduce cytosolic malonyl-CoA in rodent skeletal muscle (Park *et al.*, 2002a). In addition to phosphorylation control by AMPK, activation of MCD may also be regulated by increased protein content (Dyck *et al.*, 2000; Sakamoto *et al.*, 2000), or by the existence of a separate protein or allosteric regulator (Dyck *et al.*, 2000; Young *et al.*, 2001), although the exact nature of these regulatory mechanisms remain unknown. Gene expression may also be an important determinant of MCD activity, since MCD mRNA is most abundant in human cardiac and skeletal muscles, tissues in which cytoplasmic malonyl-CoA is a potent inhibitor of CPT I and, thus, of mitochondrial LCFA oxidation (Sacksteder *et al.*, 1999). Indeed, elevated plasma FA levels in response to fasting, diabetes and

high-fat feeding are associated with increased skeletal muscle MCD mRNA (Young *et al.*, 2001). Consistent with these results, this study demonstrated that high FFA levels increased MCD gene expression after 6 h of post-exercise recovery by 3–4-fold, although the exercise-independent effect of elevated FAs on MCD mRNA in human skeletal muscle is unknown. In comparison with the HFA trial, this effect was abolished when FFA levels were lowered using NA and tended to be reduced when compared with CON ($P = 0.055$). These results support the hypothesis that FAs are involved in the regulation of rodent skeletal muscle MCD gene expression (Young *et al.*, 2001) and extend these data to suggest that FAs are also involved in the regulation of MCD mRNA in human skeletal muscle. However, the effect of altered MCD mRNA during recovery on MCD activation is uncertain, since MCD activity was not determined in this study or in previous studies investigating post-exercise metabolism in humans. The mechanisms by which FAs potentially regulate MCD expression remain undefined. Specific activation of peroxisome proliferator-activated receptor-alpha (PPARα) in primary cultured human skeletal muscle cells increases MCD mRNA and FA oxidation (Muoio *et al.*, 2002). Furthermore, PPARα activation increases skeletal and cardiac muscle MCD gene expression, while specific PPAR gamma (PPARγ) activation decreases muscle MCD mRNA, suggesting that FA-induced MCD expression is mediated through PPARα (Young *et al.*, 2001).

The substantial increase (15–19-fold) in PDK4 gene expression after 6 h of recovery during CON and HFA trials confirms previous findings that PDK4 is a highly exercise-responsive gene (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). Furthermore, reducing post-exercise plasma FFA concentration transiently blunted the increase in PDK4 mRNA, suggesting that FAs are involved in the regulation of PDK4 as well as MCD gene expression in human skeletal muscle. PDK4 is one of four isoenzymes present in mammalian tissues that phosphorylates and inactivates the PDH complex. Expression of PDK4 is induced in skeletal muscle by sustained increases in fat availability that occur during fasting (Wu *et al.*, 1999; Holness *et al.*, 2002; Spriet *et al.*, 2004), high fat feeding (Holness *et al.*, 2000; Peters *et al.*, 2001) and streptozotocin-induced diabetes in rats (Wu *et al.*, 1999). Similar to MCD expression, specific activation of PPARα increases the mRNA abundance of PDK4 (Muoio *et al.*, 2002) in primary cultured human skeletal muscle cells. Furthermore, PDK4 gene expression, PDK4 protein and PDK4 activity are also increased with

PPARα activation in rodent skeletal muscle (Wu *et al.*, 1999). Recently, the progressive increase in PDK4 mRNA during prolonged fasting in humans has been associated with a decline in PDHa (Spriet *et al.*, 2004). Therefore in the present study, reduced PDK4 gene expression after 3 h of recovery during LFA may have maintained PDHa, and thus in part provides a mechanism for the higher rate of CHO oxidation observed during the LFA trial. It should also be noted that in addition to lowered plasma FAs, elevated insulin decreases the abundance of PDK4 mRNA (Huang *et al.*, 2002). Since NA suppressed plasma insulin in comparison with fat infusion, a reduction in insulin concentration may account for the transient suppression PDK4 observed at 3 h during recovery. Indeed, increased plasma glucose and insulin induces a prolonged suppression of PDK4 mRNA together with increased CHO oxidation in comparison with saline infusion after a 6 h post-exercise recovery period (Chapter 5).

Although the mechanism of action of NA is not well understood, the possibility exists that an NA-induced increase in plasma epinephrine may also effect metabolic gene expression during post-exercise recovery. Suppression of plasma FFA with NA during exercise is associated with increases in plasma epinephrine (Hawley *et al.*, 2000; Howlett *et al.*, 2001; Watt *et al.*, 2004b) which coincides with a blunting of the exercise-induced increase in PPARα and PPARδ mRNA after prolonged exercise (Watt *et al.*, 2004b). Therefore, in response to NA ingestion in the final 30 min of exercise and throughout recovery, an epinephrine-mediated decline in PPARα and PPARδ activation may have indirectly contributed to the reduction of MCD and PDK4 expression in addition to the direct effect of lowered plasma FFAs during recovery. However, this hypothesis needs to be directly tested in human skeletal muscle.

In addition to plasma-derived FFAs, hydrolysis of IMTG may also be an important source of LCFA supporting the increase in post-exercise fat metabolism (Kiens & Richter, 1998). Using chemical extraction of muscle samples, no change IMTG concentration was observed during recovery despite the marked reduction in circulating FFAs with NA ingestion, supporting the previous hypothesis that plasma-derived FAs are the preferential fuel source for skeletal muscle fat metabolism during recovery from glycogen-lowering exercise (Chapter 3). Although NA

ingestion increased RER and CHO oxidation during recovery as reported previously (Walker *et al.*, 1991; Trost *et al.*, 1997), fat oxidation remained at a similar rate to that observed during CON and IMTG concentration tended to increase with NA. These results indicate that the significant blunting of post-exercise lipolysis did not limit fat oxidation, consistent with the observation that only a small fraction of the substantial adipose tissue lipolysis during post-exercise recovery is taken up by exercised muscle in the lower extremities (Mulla *et al.*, 2000). Indeed, up to 90% of the FAs released during recovery are reesterified in the liver (Wolfe *et al.*, 1990). Furthermore, the absence of a significant decrease in fat oxidation during NA compared with CON highlights the substantial reduction in FA derived energy expenditure during post-exercise recovery. For example, it is plausible that total fat oxidation was reduced by at least 75% after exercise, since maximal rates of fat oxidation measured using indirect calorimetry during exercise are 0.60 $g \cdot min^{-1}$ (Achten *et al.*, 2002) compared with a maximum of only 0.16 $g \cdot min^{-1}$ during the fat infusion trial. Therefore, the large decrease in FA requirement after exercise appears to account for the negligible effect of FA availability on fat oxidation and IMTG concentration during LFA and HFA compared with CON. Surprisingly, IMTG content was not significantly increased in response to high FFA concentrations during Intralipid and heparin infusion. In resting skeletal muscle, increased IMTG concentration has been observed with elevated plasma FAs (Boden *et al.*, 2001), however this difference is most likely attributable to the reduced sensitivity of the muscle biopsy technique in comparison with the non-invasive method of ^{1}H-NMR (Boden *et al.*, 2001).

In conclusion, altering the availability of plasma FFA during recovery induces changes in whole-body substrate oxidation that are unrelated to differences in skeletal muscle malonyl-CoA. Furthermore, fat oxidation and ACCβ phosphorylation appear to be dissociated after exercise, suggesting mechanisms other than phosphorylation-mediated changes in ACC activity have an important role in regulating malonyl-CoA and fat metabolism in human skeletal muscle during post-exercise recovery. Alternative mechanisms regulating post-exercise fat metabolism include citrate and LCFA-CoA mediated changes in ACC activity, or differences in MCD activity. Reducing plasma FFA concentrations with NA attenuated the post-exercise increase in MCD and PDK4 gene expression, suggesting that FAs and/or other factors induced by NA are involved in the regulation of these genes. Despite

marked changes in plasma FFA availability, no significant change in IMTG concentration was detected, providing further evidence that plasma-derived FAs are the preferential fuel source contributing to the enhanced fat oxidation during recovery from glycogen-lowering exercise.

