THE ROLE OF THE PYRUVATE DEHYDROGENASE COMPLEX IN THE REGULATION OF HUMAN SKELETAL MUSCLE FUEL METABOLISM

by

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Abstract

The pyruvate dehydrogenase complex (PDC) is the rate limiting step in the entry of glucose derived pyruvate into the tricarboxylic acid (TCA) cycle. As such it plays an important role in the control of the use of carbohydrate as the source of oxidative energy for skeletal muscle contraction.

The first experimental chapter investigates the effect of dichloroacetate pre-treatment during low-intensity (<60% VO_2max) exercise, below which it is suggested that increasing PDC activation and resting acetyl group availability via dichloroacetate (DCA) pre-treatment will be ineffective at reducing non-oxidative ATP production and improving contractile function. Despite a significant increase in both PDC activation ($p<0.01$) and acetylcarnitine availability ($p<0.01$) prior to the onset of exercise following DCA infusion, there was no difference in substrate level phosphorylation detected during exercise.

The following experimental chapter examines the link between blood lactate concentration and the onset of the ventilatory threshold. Infusion of DCA (50mg.kg^{-1}) prior to the onset of incremental exercise lead to a significant reduction in resting blood lactate ($p<0.05$), but this was not preserved during the following bout of incremental cycling exercise commencing at 50% VO_2max. There was also no alteration in the onset of the ventilatory threshold detected after DCA pre-treatment.

The final experimental chapter has investigated the effect of DCA infusion (50 $mg.kg^{-1}$) upon high-fat diet induced PDC inhibition during exercise. During moderate cycling exercise (75% VO_2max) DCA infusion reversed the high-fat induced inhibition of PDC activation. DCA infusion reduced the metabolic inertia present at the onset of contraction, through both PDC activation and pooling of acetyl groups prior to contraction.

This thesis has highlighted the role of the PDC as an important site of regulation of human skeletal muscle fuel metabolism, which may provide a novel target for the treatment of the metabolic syndrome.

293 words.

Declaration.

Work presented in this thesis was carried out at the School of Biomedical Sciences, University of Nottingham between February 2003 and 2008.

This thesis represents my own work, except where assistance has been acknowledged.

The analysis of muscle metabolites in **Chapters 3 and 5** was performed by Dr Tim Constantin-Teodosiu at the School of Biomedical Sciences, University of Nottingham.

The analysis of plasma ammonia concentration in **Chapter 4** was performed by Mr John Fox at the School of Biomedical Sciences, University of Nottingham.

The analysis of plasma free fatty acid concentration in **Chapter 5** was performed by Dr. Francis Stephens at the School of Biomedical Sciences, University of Nottingham.

No part of this thesis has been submitted in any previous application for a higher degree. All sources of information have been specifically referenced.

David Laithwaite.

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Abbreviations

Acetate	Sodium acetate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CoA	Co-enzyme A.
DCA	Sodium dichloroacetate
dm.	Dry muscle
G-6-P	Glucose -6-phosphate
H^+	Hydrogen ion
iv	Intravenous
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
PCr	Phosphocreatine
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
P_i	Inorganic phosphate
SLP	Substrate level phosphorylation
TCA	Tricarboxylic acid cycle
wm.	Wet muscle

Chapter 1.

GENERAL INTRODUCTION

Overview.

The pyruvate dehydrogenase complex (PDC) catalyses the physiologically irreversible reaction that links glycolysis with several other biochemical pathways. Within muscle, both skeletal and cardiac, and other tissues with high energy demands the Acetyl-CoA derived from this reaction enters the tricarboxylic acid (TCA) cycle for the subsequent synthesis of ATP (Randle, 1995). Within tissues with a low energy demand (e.g. liver and adipose tissue) the acetyl-CoA generated is utilised for fatty acid and steroid synthesis.

Within skeletal and cardiac muscle strict regulation of the actions of PDC are required, to ensure both efficient use of carbohydrate derived fuel for contraction and to meet the sudden rise in demand for ATP production during the rest-to-work transition.

Work performed by Roberts and co-workers (Roberts *et al*, 2002b) has suggested a maximal exercise intensity at which pre-treatment with DCA is not of benefit to muscle metabolism, although it is unclear whether a lower threshold of exercise intensity exists. It has been assumed by some that the increase in ventilation measured at the ventilatory threshold is related to the similar increase in blood lactate concentration measured at that time. Using dichloroacetate to reduce blood lactate concentration will allow for investigation of the relationship in healthy human volunteers between lactate concentration and the ventilatory threshold. Putman and co-workers (Putman *et al*, 1993) identified that a reduction in dietary carbohydrate intake, or increase in dietary fat intake, leads to a blunting of PDC activation or inhibition of flux through PDC during

subsequent exercise. Given that DCA is known to increase skeletal muscle PDC activation at rest and during exercise, its effects in this population have not yet been investigated.

Based upon the above research, the present thesis has examined the effects of PDC activation using the PDK inhibitor dichloroacetate (DCA) in three circumstances:

i. during the rest-to-work transition in human skeletal muscle at low intensity exercise,
ii. in an attempt to further investigate the mechanisms behind the onset of the ventilatory threshold and
iii. in an attempt to overcome the blunting of muscle PDC activation seen after a reduction in dietary carbohydrate intake.

To introduce the background to this project the literature review will initially describe the structure and function of PDC further, including its mechanism of regulation and the isoforms of pyruvate dehydrogenase kinase and phosphatase. This will be followed by a discussion of the concept of metabolic inertia in skeletal muscle. An overview of the different fuels used at different exercise intensities and carnitine's function is presented. The review will then discuss the use of DCA as both an investigative tool in the exploration of metabolic inertia and a therapeutic tool in the treatment of conditions associated with hyperlactataemia. Finally this review will consider FOXO and PPAR and their role in the regulation of PDC activity in skeletal muscle.

Literature Review.

Pyruvate dehydrogenase complex - structure and function.

The pyruvate dehydrogenase complex (PDC) is located on the mitochondrial inner membrane (Schnaitman and Greenawalt, 1968; Addink *et al*, 1972), where it oxidatively decarboxylates pyruvate to acetyl-CoA and carbon dioxide coupled with the reduction of NAD+ to NADH (see below).

$$CH_3COCOO^- + CoASH + NAD^+ \xrightarrow{PDC} CH_3CO.CoA + CO_2 + NADH$$

PDC consists of three catalytic components, pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3) (Reed and Hackert, 1990). Each complex consists of a core of sixty E2 subunits, to which thirty E1 subunits are attached (at the E1-binding domain of E2). Six to twelve E3 subunits are tightly bound to the E2 core through E3-binding protein (see Figure 1.1)

These three enzymes decarboxylate and dehydrogenate pyruvate, in the presence of the essential cofactors thiamine pyrophosphate (TPP), free CoASH and NAD^+. The E1 enzyme catalyses the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoyl moiety of E2. This enzyme then catalyses the transfer of the acetyl group to the free CoASH cofactor. The reduced lipoyl moiety of E2 is then re-oxidised by E3, with NAD^+ acting as the final acceptor of the reduced electron (Figure 1.2).

Figure 1.1 – Scheme of a fragment of the mammalian pyruvate dehydrogenase complex structure (from Strumilo, 2005). Only 2 subunits of E2 with E1 and E3 components are shown.

Key:

ID	Inner domain.
BD	Binding domain.
LD	Lipoyl domain.
TDP	Thiamine diphosphate.
FAD	Flavin adenine dinucleotide (oxidised form).
PDK	Pyruvate dehydrogenase kinase.
PDP	Pyruvate dehydrogenase phosphatase.