CHAPTER FIVE

EFFECT OF GLUCOSE AVAILABILITY ON THE REGULATION OF FAT METABOLISM IN HUMAN SKELETAL MUSCLE DURING POST-EXERCISE RECOVERY

5.1. INTRODUCTION

Recovery from exercise is characterised by numerous changes in skeletal muscle metabolism that persist for several hours after exercise. In the fasted state, there is substantial free fatty acid (FFA) mobilisation from adipose tissue following exercise (Mulla *et al.*, 2000), resulting in elevated rates of plasma-derived fat oxidation (Devlin *et al.*, 1989; Wolfe *et al.*, 1990; Tuominen *et al.*, 1997; Mulla *et al.*, 2000). Insulin stimulated glucose uptake and glycogen synthase activity are also increased during post-exercise recovery in human skeletal muscle (Mikines *et al.*, 1988; Wojtaszewski *et al.*, 2000a) and this appears to be influenced by the extent of muscle glycogen depletion (Nielsen *et al.*, 2001). Furthermore, skeletal muscle insulin sensitivity is increased at least 48 h after a bout of exercise (Mikines *et al.*, 1988), leading to a rapid rate of glycogen resynthesis when high-carbohydrate foods are consumed following glycogen-depleting exercise (Chapter 3). Collectively, these findings clearly indicate that recovery from prolonged exercise induces a number of changes in skeletal muscle that favour fat oxidation and allow for a high metabolic priority towards glycogen resynthesis.

Potential mechanisms that facilitate an increase in fat metabolism whilst allowing the partitioning of glucose towards glycogen resynthesis in human skeletal muscle remain poorly understood. In the presence of elevated glucose and insulin, a decline in PDHa during post-exercise recovery coincides with a rapid increase in glycogen synthesis and lowered RER values (Chapter 3). In rodent skeletal muscle, an increasing body of evidence suggests that AMPK activation increases post-exercise skeletal muscle fat oxidation by lowering the concentration of malonyl-CoA and thus allowing for enhanced CPT I mediated long-chain FA (LCFA) transport into the mitochondria (Ruderman *et al.*, 2003). AMPK is postulated to decrease malonyl-CoA levels in rodent skeletal muscle through two mechanisms: 1) inactivating acetyl-CoA carboxylase (ACC), the enzyme facilitating an increase in malonyl-CoA synthesis, and 2) increasing malonyl-CoA decarboxylase (MCD) activity, the

enzyme involved in the degradation of malonyl-CoA (Park *et al.*, 2002a). However, in response to altered fat availability in human skeletal muscle, ACCβ and AMPKα phosphorylation appear to be dissociated after exercise, whilst changes in whole-body substrate oxidation are unrelated to differences in malonyl-CoA during the immediate post-exercise recovery period (Chapter 4). To further examine the hypothesis that AMPK regulates post-exercise skeletal muscle fat oxidation, this study examined whether glucose availability induced changes in substrate oxidation during post-exercise recovery that can be explained by an AMPK-mediated change in ACC.

During recovery from glycogen-depleting exercise, intramuscular triglyceride (IMTG) has been identified as an important source of FAs to support the enhanced rate of skeletal muscle fat oxidation (Kiens & Richter, 1998). However, during similar conditions of elevated plasma glucose and insulin (Chapter 3), IMTG appears to have a negligible role in contributing to the enhanced fat oxidation during recovery from exhaustive exercise. Furthermore, post-exercise IMTG concentration does not decline in the presence of reduced FA availability (Chapter 4), supporting the previous hypothesis that plasma-derived FAs are the preferential fuel source for skeletal muscle fat metabolism after glycogen-lowering exercise (Chapter 3). Despite these findings, it is unknown whether IMTG is an important energy substrate for post-exercise skeletal muscle metabolism in the presence of elevated glucose without an associated hyperinsulinemic response.

Increases in metabolic gene transcription relevant to fat and CHO oxidation are well documented during exercise and appear to increase further during the post-exercise recovery period (Kraniou *et al.*, 2000; Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). For many genes, transcriptional activity peaks during the initial recovery phase after endurance exercise and returns to basal levels within a ~24 h period (Pilegaard *et al.*, 2000). Transcriptional activation is followed by increases in mRNA that are observed to peak after 1–4 h of recovery (Kraniou *et al.*, 2000; Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2002). Substrate availability during post-exercise recovery may also be linked with the transcriptional activation of metabolic genes in response to exercise. Reduced muscle glycogen after prolonged exercise is associated with enhanced transcriptional activation and mRNA of PDK4, raising the possibility that signalling mechanisms sensitive to muscle glycogen content may be linked to the

transcriptional control of exercise-responsive genes (Pilegaard *et al.*, 2002). Furthermore, increased PDK4 gene expression is associated with reduced pyruvate dehydrogenase activity (PDHa) during prolonged fasting (Spriet *et al.*, 2004) and may be an important long-term regulator of the PDH complex when there is sustained changes in tissue fat delivery and/or handling (Peters *et al.*, 1998; Sugden & Holness, 2003).

The objectives of this study were to examine the effect of plasma glucose availability during recovery from glycogen-lowering exercise on: (1) the phosphorylation state of ACC and its key regulator AMPK and, (2) the importance of IMTG as an energy substrate, (3) the mRNA abundance of proteins regulating fat (MCD, LPL) and carbohydrate (PDK4, GLUT4) oxidation. In the first of two trials, plasma glucose was elevated using glucose infusion, a physiological intervention that facilitates a large increase in glycemia without an associated hyperinsulinemic response (Perley & Kipnis, 1967). During a second trial, saline was infused as a control over a 6 h recovery period after a strenuous bout of exercise. On the basis that elevated plasma glucose is associated with decreased AMPK activity (Itani *et al.*, 2003), it was hypothesised that post-exercise glucose infusion would reduce both total AMPKα and ACCβ phosphorylation leading to a decrease in fat oxidation compared with the control trial.

5.2. METHODS

Subjects

Seven endurance-trained males, age, 29.4 ± 1.5 years; body mass, 78.1 ± 3.6 kg; peak power output, 5.1 ± 0.2 watts·kg^{-1} and $\dot{V}O_{2\,max}$, 61.6 ± 2.0 ml·kg^{-1}·min^{-1} (mean ± SEM) volunteered to participate in this study. All subjects were considered to be healthy, did not smoke or take any medications, and had no evidence of cardiovascular or metabolic diseases. The experimental procedures and possible risks of the study were explained verbally and in writing to all participants before obtaining their informed and written consent to participate. The study protocol was approved by the Deakin University Human Research Ethics Committee and was performed according to the Declaration of Helsinki.

Pre-experimental protocol

Daily energy intake and composition of each subject's habitual diet was assessed using a 4-day food record (3 weekdays and 1 weekend day). Dietary data (Table 5.1) was analysed for energy intake and macronutrient composition (Food Processor Nutrition Analysis Software, version 7, ESHA Research, Salem OH, USA). One to two weeks prior to testing, maximal oxygen uptake ($\dot{V}O_{2\,max}$) was determined on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Expired air was analysed by O_2 and CO_2 analysers (AEI Technologies, Pittsburgh, PA, USA) and $\dot{V}O_{2\,max}$ determined from a plateau in $\dot{V}O_2$ (increase <2 ml·kg^{-1}·min^{-1}) with a further increase in power output. From the $\dot{V}O_{2\,max}$ test, power outputs corresponding to submaximal $\dot{V}O_2$ values to be used during the experimental trial were determined using linear regression analysis for each subject. All subjects were familiarised with the intensity of the glycogen-lowering protocol and the experimental power outputs verified ~1 week before the experiments. For 2 days prior to experimental trials, subjects abstained from exercise and the consumption of caffeine and alcohol. In addition, on the day before each experimental trial subjects were provided with a high-CHO diet to maximise glycogen stores (Table 5.1).

Experimental protocol

Subjects participated in two randomised, counterbalanced experimental trials [saline infusion (CON) vs. glucose infusion (GLU)], each separated by at least a 1 week period. On each occasion, subjects reported to the laboratory at 0715 after a 10-12 hr

overnight fast. After voiding, body mass was recorded and a teflon catheter inserted into an antecubital vein for blood sampling. Following 30 min of rest in a supine position, resting $\dot{V}O_2$ and $\dot{V}CO_2$ were measured (for determination of the respiratory exchange ratio – RER), and a 5 ml venous blood sample was drawn. Subjects then consumed a light, high-CHO breakfast (Table 5.1). During a subsequent 90 min rest period, three incisions were made over the vastus lateralis muscle under local anesthesia (1% xylocaine solution). Expired gas and blood samples were then obtained and exercise initiated on a Lode Excalibur cycle ergometer. The glycogen-lowering protocol was performed as previously described (Kuipers *et al.*, 1987). This consisted of cycling at 75% $\dot{V}O_{2\,max}$ for 20 min, followed by alternating 2 min bouts of 90% and 50% of $\dot{V}O_{2\,max}$ for 5-10 intervals, then decreasing intensity to 80% and 50% 2-min bouts for another 4-5 intervals, and finishing with 2 min 70% and 50% bouts until 90-min of exercise had been completed. During exercise subjects ingested water *ad libitum*. A blood sample was also taken ~5 min prior to exhaustion. After cessation of exercise, subjects moved from the cycle ergometer to a bench where a percutaneous needle muscle biopsy with suction was obtained from the thigh. Approximately 30 sec lapsed between the termination of the exercise, and the procurement of the muscle sample. Additional muscle biopsies were taken from separate incisions on the same thigh at 3 h and at completion of the 6 h recovery period.

Immediately after the initial muscle sample, a second teflon catheter was inserted into the opposite antecubital vein. Infusion of either a 25% glucose solution at 4 $mg \cdot kg^{-1} \cdot min^{-1}$ (Viaflex Baxter Healthcare, NSW, Australia) (GLU) or 0.9% saline at 1.5 $ml \cdot min^{-1}$ (CON) was then commenced for 6 hours. Blood samples were also obtained at 30 min intervals and breath samples collected every hour according to procedures outlined in Chapter 2. Due to difficulties in collecting respiratory measurements for one subject, RER and substrate oxidation data are presented for six subjects. Water ingestion was allowed *ad libitum* during the recovery period.

Analyses

Blood samples were analysed for whole blood glycerol and plasma FFA, insulin, glucose and lactate according to procedures outlined in Chapter 2. Muscle glycogen,

IMTG, ATP, PCr, creatine, gene expression and AMPKα and ACCβ phosphorylation were analysed as previously described in Chapter 2.

Statistics

Results were analysed using a two-way ANOVA with repeated measures (time x trial) and specific differences were located using a Tukey post hoc test (SigmaStat, version 3). Statistical significance was accepted at $P \leq 0.05$. Values are presented as the mean ± SEM.

5.3. RESULTS

Diet Analysis

Total energy intake in the 2 days immediately prior to each trial was not different from habitual energy intake (Table 5.1). During the day prior to each trial, CHO intake was higher ($P < 0.001$) and fat intake lower ($P < 0.001$) to elevate levels of muscle glycogen.

RER and substrate oxidation

Resting and pre-exercise RER were not different between trials (Table 5.2). During the initial hour of recovery, RER was significantly lower ($P < 0.01$) than rest for the GLU trial only. During recovery, RER was significantly higher at 3 and 6 h ($P < 0.05$) and 5 h ($P < 0.01$) in the GLU trial compared with CON. Fat oxidation during the entire recovery period was significantly higher than CHO oxidation for the CON trial ($P < 0.01$) (Figure 5.1). When compared with CON, CHO oxidation during GLU was significantly greater ($P < 0.01$) and fat oxidation significantly lower ($P < 0.05$) throughout recovery.

Blood responses

Plasma glucose levels were similar at rest, prior to and after exercise for both trials (Figure 5.2B). During recovery, glucose concentrations were significantly greater during GLU compared with CON ($P < 0.01$) and significantly above rest after 2 h ($P < 0.01$) in the GLU trial. Pre-exercise plasma insulin was significantly higher ($P < 0.01$) than rest for both trials as a result of the high-CHO breakfast (Figure 5.2A). Glucose infusion increased plasma insulin throughout recovery in comparison with CON ($P < 0.05$), although the magnitude of this response was small compared with the rapid increase in insulin after oral ingestion of glucose at breakfast. Plasma FFA levels were similar at rest, prior to and immediately after exercise for both trials (Table 5.3). During CON, plasma FFAs increased significantly above rest after 1 h of recovery ($P < 0.001$), whilst elevated glucose levels during GLU significantly reduced plasma FFAs compared with CON ($P < 0.01$). Plasma glycerol responses at the end of exercise were significantly higher than rest for both trials ($P < 0.001$) (Table 5.3). Consistent with FFA responses, glycerol remained elevated above rest throughout recovery in the CON trial ($P < 0.001$, 1, 3 and 6 h; $P < 0.05$, 2 and 5 h) except at 4 h. No change in plasma glycerol was detected during recovery in the GLU trial as a result of glucose infusion. Plasma lactate increased significantly ($P <$

0.01) above rest in response to the high intensity exercise and returned to baseline levels throughout recovery in both trials (Table 5.3).

Muscle metabolites

No difference in muscle glycogen content between trials was observed upon completion of the exercise bout (Table 5.4). During the GLU trial, muscle glycogen increased significantly after 3 h ($P < 0.05$) and 6 h ($P < 0.01$) compared with 0 h. No change in IMTG concentration was detected during recovery for both trials (Table 5.4). Muscle ATP, PCr and creatine also remained unchanged throughout the recovery period (Table 5.4).

ACCβ and AMPK phosphorylation

Immediately after exercise (0 h), ACCβ-P was ~5-fold higher than 3 or 6 h during CON ($P < 0.01$) (Figure 5.3A). An exercise-induced increase in ACCβ-P was also observed for the GLU trial (~3-fold) compared with 3 h ($P < 0.001$) and 6 h ($P < 0.01$), although no treatment effect for ACCβ-P was detected during recovery. No exercise or treatment effect was observed for AMPK-P throughout the recovery period (Figure 5.3B).

Gene expression

Gene expression of the four genes measured during post-exercise recovery relative to the housekeeping gene CYC are presented in Figure 5.4. mRNA abundance of MCD increased by ~3-fold during CON and ~5-fold during GLU ($P < 0.001$) (Figure 5.4A). A ~4.5-fold increase ($P < 0.05$) in LPL mRNA was observed after 6 h of glucose infusion, however this effect was not observed during CON (Figure 5.4B). Glucose infusion induced a ~3-fold increase ($P < 0.05$) in GLUT4 gene expression after 6 h of recovery (Figure 5.4C). No change in GLUT4 mRNA was observed during CON. In response to the exercise bout and elevated FA levels during CON, PDK4 gene expression was markedly increased after 3 and 6 h of recovery (~11-fold, $P < 0.001$) (Figure 5.4D). During glucose infusion, a significant increase in PDK4 mRNA after 3 h was also observed (~8-fold, $P < 0.01$), however PDK4 expression was significantly reduced compared with mRNA levels at 3 h during CON ($P < 0.05$). Furthermore, the exercise induced increase in PDK4 mRNA was abolished during GLU, resulting in significantly higher PDK4 mRNA abundance in CON compared to GLU after 6 h ($P < 0.05$).

Table 5.1. Daily energy and macronutrient intake before post-exercise recovery.

	Habitual	2 days before	1 day before	Trial breakfast
Energy, kJ	14536 ± 1109	13868 ± 1331	14150 ± 571	1281 ± 0
CHO, %	59 ± 0.8	60 ± 1.4	75 ± 1.1*	82 ± 0
Fat, %	25 ± 1.2	25 ± 1.8	11 ± 0.5*	13 ± 0
Protein, %	14 ± 0.4	15 ± 1.4	14 ± 0.8	5 ± 0

Values are means ± S.E.M., $n = 7$. CHO, carbohydrate. * Significantly different from 2 days before and habitual, $P < 0.001$.

Table 5.2. Respiratory exchange ratio values before exercise and during a 6 hour post-exercise recovery period.

Trial	Rest	Pre-exercise	1 h	2 h	3 h	4 h	5 h	6 h
CON	0.77 ± 0.01	0.84 ± 0.03	0.72 ± 0.02	0.77 ± 0.01	0.77 ± 0.02	0.76 ± 0.01	0.73 ± 0.01	0.76 ± 0.01
GLU	0.80 ± 0.01	0.81 ± 0.02	0.71 ± 0.03*	0.80 ± 0.02	0.83 ± 0.01#	0.79 ± 0.01	0.81 ± 0.01†	0.82 ± 0.02#

Values are means ± S.E.M., $n = 6$. * Significantly different from the resting value of the same trial, $P < 0.05$; significantly different from control (CON), # $P < 0.05$, † $P < 0.01$.

Table 5.3. Plasma fatty acids, glycerol and lactate at rest, before exercise, end of exercise and during a 6 hour post-exercise recovery period.