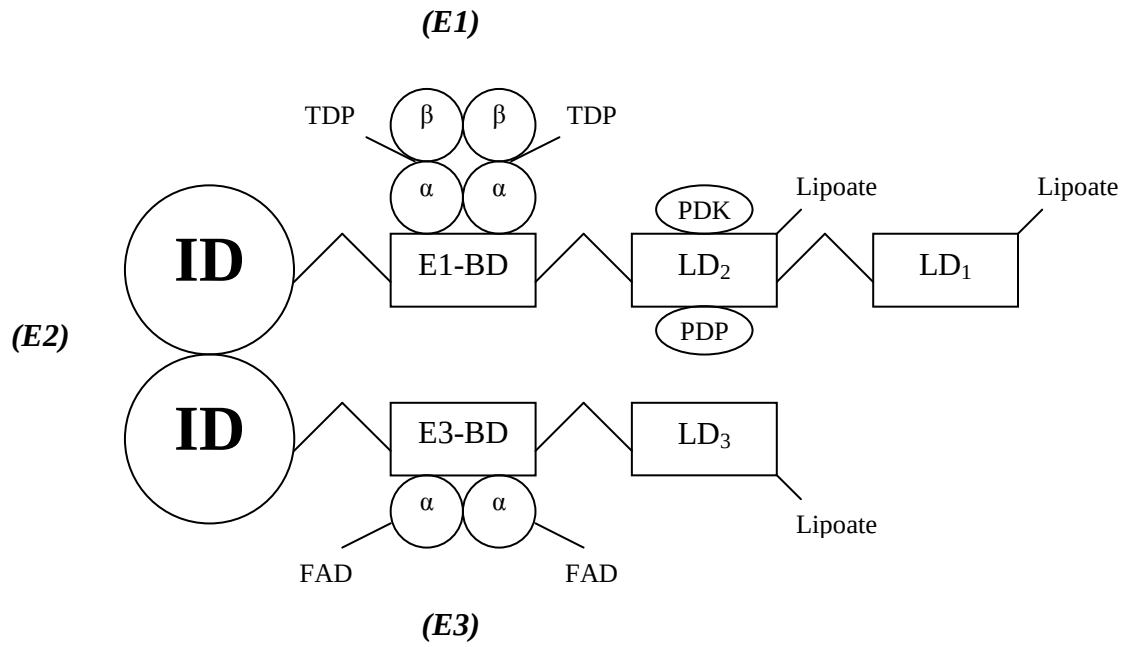

(E1)
TDP
β
β
TDP
α
α
PDK
Lipoate
Lipoate
ID
E1-BD
LD2
LD1
PDP
(E2)
ID
E3-BD
LD3
α
α
Lipoate
FAD
FAD
(E3)

Figure 1.2 – Sequence of reactions catalysed by the pyruvate dehydrogenase complex.

Key:

TPP Thiamine pyrophosphate.

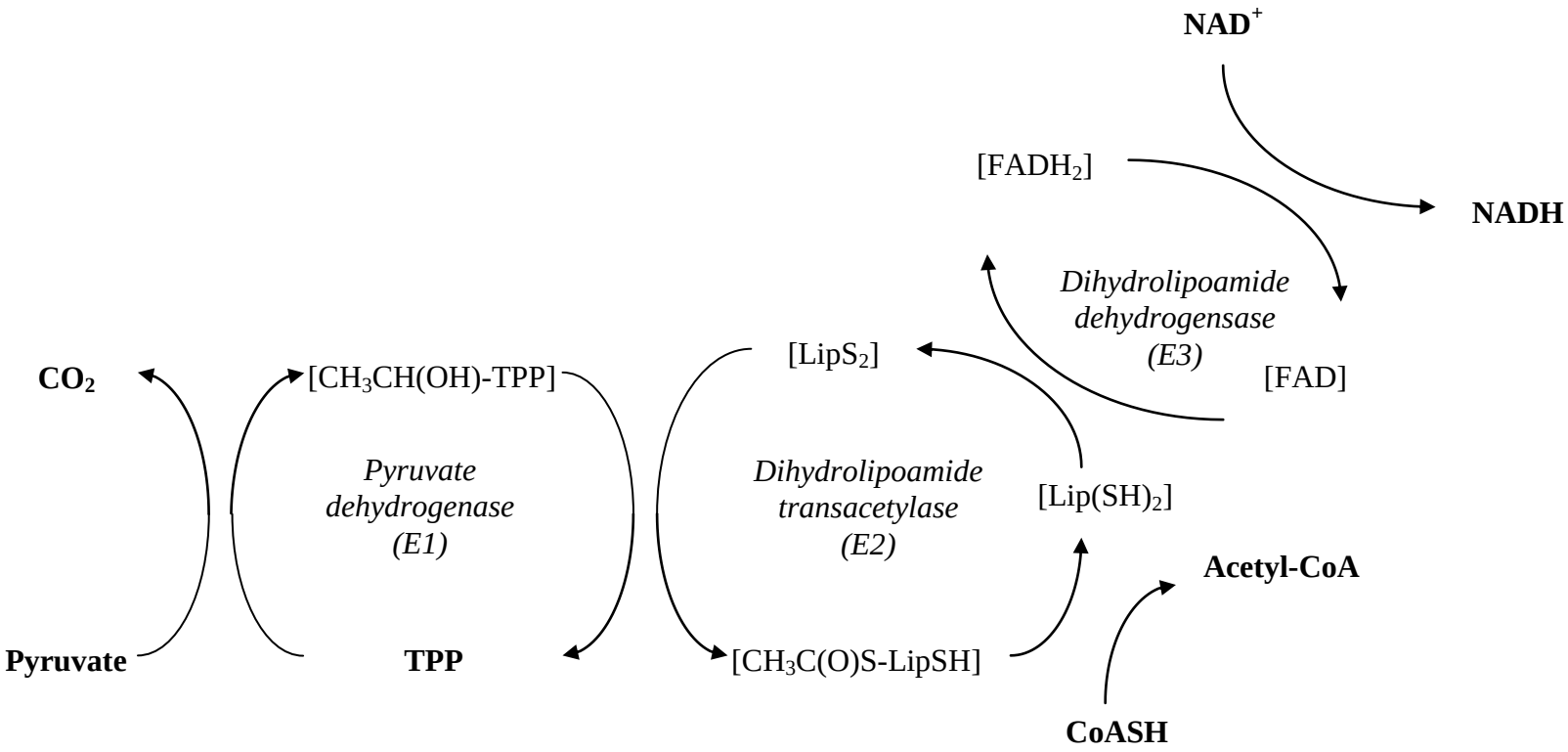
Lip Lipoic acid.

$FADH_2$ Flavin adenine dinucleotide (reduced form).

FAD Flavin adenine dinucleotide (oxidised form).

NADH Nicotinamide adenine dinucleotide (reduced form).

NAD Nicotinamide adenine dinucleotide (oxidised form).


NAD+
[FADH2]
NADH
Dihydrolipoamide dehydrogensase (E3)
[FAD]
CO2
[CH3CH(OH)-TPP]
[LipS2]
Pyruvate dehydrogenase (E1)
Dihydrolipoamide transacetylase (E2)
[Lip(SH)2]
Acetyl-CoA
Pyruvate
TPP
[CH3C(O)S-LipSH]
CoASH

Pyruvate dehydrogenase is tetrameric, consisting of equal numbers of α and β subunits ($\alpha_2\beta_2$). The roles and integration of these subunits is poorly understood but the α-residue is known to possess the serine residues that are the target for covalent modification by the complex's intrinsic regulatory kinase and phosphatase system, responsible for regulation of PDC activity and discussed later in this review. The E1 component is responsible for catalysing the rate-limiting step of the overall PDC reaction (Cate *et al*, 1980). Within mammals no pathways exist for the synthesis of pyruvate from Acetyl-CoA, therefore the control of this reaction is crucial to avoid depletion of carbohydrate derived carbon skeletons which cannot be re-synthesised by the body. Regulation of PDC in skeletal muscle will be discussed later in this review.