Time (h)	CON			GLU		
	Fatty acids (mmol l^{-1})	Glycerol (mmol l^{-1})	Lactate (mmol l^{-1})	Fatty acids (mmol l^{-1})	Glycerol (mmol l^{-1})	Lactate (mmol l^{-1})
Rest	0.29 ± 0.07	0.06 ± 0.02	1.3 ± 0.2	0.24 ± 0.05	0.07 ± 0.02	1.1 ± 0.1
Pre-exercise	0.16 ± 0.04	0.06 ± 0.02	1.7 ± 0.1	0.15 ± 0.03	0.06 ± 0.02	1.6 ± 0.1
End-exercise	0.50 ± 0.15	0.21 ± 0.04‡	5.6 ± 0.9‡	0.58 ± 0.14	0.21 ± 0.03‡	5.2 ± 0.7‡
1	1.11 ± 0.12‡	0.15 ± 0.04‡	2.0 ± 0.3	0.52 ± 0.07†	0.09 ± 0.02	1.9 ± 0.1
2	1.13 ± 0.10‡	0.12 ± 0.02*	1.3 ± 0.2	0.52 ± 0.05†	0.09 ± 0.02	1.3 ± 0.2
3	1.24 ± 0.07‡	0.14 ± 0.03‡	1.4 ± 0.2	0.49 ± 0.10†	0.09 ± 0.02	1.3 ± 0.1
4	0.82 ± 0.08‡	0.06 ± 0.02	1.2 ± 0.1	0.28 ± 0.02†	0.08 ± 0.02	1.2 ± 0.1
5	0.85 ± 0.09‡	0.11 ± 0.03*	1.1 ± 0.2	0.37 ± 0.04†	0.09 ± 0.02	1.3 ± 0.2
6	1.04 ± 0.11‡	0.13 ± 0.03‡	1.2 ± 0.2	0.53 ± 0.14†	0.10 ± 0.02	1.1 ± 0.1

Values are means ± S.E.M., $n = 7$. Significantly different from the resting value of the same trial, * $P < 0.05$, ‡ $P < 0.001$; † significantly different from CON trial, $P < 0.01$.

Table 5.4. Muscle metabolite concentrations immediately after exercise and during a 6 hour post-exercise recovery period.

	CON			GLU		
Metabolite	0 h	3 h	6 h	0 h	3 h	6 h
ATP	24.4 ± 1.0	24.5 ± 0.9	25.8 ± 1.1	24.1 ± 0.6	24.7 ± 0.5	24.3 ± 0.7
PCr	91.4 ± 5.6	90.6 ± 3.3	88.3 ± 3.2	82.2 ± 4.3	85.2 ± 3.3	85.7 ± 1.4
Creatine	52.5 ± 4.9	53.3 ± 3.4	55.6 ± 2.7	61.8 ± 3.4	58.6 ± 2.8	58.1 ± 2.7
Glycogen	153 ± 26	155 ± 34	152 ± 25	125 ± 19	171 ± 28*	185 ± 27†
IMTG	47.9 ± 7.6	51.0 ± 7.1	51.9 ± 7.6	49.0 ± 8.1	42.5 ± 4.7	39.2 ± 6.3

Values are means ± S.E.M., $n = 7$. All values are expressed as mmol kg^{-1} d.m. Significantly different from 0 h, * $P < 0.05$, † $P < 0.01$.

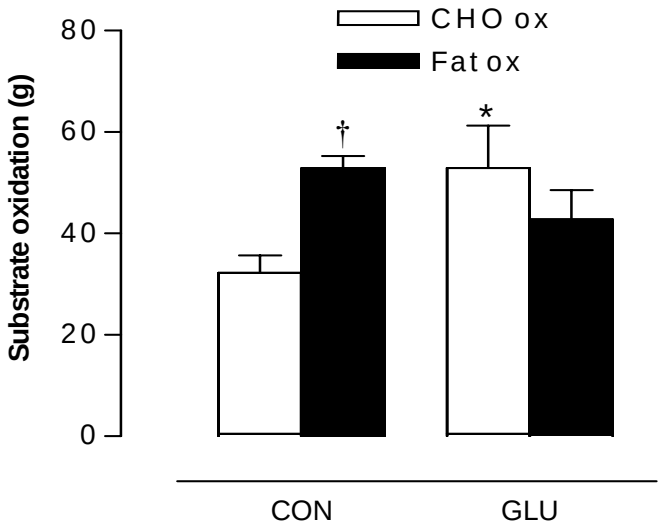

Figure 5.1. Carbohydrate (CHO) and fat oxidation during a 6 h recovery period with either saline (CON) or glucose infusion (GLU). Values are means ± S.E.M. for 6 subjects. * Significantly different from CON trial, $P < 0.01$; † significantly different from CHO oxidation, $P < 0.01$.

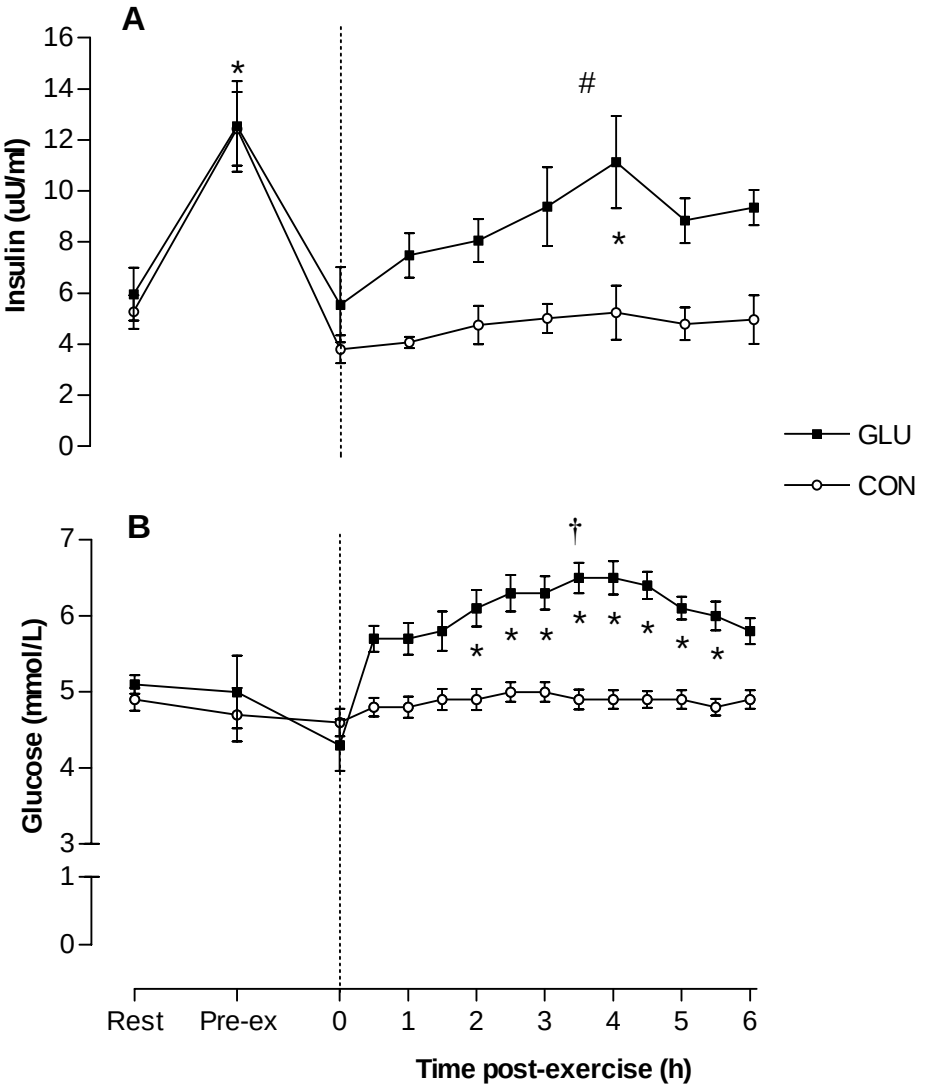

Figure 5.2. Plasma insulin (A) and glucose (B) concentrations at rest, before and after exercise and during a 6 h recovery period with either saline (CON) or glucose infusion (GLU). Values are means ± S.E.M. for 7 subjects. * Significantly different from the resting value of the same trial, $P < 0.01$; significantly different from CON trial, # $P < 0.05$, † $P < 0.01$.