Dihydrolipoamide transacetylase forms the structural hub to which the E1, E3 and the intrinsic regulatory phosphatase and kinase bind. The E2 enzyme catalyses the reversible acetyltransferase reaction and contains the lipoic acid residue that interacts with the active sites of the enzyme complex (Yeaman, 1989).

Dihydrolipoamide dehydrogenase is a FAD-containing flavoprotein which contains the single disulphide bridge that undergoes oxidation and reduction during catalysis. The E3 enzyme is also linked to E3 binding protein which is thought to be responsible for linking PDC to the mitochondrial membrane protein.

Pyruvate dehydrogenase complex - regulation in skeletal muscle.

At rest PDC activation is required to remain low, to prevent the utilisation of carbohydrate unnecessarily. However at the onset of exercise a rapid increase in activation is required to provide acetyl units for entry into the TCA cycle. Regulation of this enzyme is therefore essential for both the prompt use of carbohydrate derived energy stores within skeletal muscle at the onset of contraction and to avoid the consumption of carbohydrate stores when rapid ATP production is not required.

There are two methods by which the activity of PDC can be regulated. The first is by inhibition of the enzyme complex by its products NADH and acetyl-CoA, as well as activation of the enzyme complex by pyruvate, NAD^+ and CoASH. The second is by the covalent modification of the enzyme complex by its intrinsic kinase (pyruvate dehydrogenase kinase, PDK) and phosphatase (pyruvate dehydrogenase phosphatase, PDP) system, which will be discussed later.

At rest PDC in skeletal muscle exists mainly in its phosphorylated (deactivated) state. At the commencement of contraction the initial activation of PDC is thought to be through calcium ion stimulation of PDP activation. This initially crude activation is then further 'fine tuned' by the action of substrate and product feedback (e.g. [ATP]/[ADP], [NADH]/[NAD^+] and [acetyl-CoA]/[CoASH] ratios). This 'fine tuning' is believed to be mediated through the stimulation and inhibition of the PDK isoenzymes, as PDP seems relatively insensitive to physiological or pathological disturbances, although there is some effect of acetyl-CoA and NADH directly upon the E2 subunit, through reducing the

availability of oxidised lipoyl groups on the E2 PDC component. This would reduce the formation of E1-[TPP], leading to a reduction of pyruvate decarboxylation at the rate limiting step in the whole PDC reaction (Denton *et al*, 1975).

PDC's regulatory enzymes (PDK and PDP) are constantly active in the mitochondria, controlling the amount of phosphorylated, and therefore inactive, PDC through their action whilst bound mainly at the E2 subunit lipoyl domains (Figure 1.3).

Pyruvate dehydrogenase phosphatase dephosphorylates the serine residues on the E1 subunit and actives PDC as a whole. It is only weakly bound to the E2 hub of the enzyme complex (Wieland *et al*, 1983) and requires the presence of magnesium ions to enable tighter binding of the enzyme to the E2 subunit. PDP activity is reduced in the presence of a high [NADH]/[NAD^+] ratio, in contrast to PDK. Two isoenzymes of pyruvate dehydrogenase phosphatase (PDP) have so far been identified, both of which require magnesium ions for their action. These also show differences in distribution, kinetic parameters and regulation between one another. PDP1 is found predominantly in skeletal muscle mitochondria with an almost undetectable amount present in mammalian liver, whilst PDP2 seems to reside in liver and adipocyte mitochondria (Huang B *et al*, 1998). PDP1 needs calcium ions for full activity (as the presence of calcium ions promotes the association of PDP with PDC), whilst PDP2, which appears to be calcium independent, seems to have a similar activity to a fully activated PDP1. PDP2 is also stimulated by the presence of spermine, and although this is unlikely to be a likely stimulus *in vivo* it does suggest the presence of another regulatory substance. There is

some evidence that PDP2 is sensitive to insulin, resulting in stimulation of the pyruvate dehydrogenase complex within tissues with a predominantly biosynthetic function (Huang *et al*, 1998).

Pyruvate dehydrogenase kinase (PDK) is tightly bound to the E2 hub of the pyruvate dehydrogenase complex (Linn *et al*, 1969), and inactivates the PDC by phosphorylating one or all of three serine residues on the α chain of the E1 component (Serine-264 (site 1), Serine-271 (site2) and Serine-203 (site3)) (Linn *et al*, 1969). Phosphorylation of the serine residue at site 1 has been demonstrated to fully inactivate PDC (Sugden *et* al, 1979) and it has been suggested that phosphorylation at the other two sites may slow reactivation by PDP. As mentioned above, PDK activation is stimulated by an increase in the [acetyl-CoA]/[CoASH] and [NADH]/[NAD^+] ratios ensuring that PDC flux is reduced when acetyl group availability is high. PDK activity is reduced by the presence of pyruvate, as well as a reduction in the [acetyl-CoA]/[CoASH] and [ADP]/[ATP] ratios. Dichloroacetate (discussed later), and other halogenated carboxylic acids (Whitehouse and Randle, 1973), also markedly reduce PDK activation through an effect similar to the inhibition from pyruvate.

Four discrete PDK isoenzymes have been identified in human skeletal muscle (PDK 1-4) (Gudi *et al*, 1995; Bowker-Kinley *et al*, 1998) with varying concentrations, specific activities and kinetic properties. As the changes in activation state of PDC appear to correlate with changes in PDK activity under a number of circumstances (Hansford *et al*, 1976; Cate and Roche, 1978) there has been much interest in these isoforms.

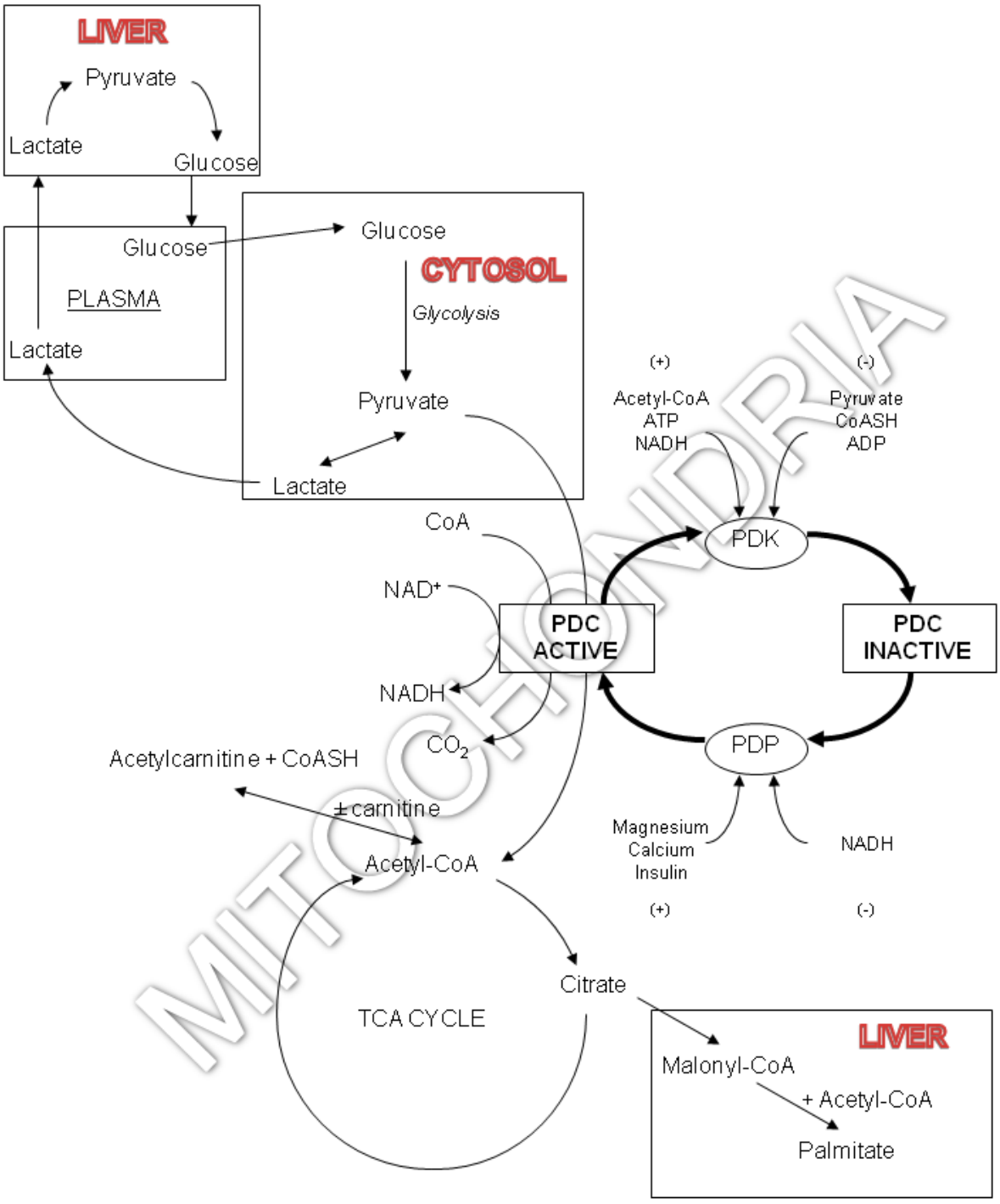
Figure 1.3 – Mechanisms regulating the pyruvate dehydrogenase complex (PDC) by phosphorylation-dephosphorylation, together with the reaction catalysed by PDC and its links with other metabolic pathways.