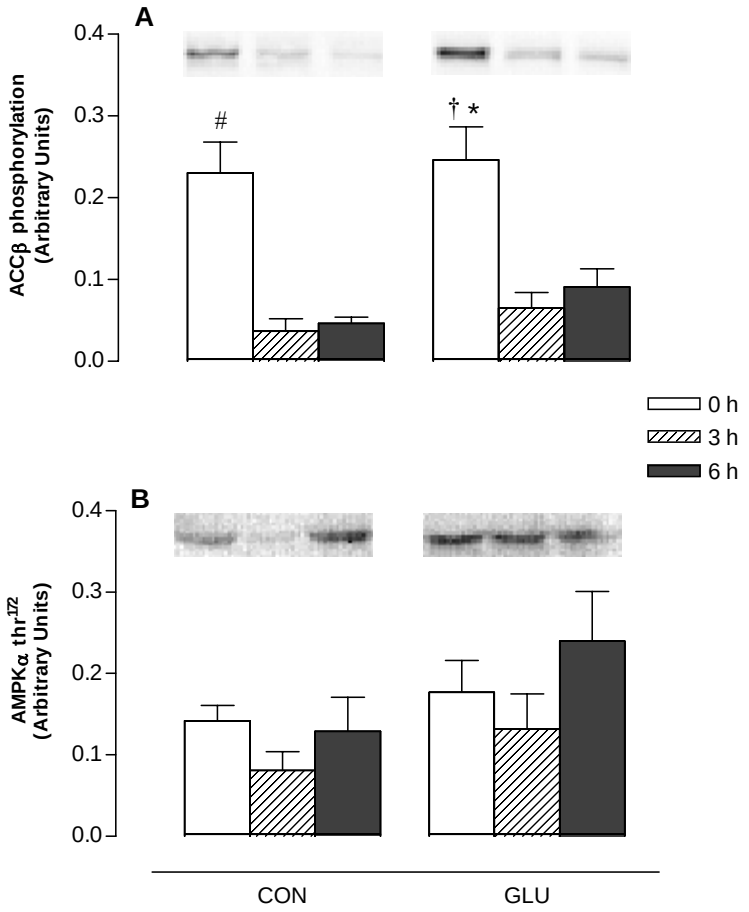

Figure 5.3. Acetyl-CoA carboxylase-β (ACCβ) (A) and AMP-activated protein kinaseα threonine 172 phosphorlyation (AMPK) (B) immediately after exercise (0 h) and during a 6 h recovery period with either saline (CON) or glucose infusion (GLU). Values are means ± S.E.M. for 7 subjects. # Significantly different from 3 and 6 h, $P < 0.001$, † significantly different from 3 h, $P < 0.001$; * significantly different from 6 h, $P < 0.01$.

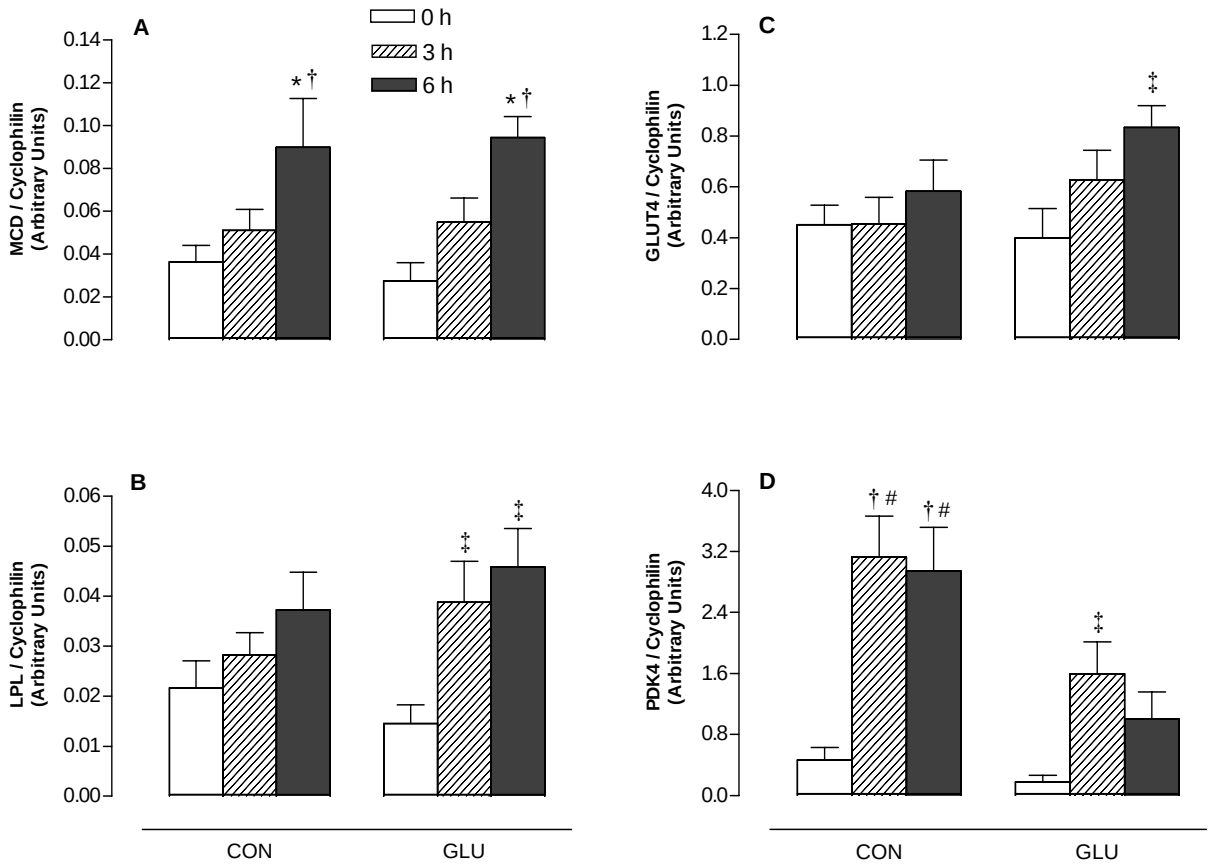

Figure 5.4. mRNA abundance of MCD (A), LPL (B), GLUT4 (C) and PDK4 (D) immediately after exercise (0 h) and during a 6 h recovery period with either saline (CON) or glucose infusion (GLU).Values are means ± S.E.M. for 7 subjects. Significantly different from 0 h, ‡ $P < 0.05$, † $P < 0.001$; * significantly different from 3 h, $P < 0.05$; # significantly different from GLU trial, $P < 0.05$.

5.4. DISCUSSION

After a single bout of intense, dynamic exercise in trained and untrained humans, the recovering skeletal muscle relies primarily on FAs as an oxidative energy source. However, the cellular and molecular mechanisms facilitating the increase in skeletal muscle fat oxidation following prolonged exercise remain poorly defined. This study investigated the effect of glycogen-lowering exercise on the regulation of fat metabolism with or without hyperglycemia. In response to increased glucose availability (GLU), CHO oxidation was significantly higher during recovery compared with the fasted state (CON). However, in contrast with the study hypothesis, a decrease in fat oxidation or an associated reduction in total AMPKα or ACCβ phosphorylation was not observed during GLU in comparison with the CON trial. Despite the relatively high rates of fat oxidation and small rise in plasma insulin during GLU and suppression of plasma FAs in comparison with CON, a significant decline in IMTG concentration was not detected. In addition, novel changes in gene expression during post-exercise recovery were observed, of which the most notable was the significant reduction in PDK4 gene expression in the presence of elevated plasma glucose.

In the immediate recovery period after prolonged exercise, skeletal muscle glycogen resynthesis is of high metabolic priority. In accordance with this, muscle glycogen content appears to be an important regulator of contraction- and insulin-induced muscle glucose transport in addition to its well known effect on glycogen synthase activity (Richter *et al.*, 2001). Furthermore, the magnitude of muscle glycogen depletion during exercise influences the utilisation of long-chain FAs as an energy substrate (Blomstrand & Saltin, 1999). However, the mechanisms behind these exercise- and glycogen-induced effects are not well characterised. AMPK is increasingly being recognised as an important energy-sensing molecule with putative effects on skeletal muscle metabolism. In rodent skeletal muscle, AMPK is considered to play a major role in regulating fat metabolism during post-exercise recovery (Ruderman *et al.*, 2003). Following high-intensity or prolonged exercise, isoform-specific changes in AMPK activity are observed in human skeletal muscle. These include a rapid decrease in AMPKα_2 activity to near resting levels together with unchanged AMPKα_1 activation (Wojtaszewski *et al.*, 2000b; Wojtaszewski *et al.*, 2002; Wojtaszewski *et al.*, 2003), whilst total AMPKα phosphorylation also decreases (Wojtaszewski *et al.*, 2002). In this study, AMPKα-P, which is correlated

with total AMPKα activity in rodent (Park *et al.*, 2002b) and human (Wojtaszewski *et al.*, 2002; Clark *et al.*, 2004) skeletal muscle, did not decline during a 6 h recovery period in either trial. Although a resting biopsy was not obtained in this study, it can be inferred from previous studies that AMPKα-P was elevated immediately after exercise (Wojtaszewski *et al.*, 2000b; Wojtaszewski *et al.*, 2003; Yu *et al.*, 2003; Clark *et al.*, 2004). Therefore, in accordance with Chapter 4, although AMPKα-P was similar throughout recovery in both trials, AMPKα activation is unlikely to have remained above resting levels during recovery. In support of this hypothesis, a reduced AMP/ATP ratio and stable ATP demand under resting conditions during recovery would be expected to keep AMPK in its inactive (dephosphorylated) form. ACCβ-P, which is inversely related to ACC activity in rat muscle (Park *et al.*, 2002b), rapidly declined after 3 h and remained unchanged after 6 h of recovery in both trials. These findings demonstrate that ACCβ-P is very exercise responsive (Chen *et al.*, 2003; Clark *et al.*, 2004), particularly in the presence of reduced muscle glycogen (Wojtaszewski *et al.*, 2003). Furthermore, in contrast to Chapter 4, no dissociation between ACCβ-P and fat oxidation was observed in either trial, since no difference in fat oxidation between trials was apparent.

After prolonged, glycogen-lowering exercise with elevated glucose and insulin to facilitate rapid glycogen resynthesis, the role of IMTG for energy provision during post-exercise recovery remains equivocal. Using glucose infusion to induce hyperglycemia without hyperinsulinemia during recovery, a small but non-significant decline in IMTG concentration was observed, whilst no change was detected in the CON trial. These results concur with chapters 3 and 4 and support the hypothesis that plasma-derived FAs are the preferential fuel source for fat oxidation during recovery from glycogen-lowering exercise. Evidence from isolated rodent skeletal muscle suggests IMTG is a dynamic substrate (Dyck & Bonen, 1998) that is regulated by the rate-limiting enzyme hormone-sensitive lipase (HSL) (Langfort *et al.*, 1998). The regulation of skeletal muscle HSL during exercise is complex and is thought to involve both β-adrenergic and perhaps more importantly AMPK-mediated mechanisms (Watt *et al.*, 2004c). Indeed, the physiological importance of activating HSL for triacylglycerol hydrolysis is uncertain (Watt *et al.*, 2004c) and requires further investigation during exercise and the immediate recovery period. It should also be noted that the muscle biopsy technique is unable to account for triacylglycerol (TG) and/or FA cycling. Thus, the possibility exists that TG

resynthesis during recovery may have masked the utilisation of TG as an oxidative fuel. Furthermore, variation in IMTG storage and contamination from TG between muscle fibres must also be considered, although the use of endurance-trained subjects in this study is likely to markedly reduce these measurement errors (Watt *et al.*, 2002).

To further understand the molecular mechanisms regulating substrate metabolism after glycogen-lowering exercise, genes pertinent to CHO and fat oxidation were measured. The major finding was the significant reduction in exercise-induced PDK4 gene expression after 3 and 6 h of recovery during glucose infusion. PDK is a regulatory enzyme in skeletal muscle that phosphorylates and inactivates the E1α-subunit of the PDH complex. During conditions of reduced glycolytic flux and increased FA oxidation, PDK activity and PDK4 mRNA are elevated in resting human skeletal muscle (Peters *et al.*, 1998). Indeed, PDK4 is modified when there is a sustained change in tissue FA delivery and/or handling and is hypothesised to be a "lipid status"-responsive PDK isoform (Sugden *et al.*, 2001). In support of this hypothesis, reducing plasma FFA levels with glucose infusion lowered the exercise-induced increase in PDK4 mRNA observed during the CON trial. Interestingly, despite an enhanced suppression of plasma FAs with nicotinic acid (NA), a transient reduction in PDK4 expression during post-exercise recovery was observed (Chapter 4). This discrepancy is most likely a result of the elevated insulin in the present study which is known to decrease the abundance of PDK4 mRNA (Huang *et al.*, 2002). Although insufficient muscle tissue prevented the measurement of PDHa in this study, the progressive increase in PDK4 mRNA during prolonged fasting in humans has been associated with a decline in PDHa (Spriet *et al.*, 2004). Therefore, the possibility exists that attenuated expression of PDK4 also reduced PDK activity and thus maintained PDHa to account for the higher rates of CHO oxidation observed during GLU compared with the CON trial. In support of this postulate, Chapter 3 demonstrated that reduced FFA levels in response to high-CHO ingestion prevented a significant decline in PDHa after 3 h of post-exercise recovery.

Elevated plasma FA levels are associated with increased skeletal muscle MCD mRNA and activity in oxidative (soleus) muscle (Young *et al.*, 2001). In human skeletal muscle, lowering plasma FAs using NA reduces the expression of MCD compared with fat infusion and control trials during post-exercise recovery (Chapter

4). Therefore, FAs appear to be involved in the regulation of MCD as well as PDK4 gene expression in human skeletal muscle. Consistent with these data, the present study indicates that high FFA levels during 6 h of post-exercise recovery in the fasted state increased skeletal muscle MCD gene expression by ~2.5-fold. However, reducing FFA availability with glucose infusion did not suppress the post-exercise increase in MCD mRNA abundance as observed with NA ingestion (Chapter 4). Since plasma FAs were significantly lower with NA compared to GLU throughout recovery except at 4 and 5 h (data not shown), the regulation of MCD expression may be sensitive to plasma FAs above resting levels or NA may suppress MCD mRNA through a mechanism unrelated to reduced FA availability. In support of the latter postulate, NA also increases plasma epinephrine which coincides with a blunting of the exercise-induced increase in PPARα and PPARδ mRNA after prolonged exercise (Watt *et al.*, 2004a). Therefore, one inviting hypothesis is that an epinephrine-mediated decline in PPARα and PPARδ activation may have indirectly contributed to the reduction of MCD expression in Chapter 4, together with an additive effect of lowered plasma FFA levels during recovery. However, this hypothesis needs to be directly tested in human skeletal muscle.

GLUT4 is a major regulatory protein involved in skeletal muscle glucose uptake and disposal. The increased post-exercise GLUT4 protein expression is controlled by both pretranslational and translational mechanisms that leads to an increase in muscle glucose uptake in accordance with glucose availability (Kuo *et al.*, 1999a; Kuo *et al.*, 1999b). After a single bout of moderate-intensity exercise, GLUT4 mRNA is increased and remains elevated for up to 3 hours in recovery (Kraniou *et al.*, 2000). This study has also demonstrated that GLUT4 gene expression is unchanged after 6 h of post-exercise recovery and extend these data to show that despite an upregulation of GLUT4 mRNA with increased glucose availability, no difference was observed between GLU or CON. In rodent skeletal muscle, CHO supplementation attenuates the exercise-induced GLUT4 gene expression for up to 42 h after exercise (Kuo *et al.*, 1999b; Garcia-Roves *et al.*, 2003). The discrepancy in these findings may result from a lowered insulin response during glucose infusion compared with oral CHO feeding, although this hypothesis needs verifying in human skeletal muscle. Based on the absence of a treatment effect, it appears unlikely that changes in GLUT4 gene expression contributed to the enhanced glycogen resynthesis observed during GLU.

Acute exercise transiently increases LPL gene expression in untrained individuals (Seip *et al.*, 1997), whilst no difference in exercise-induced LPL activity was reported during a euglycemic hyperinsulinemic clamp after exercise compared with saline infusion (Kiens *et al.*, 1989). No effect of hyperglycemia on LPL mRNA relative to saline infusion was demonstrated in this study, although a time effect was observed during GLU. Muscle LPL activity is increased in well-trained individuals after glycogen-depleting exercise that coincides with the use of IMTG as an energy source during recovery (Kiens & Richter, 1998). Although this study found no treatment effect of elevated plasma glucose on LPL gene expression, the possibility exists that LPL-mediated breakdown of very-low density lipoprotein triglyceride (VLDL TG) in muscle provide supplementary long-chain FAs as an oxidative fuel in addition to plasma-derived FAs during recovery.

In summary, increased glucose availability after high-intensity exercise induces a significant increase in CHO oxidation compared with the fasted state, although no differences in whole-body fat oxidation were apparent. Although AMPKα phosphorylation remained elevated during recovery, it is unlikely that AMPK activity was sustained throughout the 6 h recovery period. No change in IMTG concentration was observed, supporting the hypothesis that plasma-derived FAs are the preferential fuel source for fat oxidation during recovery from glycogen-lowering exercise. The large exercise-induced PDK4 gene expression was attenuated when plasma FFAs were reduced during GLU, supporting the hypothesis that PDK4 is responsive to sustained changes in lipid availability and/or changes in plasma insulin. Furthermore, the possibility exists that the suppression of PDK4 mRNA also reduced PDK activity and thus maintained PDHa to account for the higher rates of CHO oxidation observed during GLU compared with the CON trial.