Key:

PDK Pyruvate dehydrogenase kinase.

PDP Pyruvate dehydrogenase phosphatase.

TCA Tricarboxylic acid cycle.



The commonest isoforms identified in skeletal muscle are PDK2 and 4, thought to be responsible for the inactivation of PDC in response to alterations in pyruvate and lipid availability respectively. The distribution of the four PDK isoforms between tissues suggests that there exists a need for a differing activation of PDC between these tissues.

PDK1 has been demonstrated to mainly reside in human heart tissue, with low levels of expression in skeletal muscle (Popov *et al*, 1994). The functional role of PDK 1 is currently unclear, but Bowker-Kinley *et al* (1998) have suggested that it may be responsible for detecting changes in the concentrations of intra-mitochondrial pyruvate and ADP in the heart.

PDK2 mRNA has been found to be highly expressed in most human tissues (Popov *et al*, 1994). It is the most sensitive isoenzyme to ATP and also the most sensitive to inhibition by dichloroacetate (Bowker-Kinley *et al*, 1998). It has been suggested, by the same group, that PDK2 is the major PDK isoform involved in control of mammalian PDC activity, due to its profuse expression within most tissues.

PDK3 is mostly found in testes, and possesses the highest specific activity of all the PDK isoforms (1250 $nmol.min^{-1}.mg$; Bowker-Kinley *et al*, 1998). It has been suggested to have a specific role within the testes, although that has not yet been clarified.

PDK4 is mostly found within cardiac and skeletal muscle and is relatively insensitive to changes in [ADP], [ATP], the [NADH]/[NAD^+] ratio and acetyl-CoA concentration (Bowker-Kinley *et al*, 1998). Due to its relative insensitivity to the presence of pyruvate, it allows for the inhibition of PDC action without a reduction in the concentration of pyruvate required for other processes, such as transamination to alanine prior to

metabolism in situations of chronic negative nitrogen balance. PDK4 seems to possess more of a role in the long term regulation of PDC activation within skeletal muscle, which is supported by the finding in healthy human volunteers fed a high fat/low carbohydrate diet an increase in PDK4 mRNA expression was found (Peters *et al*, 2001) and this may be responsible for the suppression of carbohydrate utilisation observed in a similar population (Putman *et al,* 1993). These findings are supported by findings in the rat model that the PDK4 mRNA expressed in heart and skeletal muscle is selectively upregulated by starvation, insulin resistance, diabetes mellitus and hyperthyroidism (Huang *et al,* 2002; Wu *et al,* 1999; Sugden *et al*, 1998).

Further work (Peters *et al*, 2001) investigating the effect of high and low carbohydrate diets administered to glycogen depleted individuals noted a significant reduction in PDK4 mRNA abundance following the high carbohydrate diet (0.068±0.025 with low carbohydrate versus 0.021±0.007 after high carbohydrate feeding). This evidence supports the previous work in demonstrating an increase in PDK4 activation in response to low carbohydrate stores. It is uncertain if this response is a result of an increase in circulating fatty acid concentrations, or as a response to the reduction of glycogen stored within the muscle fibre.

Another hypothesis states that the reduction in plasma insulin concentration seen after high fat (73%)/low carbohydrate (5%) feeding exerts an influence over PDK4 activity. Following three days of this diet the change in PDK activity was found to be proportional to the subject's normal level of physical activity (Peters *et al*, 2001). As the skeletal

muscle of trained individuals is more sensitive to the effects of insulin, it may be that the larger increase in PDK4 activity seen is a reflection of this effect.

Similar effects are seen with insulin resistance, and the resultant reduced availability of carbohydrate for skeletal muscle metabolism. This up-regulation of PDK isoforms, specifically PDK4, arises in part due to activation of peroxisome-proliferator-activated receptor α (PPARα). PPARα is activated by non-esterified fatty acids (NEFA) and is likely to account for their inhibitory action upon PDC activation, likely through the stimulation of PDK4 activity (Pilegaard and Neufer, 2004). Peroxisome-proliferator-activated receptor (PPARs) and FOXO1 are the molecular signalling pathways that have been suggested to be involved with the expression of PDK4 mRNA (Wu *et al*, 2001; Degenhardt *et al*, 2007) or PDK4 protein in skeletal muscle (Constantin *et al*, 2007).

PPARα expression increases with aerobic training in both men (Russell *et al*, 2003) and women (Horowitz *et al*, 2000). As well as influencing the rate of carbohydrate oxidation in skeletal muscle, PPARα regulates the expression of genes coding for muscle enzymes involved in fat metabolism. The increased PPARα activity seen with aerobic training therefore accounts for the increased potential for fat oxidation, and relative contribution from this source during sub-maximal exercise, seen in the skeletal muscle of trained subjects.

Metabolic inertia at the onset of skeletal muscle contraction.

PDC activity is low in resting muscle, to prevent the use of carbohydrate stores during time of low muscle ATP demand, but is required to increase rapidly at the onset of contraction. However the increase in mitochondrial ATP re-synthesis follows an exponential increase (Tschakovsky and Hughson, 1999), within which period the increased requirements for ATP regeneration is met via oxygen independent routes. During the first 30 seconds of single leg extension exercise at 65 W Bangsbo *et al* (1990) noted that the contribution to ATP generation from glycolysis and phosphocreatine breakdown accounted for 80% of total ATP generation. This proportion decreased to 45% during the following 60-90 seconds and then 30 % after 120 seconds of exercise and appeared to be mirrored by an increase in the contribution from mitochondrial ATP generation. The delay in mitochondrial ATP regeneration has classically been attributed to a corresponding delay in skeletal muscle blood flow and therefore oxygen delivery to contracting muscle fibres (Richardson *et al*, 1995). The increases in skeletal muscle oxygen utilisation correspond to the increase in total limb blood flow during the early stages of the rest-to-work transition, and the term 'oxygen deficit' was used to describe this phenomenon. Following the work of Grassi *et al* (1998a; b), who demonstrated that abolishing the delay in muscle blood flow at the onset of exercise did not alter limit the rate of oxygen consumption at this time, the conclusion of the authors was that the delay arose due to an inertia in microvascular oxygen delivery or mitochondrial energy provision.