CHAPTER SIX

DISCUSSION OF FINDINGS AND FUTURE DIRECTIONS

6.1 DISCUSSION OF FINDINGS

Recovery from prolonged or high-intensity exercise is characterised by an increased reliance on plasma-derived FAs as an oxidative fuel and a partitioning of glucose towards glycogen resynthesis. In response to altering fat and CHO availability after glycogen-lowering exercise in this thesis, RER and calculated rates of whole body substrate utilisation indicated FAs were a major fuel source. This finding is consistent with previous studies that have examined fat oxidation in the presence of reduced glycogen after exercise (Bielinski *et al.*, 1985; Devlin *et al.*, 1989; Wolfe *et al.*, 1990; Schrauwen *et al.*, 1997; Tuominen *et al.*, 1997; Kiens & Richter, 1998). Furthermore, the results of this thesis support the hypothesis that recovery from prolonged exercise induces a number of changes in skeletal muscle that allow for a high metabolic priority towards glycogen resynthesis (Richter *et al.*, 2001). Despite the well reported finding that skeletal muscle fat oxidation is enhanced after exercise, the cellular and molecular mechanisms facilitating this effect remain poorly defined. To further our understanding of the primary factors regulating both fat and CHO metabolism after exercise, this thesis examined alterations in metabolic protein activity, content and gene expression in human skeletal muscle.

An increasing body of evidence suggests that exercise-induced activation of AMPK appears to co-ordinately regulate a decrease in ACC and increase in MCD activity during post-exercise recovery (Park *et al.*, 2002a; Ruderman *et al.*, 2003). Such coordinated changes in MCD and ACC attributable to AMPK are suggested to lower malonyl-CoA concentration and thus allow for enhanced CPT I mediated LCFA transport into the mitochondria (Park *et al.*, 2002a; Ruderman *et al.*, 2003). However, whilst this mechanism is evident in rodent muscle (Ruderman *et al.*, 2003), it is unknown whether AMPK mediates an increase post-exercise fat oxidation in human skeletal muscle. Therefore, one aim of this thesis was to examine malonyl-CoA concentration together with ACCβ and AMPKα phosphorylation after exercise in response to altered plasma FFA availability in human muscle.

The first important finding of these studies is that differences in whole-body fat oxidation in response to acute alterations in plasma FFA availability after exercise are unrelated to changes in malonyl-CoA concentration. This result is in contrast to the important role of malonyl-CoA in regulating FA oxidation in resting human muscle (Bavenholm *et al.*, 2000; Rasmussen *et al.*, 2002) and in rodent muscle after exercise (Rasmussen *et al.*, 1998; Park *et al.*, 2002a). Thus, the data suggest that in human skeletal muscle, malonyl-CoA is not a primary regulator of fat metabolism during the immediate post-exercise recovery period. During exercise, available evidence also suggests that changes in malonyl-CoA concentration in human skeletal muscle do not play a major regulatory role in controlling LCFA oxidation (Kiens & Roepstorff, 2003). Furthermore, the regulation of CPT I activity appears more complex than simple changes in malonyl-CoA levels and/or that malonyl-CoA inhibition is overridden during exercise (Bezaire *et al.*, 2004). Clearly, the role of malonyl-CoA as a regulator of human skeletal muscle fat metabolism after exercise remains uncertain and requires further study.

During recovery from glycogen-lowering exercise, no change in AMPKα phosphorylation (AMPKα-P) was observed with altered fat or CHO availability. Although pre-exercise measurements were not taken, it can be inferred from previous studies that both AMPKα-P and ACCβ phosphorylation (ACCβ-P) were increased in response to glycogen-lowering exercise (Wojtaszewski *et al.*, 2000; Wojtaszewski *et al.*, 2003; Yu *et al.*, 2003). However, previous studies suggest that AMPK activation is unlikely to have remained above resting levels during recovery (Wojtaszewski *et al.*, 2000; Wojtaszewski *et al.*, 2002; Wojtaszewski *et al.*, 2003) and thus may account for the lack of increase in ACCβ-P (Chapters 4 and 5). Furthermore, the low and stable ATP demand observed during these studies would be expected to keep AMPK in its inactive form. Of further significance in this thesis was the absence of change in ACCβ-P after 3 h of recovery despite the difference in fat oxidation between fat infusion and NA ingestion in Chapter 4. It appears evident from this finding that ACCβ-P and fat oxidation are dissociated in human skeletal muscle during post-exercise recovery. Thus, factors other than the phosphorylation state of ACCβ appear to regulate ACC activity and subsequently malonyl-CoA in human skeletal muscle. These factors may include the cytosolic concentration of citrate and LCFA-CoA, or that ACCβ-P is affected by other inhibitors (e.g., by phosphatase activity).

It should also be noted that although AMPK and ACC activity was not measured in this thesis, AMPKα-P is correlated with total AMPK activity in rodent (Park *et al.*, 2002b) and human (Wojtaszewski *et al.*, 2002; Clark *et al.*, 2004) skeletal muscle, whilst ACCβ-P is inversely related to ACC activity in rat muscle (Park *et al.*, 2002b). Therefore, the AMPKα and ACCβ phosphorylation data presented in this thesis appears to be a valid representation of AMPK and ACC activity during post-exercise recovery. Furthermore, AMPK and ACC total protein concentrations were not determined due to technical difficulties. However, total protein for AMPK and ACC remains stable in response to exercise and substrate availability interventions (McGee *et al.*, 2003; Wojtaszewski *et al.*, 2003; Clark *et al.*, 2004), providing further support that AMPK and ACC phosphorylation measures are indicative of protein activity and not changes in protein content.

In addition to plasma-derived FAs, IMTG hydrolysis is thought to be an important source of LCFAs supporting the increase in fat metabolism after exercise in the presence of elevated glucose and insulin (Kiens & Richter, 1998). However, using a protocol that essentially replicated this study, IMTG concentration was demonstrated to have a negligible role in contributing to the enhanced fat oxidation after exhaustive exercise (Chapter 3). Furthermore, despite the marked reduction in plasma FFA concentration with NA ingestion, fat oxidation remained similar to that observed during the control trial and IMTG concentration tended to increase (Chapter 4). These results suggest that FA requirement is substantially reduced after exhaustive exercise and this accounts for the negligible effect of reduced FA availability on lowering fat oxidation. Interestingly, elevated plasma FFAs during Intralipid and heparin infusion did not increase IMTG concentration as previously demonstrated in human skeletal muscle using the non-invasive method of ^{1}H-NMR (Boden *et al.*, 2001). No change in IMTG concentration was also observed during conditions of hyperglycemia without hyperinsulinemia after exercise (Chapter 5). Therefore, these data support the hypothesis that plasma-derived FFAs are the preferential fuel source contributing to the enhanced fat oxidation during recovery from glycogen-lowering exercise. Mechanisms regulating IMTG hydrolysis were not investigated in this thesis, although they are complex and involve both β-adrenergic and perhaps more importantly AMPK-mediated mechanisms (Watt *et al.*, 2004b).

Regulation of skeletal muscle CHO metabolism following glycogen-lowering exercise has also been studied in this thesis. In response to increased glucose availability, CHO oxidation was significantly higher during recovery compared with the fasted state (Chapter 5). Of particular significance was the novel measurement of skeletal muscle PDHa during post-exercise recovery (Chapter 3). After a 3 h recovery period, PDHa was lower but not significantly different from immediately post-exercise, followed by a return to basal levels after 6 h with high-CHO ingestion. Although PDHa remained elevated during early post-exercise recovery, the decline was consistent with a rapid increase in glycogen resynthesis and high rates of fat oxidation, supporting the theory that muscle glycogen replenishment has high metabolic priority. The possibility also exists that maintenance of PDHa during the early phase of recovery facilitated the increase in CHO oxidation during glucose infusion compared with control (Chapter 5). Furthermore, the importance of PDHa as a regulator of skeletal muscle substrate oxidation is demonstrated by the ability of short-term (2-3 days) high-CHO feeding to increase PDHa and CHO oxidation at rest and during moderate-intensity exercise compared with a high-fat intake (Putman *et al.*, 1993; Arkinstall *et al.*, unpublished data).

To further understand the molecular mechanisms regulating substrate metabolism after glycogen-lowering exercise, genes pertinent to CHO and fat oxidation were measured. Novel changes in gene expression during post-exercise recovery were observed, of which the most notable were a significant reduction in MCD and PDK4 gene expression in the presence of reduced FFA availability. Indeed, it was demonstrated that lowering plasma FAs after exercise with NA ingestion suppresses MCD mRNA compared with elevated plasma FFAs during control and fat infusion trials (Chapter 4). These results confirm the hypothesis that FAs are involved in the regulation of rodent skeletal muscle MCD gene expression (Young *et al.*, 2001) and extend these data to suggest that FAs are also involved in the regulation of MCD mRNA in human skeletal muscle. However, the effect of altered MCD mRNA during recovery on MCD activation is uncertain, since MCD activity was not determined in this thesis or in previous studies investigating post-exercise metabolism in humans. Interestingly, reducing FFA availability with glucose infusion did not suppress the post-exercise increase in MCD mRNA abundance (Chapter 5). This raises the possibility that NA may have suppressed MCD mRNA through a mechanism unrelated to reduced FA availability. In support of this

hypothesis, NA also increases plasma epinephrine which coincides with an attenuation of the exercise-induced increase in PPARα and PPARδ mRNA after prolonged exercise (Watt *et al.*, 2004a). Therefore, one inviting hypothesis is that an epinephrine-mediated decline in PPARα and PPARδ activation may have indirectly contributed to the reduction of MCD expression in Chapter 4, together with an additive effect of lowered plasma FFA levels during recovery. Clearly, further study is required to directly test this hypothesis in human skeletal muscle.

Expression of PDK4 is modified when there is a sustained change in tissue FA delivery and/or handling (Wu *et al.*, 1999; Holness *et al.*, 2002; Spriet *et al.*, 2004) and is hypothesised to be a "lipid status"-responsive PDK isoform (Sugden *et al.*, 2001). Consistent with these data, lowering plasma FFA levels with glucose infusion induced a prolonged suppression of PDK4 mRNA during recovery (Study III), whilst this effect was only transient with NA ingestion (Chapter 4). Since insulin lowers PDK4 gene expression (Huang *et al.*, 2002), these are the first data in human skeletal muscle to demonstrate that a sustained reduction in PDK4 mRNA after exercise results from an additive effect of decreased FA availability and increased insulin secretion. Furthermore, the progressive increase in PDK4 mRNA during prolonged fasting in humans has been associated with a decline in PDHa (Spriet *et al.*, 2004). This finding raises the possibility that attenuated expression of PDK4 also reduced PDK activity and thus maintained PDHa to account for the higher rates of CHO oxidation observed during glucose infusion and NA ingestion. In support of this postulate, Chapter 3 demonstrated that reduced FFA levels in response to high-CHO ingestion prevented a significant decline in PDHa after 3 h of post-exercise recovery.

Collectively, these findings demonstrate that alterations in fat and glucose availability after glycogen-lowering exercise induce significant changes in substrate oxidation and the transcription of important metabolic genes within skeletal muscle. However, manipulating FA and glucose availability had no additional effect on the exercise-induced phosphorylation state of AMPKα or ACCβ during recovery. Moreover, it appears that factors other than malonyl-CoA concentration have a major regulatory role in controlling LCFA oxidation during post-exercise recovery in human skeletal muscle. Large differences in FA, glucose and insulin availability had no impact on the degradation of IMTG after exercise. This highlights the reliance on

plasma-derived FAs to support the enhanced rate of fat oxidation after glycogen-lowering exercise.

6.2 FUTURE DIRECTIONS

Although the mechanisms regulating fat metabolism during short and long-term exercise in humans have received considerable attention, relatively little is known about the regulation of FA oxidation during post-exercise recovery in human skeletal muscle. Clearly further studies are required to examine the transcriptional, translational and post-translational events involved in the metabolic adaptive process to acute exercise and nutritional stimuli. This series of studies has focused on the effect of substrate manipulation after glycogen-lowering exercise and attempted to integrate changes in whole body CHO and fat oxidation with changes in substrate concentration, protein content and activity. The abundance of selected genes was also determined in this thesis, although whether changes in gene expression translate to changes in protein content and activity remains unknown.

Future studies are required to determine the extent to which the activities of key enzymes regulating fat metabolism such as MCD, ACC, AMPK, HSL, and CPT I are altered in response to substrate availability after exercise in human skeletal muscle. The importance of key factors regulating these enzymes such as malonyl-CoA, carnitine, citrate and LCFA-CoA also require further investigation. Integrating changes in mRNA abundance with changes in protein content and activity would also provide further information on the immediate and long-term regulation of these proteins by exercise and substrate provision. Furthermore, signalling mechanisms sensitive to muscle glycogen content may be linked to the transcriptional control of exercise-responsive genes (Pilegaard *et al.*, 2002) and to the activity of AMPKα (Wojtaszewski *et al.*, 2003). Therefore, further studies are warranted to examine the effect of post-exercise muscle glycogen content on metabolic gene expression and the activities of key enzymes regulating LCFA oxidation in humans. The effect of endurance exercise training on metabolic responses during recovery is another area of future study, since differences in gene transcription are observed in trained skeletal muscle after exercise (Pilegaard *et al.*, 2003). Examining the mechanisms for FA-induced changes in metabolic gene transcription during post-exercise recovery would also provide valuable information about the molecular regulation of human skeletal muscle metabolism. In particular, determining the importance of FA

sensitive coactivator molecules such as PPARα and δ and forkhead type transcription factors (FOXO), together with the PPARγ coactivator 1α (PGC1α) in response to exercise and substrate supply will allow a greater understanding of the cellular adaptation to exercise. Subsequent studies should also include a pre-exercise muscle biopsy to establish valid baseline measures for comparison with post-exercise data.

This series of studies utilised the muscle biopsy technique to determine the degradation or esterification of IMTG in the vastus lateralis muscle after exercise. A more sensitive measure of FA kinetics during exercise and the subsequent recovery period is stable isotope infusion, which allows for the calculation of whole body fat oxidation, lipolysis and reesterification (Wolfe *et al.*, 1990). Furthermore, isotope infusion enables the quantification of IMTG utilisation from the difference between total fat oxidation and the rate plasma FFA oxidation (Romijn *et al.*, 1993; Klein *et al.*, 1994; Romijn *et al.*, 1995; van Loon *et al.*, 2001), since the contribution from circulating VLDL under normal dietary conditions is typically less than 10% of total fat metabolism (Helge *et al.*, 2001). Thus, stable isotope infusion during a 5-6 h post-exercise recovery period would provide a comprehensive analysis of the relative contribution of plasma and intramuscular derived FAs towards oxidative energy production.

Recently, the use of magnetic resonance spectroscopy (^{1}H-MRS) has received considerable attention is an alternative technique for the non-invasive quantification of IMTG in human skeletal muscle in response to substrate manipulation after exercise (Boesch *et al.*, 1997; Boesch *et al.*, 1999; Decombaz *et al.*, 2000; Krssak *et al.*, 2000; Decombaz *et al.*, 2001; Larson-Meyer *et al.*, 2002; Van Loon *et al.*, 2003). Importantly, ^{1}H-MRS technology permits the accurate quantitative assessment of biologically relevant changes in IMTG for a wide spectrum of populations and also allows for the determination of IMTG across several muscle groups. A majority of ^{1}H-MRS studies have examined IMTG replenishment during a prolonged recovery period after exercise (1-3 days), however a more detailed time course of IMTG hydrolysis or resynthesis in the presence of altered CHO and fat availability requires further study. Using ^{1}H-NMR to examine whether IMTG oxidation is greater in females compared with males after exercise is also of interest, since recent evidence suggests that females utilise more IMTG during exercise than males (Steffensen *et*

al., 2002). Moreover, ^{1}H-MRS together with muscle biopsies from the vastus lateralis would enable simultaneous analysis of gene and enzyme activation that have key roles in regulating FFA and IMTG metabolism during post-exercise recovery.

6.3 CONCLUSIONS

In summary, the results of the studies presented in this thesis are as follows:

1. IMTG hydrolysis appears to make a negligible contribution to the enhanced fat oxidation during post-exercise recovery in the presence of altered fat and CHO availability.

2. Despite the maintenance of PDHa after 3 h of post-exercise recovery, muscle glycogen resynthesis is rapid and fat oxidation is increased, indicating that glycogen replenishment has high metabolic priority in human skeletal muscle.

3. Changes in whole body fat oxidation after exercise are unrelated to levels of malonyl-CoA in human skeletal muscle, suggesting that malonyl-CoA is not a primary regulator of fat metabolism.

4. ACCβ-P remained unchanged despite significant differences in fat oxidation with altered fat availability, suggesting there is dissociation between ACCβ-P and fat oxidation in humans during post-exercise recovery.

5. The lack of association between ACCβ-P and malonyl-CoA with altered fat availability suggests that factors other than the phosphorylation state of ACCβ are regulating ACC activity and thus malonyl-CoA in human skeletal muscle. These factors could include the cytosolic concentration of citrate, LCFA-CoA or differences in MCD activity.

6. Reducing plasma FFA levels with NA suppressed the post-exercise increase in MCD mRNA, suggesting that FAs and/or other factors induced by NA are involved in the regulation of MCD gene expression in human skeletal muscle.

7. Glycogen-lowering exercise induces a large increase in PDK4 gene expression that is attenuated in response to a reduction in post-exercise plasma FFA

concentration and/or elevated plasma insulin. Reduced expression of PDK4 may have also reduced PDK activity and thus maintained PDHa to account for the higher rates of CHO oxidation observed during glucose infusion and NA ingestion.

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APPENDIX 1

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