The pyruvate dehydrogenase complex as the location for this delay in mitochondrial ATP regeneration has been the focus of research within the School of Biomedical Sciences over the last 10 years, with the demonstration in 1996 of PDC activation by DCA leading to a reduction in both phosphocreatine hydrolysis and lactate accumulation during intense exercise with the blood flow (and oxygen delivery) fixed at near resting levels (Timmons *et al*, 1996). Further work exploring this finding in both human and canine skeletal muscle demonstrated that the phosphocreatine hydrolysis and lactate accumulation were, in part, due to a delay in the provision of oxygen-dependent ATP regeneration at the commencement of contraction (Timmons *et al*, 1997; 1998b). The same investigators demonstrated that DCA infusion lead to an increase in the pool of acetyl groups available for entry into the TCA cycle during contraction. From this evidence it was concluded that activation of, and corresponding flux through, PDC is limiting to acetyl-CoA availability and mitochondrial ATP re-synthesis at the onset of exercise. Pre-treatment with DCA, and the resultant accumulation of acetyl groups prior to exercise, could increase the overall contribution of oxidative pathways to total ATP production at the onset of exercise.

Research has demonstrated the accumulation of acetyl groups during moderate to intense contraction (Harris *et al*, 1987; Howlett *et al*, 1999b) in contradiction to the suggestion that the inertia during the rest-to-work period is due to an inadequacy of the supply of acetyl-CoA via PDC to meet the demands of contraction. However these investigators had not investigated the metabolic changes occurring during the initial stages of contraction, and work by Roberts *et al* (2002a) was able to demonstrate the existence of a

period of metabolic inertia in skeletal muscle at the onset of contraction using a canine model of ischaemic contraction in which five muscle biopsies were obtained during the first minute of contraction. These results demonstrated a delay in acetyl group provision that was mirrored by, and resulted from, a delay in PDC activation during the initial 20 seconds of contraction.

From this research using DCA, which both activates PDC and acetylates the free coenzyme A and acetylcarnitine pools at rest, it was not possible to determine if the location of the inertia was in acetyl-CoA delivery via PDC at the onset of contraction or due to an increase in the pool of acetyl groups available to enter the TCA cycle. Further research (Roberts *et al*, 2001; Evans *et al*, 2001), using acetate infusions to increase the provision of acetyl groups without activation of PDC, demonstrated that there was no alteration in oxidative phosphorylation following acetate pre-treatment. Further, Roberts *et al* (2001) demonstrated that it was acetyl-CoA derived from PDC, rather than the pooled acetyl groups prior to the onset of contraction, which provided the principal route of substrate entry into the TCA cycle. This research has suggested that the site of metabolic inertia at the onset of skeletal muscle contraction is at the level of the pyruvate dehydrogenase complex.

The contribution of mitochondrial ATP regeneration, and therefore the degree of metabolic inertia subject to modification by DCA pre-treatment, is dependent on the intensity of exercise (the rate of ATP degradation and therefore the rate of accumulation of ADP and AMP, which activate phosphocreatine degradation and glycolysis) and the

time taken to achieve a steady state of energy provision and utilisation (Hultman *et al*, 1967; Howlett *et al*, 1998). This, as discussed above, may explain the upper threshold of efficacy from DCA pre-treatment in overcoming metabolic inertia present at the rest-to-work transition.

'Priming' of skeletal muscle, that is increasing the availability of acetyl groups for entry into the TCA cycle, can also be achieved by an initial period of low intensity exercise. A ten minute bout of exercise at 55% VO_2max doubled the skeletal muscle acetylcarnitine concentration and trebled the muscle lactate concentration when compared to resting values (Campbell-O'Sullivan *et al*, 2002). When a subsequent ten minute bout of exercise at 75% VO_2max was performed there was no difference noted at the end of the bout of exercise in the concentrations of metabolites or the contribution from non-oxygen dependent ATP resynthesis between either the group that had performed an initial low intensity 'warm up' bout and a matched control group. However during the first minute of exercise there was significant sparing of PCr and a reduction in the formation of lactate and glucose-6-phosphate (G-6-P) in the group that had performed a 'warm up'. This group had a markedly reduced contribution to ATP resynthesis from non-oxygen dependent routes (reduced by approximately 45%) which demonstrates an attenuation of the inertia normally present at the onset of contraction in skeletal muscle.

Role of carnitine and influence of exercise intensity on fuel selection.

Carnitine plays an integral role in normal cellular metabolism, being required for the optimal utilisation of fuel substrates by skeletal muscle during exercise through two vital

roles (Brass and Hiatt, 1994). The entry of cytosolic long-chain acyl-CoA into the mitochondrion for mitochondrial β-oxidation is dependent on the formation of long chain acylcarnitines through the action of carnitine palmitoyltransferase 1 (CPT1) (Murthy and Pande, 1987). The long-chain acylcarnitine so formed is then exchanged with free intramitochondrial carnitine via carnitine acylcarnitine translocase where carnitine palmitoyltransferase 2 (CPT2) catalyses the transesterification back to acyl-CoA and free carnitine. The acyl-CoA may then enter the β-oxidation pathway to form acetyl-CoA for further metabolism.

Carnitine metabolism varies in relation to the metabolic state of the cell. At rest 80% of the total muscle carnitine pool is present as carnitine, 15% as short-chain acylcarnitines and 5% as long-chain acylcarnitines. Within 10 minutes of high-intensity exercise (75% VO_2max) the muscle carnitine pool is predominantly (45-75%) short-chain acylcarnitines, primarily acetylcarnitine, with only 20% remaining as carnitine (Harris *et al*, 1987). The concentration of skeletal muscle acetylcarnitine has been demonstrated to correlate with both muscle lactate concentration (Sahlin, 1990) and muscle acetyl-CoA content (Carlin *et al*, 1990; Constantin-Teodosiu *et al*, 1991b) further indicating its link to the metabolic state of the muscle. This correlation between acetylcarnitine concentration and muscle acetyl-CoA content is believed to occur due to buffering of acetyl groups generated by the action of PDC that are in excess of those required for utilisation by the TCA cycle enabling an adequate supply of fee CoASH for use in PDC and TCA cycle reactions. In support of this suggestion is the finding that there is no change in muscle acetylcarnitine content during exercise at lower (30-40% VO_2max)

intensity when the rate of acetyl-CoA formation from both pyruvate and fatty acid metabolism is better matched to the demand from the TCA cycle (Sahlin, 1990). In addition to buffering acetyl groups in this manner, this store of acetyl groups is also available for transacetylation back to acetyl-CoA for further metabolism.

The relative utilization of fat or carbohydrate fuel during exercise is strongly dependent on exercise intensity. The mechanisms by which this regulation occurs are not fully understood, with the 'Glucose-FFA cycle' (Randle *et al*, 1963) being suggested as an explanation for the reduction in muscle carbohydrate oxidation rate in the presence of high plasma FFA concentration. It was proposed that an increase in the mitochondrial acetyl-CoA/CoA ration would suppress PDC activation during exercise, therefore reducing carbohydrate oxidation and stimulating fat oxidation. However studies have since demonstrated no limitation of fat oxidation during high-intensity exercise (≥85% VO_2max) in association with changes of plasma FFA availability (Dyck *et al*, 1993; Romijn *et al*, 1995).

There is growing evidence to suggest that flux through PDC may indirectly regulate fatty acid oxidation during exercise, through the observation that increasing glucose availability prior to exercise at 50% VO_2max reduced the oxidation of long-chain fatty acid with a reduction in muscle long-chain acylcarnitine content (Coyle *et al*, 1997). This reduction persisted after restoration of plasma FFA concentrations (via the administration of lipid and heparin infusions) suggesting that the effect of increasing carbohydrate availability is not dependent on changes in FFA availability (Horowitz *et* al, 1997). In

support of this suggestion the increase in PDC flux seen with increased exercise intensity or increases in carbohydrate availability during exercise is associated with a corresponding decrease in long-chain fatty acid oxidation rates (van Loon *et al*, 2001; Roepstorff *et al*, 2005).

There have been two suggested mechanism through which this inhibition has been suggested to occur, via the inhibition of CPT1 activity by malonyl-CoA and the influence of PDC flux on free carnitine activity. Skeletal muscle malonyl-CoA is synthesised from acetyl-CoA via the action of acetyl-CoA carboxylase, an enzyme which is increasingly activated by an increase in the cytosolic concentration of citrate (Saha *et al*, 1995). In the presence of increased glycolytic flux, the concentrations of both citrate and acetyl-CoA are increased leading to a corresponding increase in the muscle malonyl-CoA concentration. Malonyl-CoA in known to be a potent inhibitor of CPT1 activity in vitro and changes in its concentration in resting human skeletal muscle have occurred with inverse changes in fat oxidation (Bavenholm *et al*, 2000). However it appears that this finding is not supported during exercise as studies demonstrate no association between fat oxidation rates and malonyl-CoA concentrations during prolonged moderate intensity (40% followed by 65% VO_2max) exercise (Odland *et al*, 1996) or exercise at 65%VO_2max in glycogen depleted individuals (Constantin-Teodosiu *et al*, 1991b). As carnitine is the principal substrate for CPT1, the decreases in concentration seen during high-intensity exercise may limit the ability of CPT1 to transport long-chain acyl-CoA into the mitochondrial matrix for β-oxidation (Stephens *et al*, 2006), a finding supported by the finding of van Loon *et al* (2001) that there is a 35% decrease in the rate of long-

chain fatty acid oxidation during exercise at an intensity above 75% VO_2max (Fig 1.4). Achten and Jeukendrup (2004) demonstrated that during exercise maximum and minimum fat oxidation rates are obtained at intensities of approximately 65% and over 80% VO_2max respectively, when free carnitine content has been demonstrated to decrease from approximately 11 mmol.(kg dm)$^{-1}$ at 60% VO_2max to below 5.5 mmol.(kg dm)$^{-1}$ at an exercise intensity of approximately 80% VO_2max.

The causes of fatigue during exercise depend upon the intensity and duration of exercise performed. During short duration, high-intensity (above 90% VO_2max) exercise phosphocreatine depletion leads to skeletal muscle fatigue. The accumulation of metabolic by-products (especially lactate) also result in a lowering of muscle pH, which both reduces the rate of glycolysis (in turn reducing the generation of ATP for continued contraction) and affects the binding of calcium ions to troponin. Prolonged exercise instead relies upon glycogen (both within the contracting muscle and also stores present within the liver) for continued generation of energy for contraction. As muscle glycogen is consumed there is a corresponding increased reliance on blood glucose to provide carbohydrate for muscle contraction. A fall in blood glucose will result in increased rates of liver glycogenolysis in an attempt to maintain the blood glucose concentration. When the rate of liver glycogenolysis is less than the rate of muscle glucose uptake then the blood glucose concentration and carbohydrate provision for continued contraction will become limited, leading to fatigue.

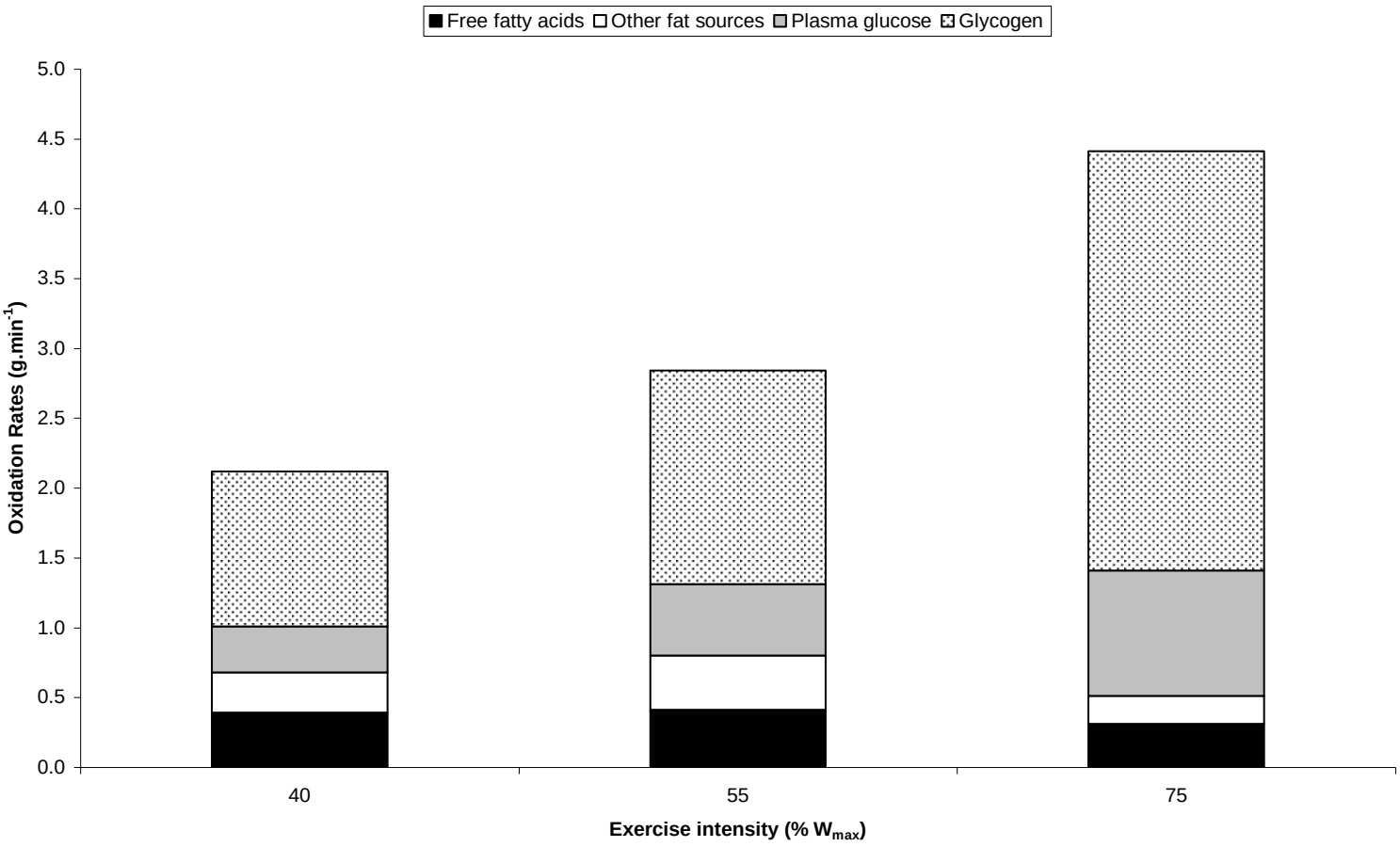


Figure 1.4 Contribution of substrates to total energy expenditure during cycling exercise at 40, 55 and 75% W_{max}. From van Loon *et al* (2001), modified.

The effect of dietary changes on PDC activation.

Lowering the availability of carbohydrate as a fuel for contracting muscle (whether through the administration of high-fat, low carbohydrate diet, or starvation) results in a reduction of PDC activation at rest and the rate of activation during exercise at 75% VO_2max. A comparison (Putman *et al*, 1993) between subjects cycling at 75% VO_2max after consumption of a low (3% carbohydrate, 51% fat and 46% protein) carbohydrate diet also demonstrated

- a reduction in VCO_2 and RQ
- reduced blood lactate concentration during exercise
- reduced muscle glycogen content
- reduced muscle pyruvate and lactate concentration

when compared to the same population after consumption of a high (86% carbohydrate, 4% fat and 10% protein) carbohydrate diet. All of these findings demonstrate a reduction in carbohydrate metabolism during exercise, as an attempt to preserve carbohydrate stores. Stellingwerff *et al* (2002) were able to demonstrate that reducing fatty acid availability stimulated carbohydrate oxidation in skeletal muscle during moderate intensity exercise (70% VO_2max). This was suggested to occur due to a reduction in the [NADH]/[NAD^+] ratio prior to contraction, which would inhibit PDK activity and therefore increase resting PDC activation.

Aerobic training, and its increased ability to utilise fatty acids as a fuel source during contraction, are of particular relevance when we consider the skeletal muscle of patients with the metabolic syndrome. This syndrome consists of impaired glucose tolerance,

insulin resistance and mild to severe lipid abnormalities and is a risk factor for the development of cardiovascular disease as well as a common co-morbidity of patients with PAD (Gorter *et al*, 2004; Olijhoek *et al*, 2004). Nine months of aerobic training in these patients induced key proteins involved in glycolytic and oxidative metabolism (PDHα-1 subunit, LDH and glutamate-oxoglutarate transaminase (GOT1))(Hittel *et al*, 2005) allowing for improved integration of the glycolysis and the TCA cycle within skeletal muscle. This beneficial response to aerobic training was preserved or even exaggerated in patients with the metabolic syndrome, but these benefits were lost within a two week period of de-training highlighting the requirement for regular exercise therapy in the treatment of this condition.

Alterations in the balance between carbohydrate and fat metabolism in non-contracting skeletal muscle are inherently linked to the activation of PDC. Low carbohydrate feeding will result in an increase in PDK activity in an attempt to preserve carbohydrate stores from their irreversible metabolism through PDC (Peters *et al*, 2001). Interaction of the regulatory pathways controlling carbohydrate and fatty acid oxidation allows control of their respective activities. Metabolites and co-factors for each pathway exert regulatory effects upon enzymes such as PDC and carnitine palmitoyltransferase (CPT) so that fluctuations in the substrate available for metabolism are adjusted to within the mitochondrion. Again alterations in the both the action of PDK and its sensitivity to its normal regulatory factors, especially pyruvate, regulate many of these changes (Spriet *et al*, 2004; Sugden and Holness, 2003). Starvation desensitises PDK to the inhibitory effects of pyruvate, in order to preserve carbohydrate stores. Exposure to fatty acids will

also stimulate PDK4 activity and alter the $NADH/NAD^+$ ratio and intramitochondrial concentration of acetyl-CoA, further inhibiting PDC and ensuring preservation of carbohydrate.

The role of PDC activation in the balance between carbohydrate and fatty acid oxidation during steady state contraction does not seem to be the same as that found in non-contracting muscle. The glucose-fatty acid cycle (GFC) which links the concentrations of NEFA to the rate of carbohydrate oxidation seems not to operate during submaximal steady state skeletal muscle contraction. After the administration of nicotinate, which limits NEFA availability to contracting muscle via its inhibition of hormone-sensitive lipolysis, to dogs the measured PDC activation during steady state contraction decreased (Timmons *et al*, 1998c). Had lipid and carbohydrate oxidation been linked, then a rise in PDC activity would be expected, and the above findings suggest that an alternative mechanism exists for the regulation of these processes in contracting skeletal muscle.

Sodium dichloroacetate.

Sodium dichloroacetate (DCA) is a systemic activator of PDC (Whitehouse and Randle, 1973), having its effects by indirectly inhibiting PDK and therefore increasing the amount of PDC in its phosphorylated, and therefore active, form. It is similar in structure to pyruvate, which is thought to account both for its ability to enter the mitochondria (Halestrap, 1975) and its action upon PDK.

As stated above, whilst DCA inhibits all of the PDK isoforms it has its greatest effect upon PDK2, resulting in a greater proportion of PDC in most tissues remaining in its active form. Administration of DCA has been demonstrated to result in the acetylation of the free carnitine and coenzyme-A pools at rest to a level seen at maximal exercise (Howlett *et al*, 1999b), as well as increasing the flow of pyruvate through PDC enabling an increase in the regeneration of ATP via oxygen dependent pathways. These effects led to a reduction in phosphocreatine breakdown, glycogenolysis and the accumulation of both P_i and lactate during the initial 2 minutes of exercise (Howlett *et al*, 1999b). The activity of DCA is most noticeable during the initial 20 seconds of contraction when inertia at this level is thought to exist due to mitochondrial enzyme delays (Greenhaff *et al*, 2002). Once this inertia has been overcome (so that PDC activation has reached levels adequate to meet the metabolic demands of the contracting muscle) the effect of DCA is minimal. This may explain why a reduced effect of DCA upon skeletal muscle metabolism during higher intensity exercise (>90% VO_2max) is seen (Howlett *et al*, 1999a), as the exercise related activation of PDC is able to match the increased activation from DCA administration. As such activation of PDC and the increased production of ATP via oxidative mechanisms, which are more efficient but slower in their delivery, is likely to have a reduced effect at these high intensities. During exercise at and above maximal intensity the delayed respiratory response has been suggested to be located at some point within the TCA cycle or electron transfer chain (Howlett *et al*, 1999a; 1999b) due to the lack of a significant effect of DCA pre-treatment.

Using the same isolated in-situ dog gastrocnemius model as their previous experiments investigating the effects of changes in conductive and diffusive O_2 upon VO_2 on-kinetics Grassi *et al* (2002) were able to demonstrate a significant preservation of the mean fatigue index (average values of force determined every 10 seconds/initial force; 0.73± 0.03 vs. 0.81 ±0.05, CON vs. DCA, p=0.02) from 80 seconds until the end (240 seconds) of exercise at approximately 70% VO_2max after the administration of DCA (300mg.(kg body mass)$^{-1}$), as well as a ~30% reduction in both PCr degradation during exercise and final muscle lactate concentration. Whilst this model failed to demonstrate the PDC as the site of metabolic inertia, the authors accepted that this model had some limitations in this respect. After DCA administration the metabolic efficiency of the muscle was increased leading to an increased force generation. The effect observed on the contribution to ATP resynthesis from oxygen-independent sources was less pronounced than previously observed in similar experiments, although the authors mentioned that a variation in muscle fibres between species may account for some of this effect (as dog gastrocnemius consists of a much higher proportion of type Ia muscle fibres when compared to human skeletal muscle).

It might be expected that when the metabolic inertia during the rest-to-work transition is prolonged, such as in patients with peripheral vascular disease (PVD) (Greenhaff *et al*, 2004), that DCA would exert a longer influence over mitochondrial ATP provision. In keeping with this suggestion research (Parolin *et al*, 2000; Timmons *et al*, 1998b) has demonstrated a prolonged effect of DCA pre-treatment on glycogenolysis and ATP provision in healthy volunteers exercising during hypoxia. Parolin *et* al (2000) were able

to demonstrate that the administration of 100 mg.(kg body weight)$^{-1}$ DCA to healthy subjects exercising in hypoxic conditions reduced the contribution of substrate level phosphorylation (phosphocreatine (PCr) breakdown and glycogenolysis) to ATP regeneration. DCA pre-treatment directed 83% of PDC flux during the first minute of exercise to oxidative mechanisms, as compared with 56% of flux under hypoxia with a control infusion. This difference occurred due to a reduction in flow towards acetylcarnitine formation during the rest-to-work transition in those subjects pre-treated with DCA, as a large pool of acetyl groups already exists available for entry into the TCA cycle. This better "matching" of ATP provision and demands resulted in preservation of muscle glycogen and PCr, and a reduction therefore in the generation of lactate and P_i. Other research has demonstrated a 50% reduction in both PCr degradation and muscle lactate accumulation during the first three minutes of ischaemic exercise at ~65% VO_2max in human volunteers (Timmons *et al*, 1998b) and a ~15% less reduction in isometric muscle tension as well as an 83% reduction in muscle lactate and 54% reduction in PCr hydrolysis in a canine model of partial ischaemia (Timmons *et al*, 1996) following the administration of DCA (50mg.(kg body mass)$^{-1}$ (Timmons *et al*, 1998b); 300mg.(kg body mass)$^{-1}$ (Timmons *et al*, 1996)). Recent researchers (Calvert *et al*, 2008) have also indicated a similar effect of DCA pre-treatment in patients with impaired skeletal muscle function secondary to chronic obstructive pulmonary disease (COPD), where a 20% reduction in peak blood lactate concentration and 15% reduction in peak blood ammonia was associated with a significant increase in mean peak exercise workload during symptom-limited maximal intensity cycling exercise.

In sepsis, which increases the flux through glycolysis, the most frequently observed metabolic disturbance is an increased blood lactate concentration (L'Her and Sebert, 2001) although the exact mechanism by which this arises is unclear. DCA administration ($20mg.(100g\ body\ mass)^{-1}$) in a rat model of sepsis resulted in a ~25% reduced concentration of muscle lactate after four hours, suggesting that PDC activity may also be reduced during sepsis (L'Her and Sebert, 2004). This would support the findings of increased PDK activation in states of negative nitrogen balance, such as sepsis, mentioned above (Vary, 1991; Sugden *et al*, 2000).

Administration of DCA in an animal model of ischaemia/reperfusion (Wilson *et al*, 2003) reduced the blood lactate concentration and the rise in end-tidal CO_2 measured 15 minutes after reperfusion following four hours of ischaemia. These investigators also noted a reduced percentage of animals having more than 10% skeletal muscle necrosis within sections of the anterior tibialis muscle following iliac artery occlusion in the group receiving DCA treatment. The hypothesis suggested by this group was that activation of PDC resulted in provision of acetyl-CoA from pyruvate for entry into the TCA cycle, and subsequent ATP production, even at low muscle oxygen tensions. As a mechanism for the avoidance of reperfusion metabolic acidosis and its associated morbidity and mortality in acute limb ischaemia, or the activation of the inflammatory cascade and the systemic inflammatory response syndrome (SIRS) from intermittent episodes of ischaemia and reperfusion (as seen in patients with intermittent claudication), the PDC is a promising therapeutic target of likely clinical benefit. Prevention of ischaemia-reperfusion endothelial injury in patients with intermittent claudication could avoid the

development of accelerated atherosclerosis and other systemic manifestations of chronic inflammation, which would be of benefit to this population.

Therapeutic PDC activation has been suggested as a means of reducing hyperglycaemia in patients with diabetes mellitus (Stacpoole and Felts, 1970), hyperlactataemia in conditions of both lactic acidosis (Stacpoole *et al*, 2006; Barshop *et al*, 2004) and ischaemia-reperfusion, such as that seen with peripheral vascular disease (Greenhaff *et al*, 2004; Wilson *et al*, 2003). The use of DCA as a therapeutic tool is limited by its toxicity after chronic administration, which has been demonstrated in both animals (Katz *et al*, 1981) and humans (Stacpoole *et al*, 1979) at a range of doses. The results of these studies have linked chronic administration of DCA with reversible neuropathies in human volunteers (Stacpoole, 1989) and with a wide variety of effects (including dyspnoea, pneumonia, conjunctivitis, diarrhoea and death) in animals (Cicmanec *et al*, 1991). Some of these effects are thought to be due to depletion of body thiamine stores (Stacpoole *et al*, 1990), which is a co-factor for both PDC and the ketoglutarate dehydrogenase complex that DCA activates. Whilst there have been attempts to manufacture a combined thiamine-dichloroacetate treatment (Henderson *et al*, 1994), it remains unclear if this will avoid the toxic effects described above.

Forkhead box O transcription factors.

The Forkhead box O (FOXO) class of transcription factors are believed to be a central metabolic regulator in glucose and lipid metabolism, as many signalling pathways converge on these factors (especially FOXO1). FOXO1 both induces or represses the

expression of many target genes involved in metabolism within insulin-responsive tissue and performs transcriptional control on signalling molecules.

FOXOs are highly expressed in both white and brown adipose tissue (Nakae *et al*, 2003) and their expression levels are altered by high fat feeding (Nakae *et al*, 2008).
Researchers have suggested that FOXO1 may suppress PPARγ expression at the transcriptional level. Dowell *et al* (2003) described the finding that FOXO1 decreased the formation of a PPARγ/Rxr/DNA complex. Furthermore it has also been reported (Armoni *et al*, 2006) that FOXO1 can also inhibit PPARγ1 and PPARγ2 promoter activity through direct interaction between FOXO1 and PPARγ promoter.

In Drosophilia during the nutrition-limited state FOXO enhances both transcription of insulin receptor ("primes for changes in nutrient availability") and expression of the translational expressor *4ebp*, which inhibits growth (and therefore spares nutrients which can then be directed towards storage). FOXO1 induces expression of *Irs2* which may improve insulin sensitivity in the insulin resistant state.

FOXOs are expressed in skeletal muscle at a level that is adjusted to changes in energy metabolism. In skeletal muscle the binding of insulin activates two signalling pathways: the Ras-Raf-MEK-ERK pathway (which does not influence muscle fibre size - Murgia *et al*, 2000) and the PI3K/AKT pathway. Activation of the PI3K/AKT pathway appears to stimulate muscle hypertrophy through stimulation of translation via regulation of GSK and mTOR kinases (Bodine *et al*, 2001). mTOR is also regulated by the supply of amino

acids and glucose to the muscle (Kim *et al*, 2003) enabling it to integrate signals from PI3K/AKT pathway with the nutritional status of the cell. One of the downstream targets of the PI3K/AKT pathway is the Forkhead box O (FOXO) class of transcription factors, of which three are found in mammalian cells (FOXO1, FOXO3 and FOXO4 (Tran *et al*, 2003)). Researchers (Brunet *et al*, 1999) have demonstrated that AKT blocks the function of all three FOXO factors by phosphorylation. FOXO factors are required for the development of insulin resistance in type II diabetes (Nakae *et al*, 2002) as well as being induced during starvation. Expression of FOXO1 and FOXO3a was increased in starvation and after glucocorticoid treatment (Furuyama *et al*, 2003). Activation of FOXO factors leads to muscle fibre atrophy through its relation with *MAFbx (muscle atrophy F box, Atrogin 1)* and *MuRF1 (muscle RING finger 1)* which conjugate ubiquitin to protein substrates. During atrophy FOXO1 and FOXO3a expression is increased. A skeletal muscle specific FOXO1 transgenic mouse showed reduced body weight due to a reduction in lean body mass (Kamei *et al*, 2004). These mice showed poor glycaemic control and reduced exercise tolerance.

Starvation reduces blood glucose and insulin concentrations, decreases insulin's actions and results in the nuclear accumulation of FOXO factors. FOXO factors can accelerate glucose production, reduce insulin secretion, decrease fat and muscle mass and generate spare energy in vivo. In humans in an insulin resistant state, such as after excessive caloric intake, the FOXO factors are active even when not in a calorie restricted state. In these circumstances this increased activity may lead to accelerated insulin resistance, eventually resulting in type 2 diabetes mellitus and hyperlipidaemia.

Muscle atrophy occurs in response to disuse, fasting and as a response to various diseases (cancer, AIDS, diabetes mellitus). Regardless of the causative factor these muscles show increased rates of protein degradation through activation of the ubiquitin-proteasome pathway (Jagoe and Goldberg, 2001). Atrophy induces the protein *atrogin-1* (*MAFbx*) most dramatically (mRNA increased 8-40 fold in all types of atrophy) and this increase precedes muscle weight loss. Although low concentrations of insulin can trigger muscle protein loss in diabetes and starvation (Mitch *et al*, 1999) the signal pathway by which this is achieved was unclear until recently.

Sandri *et al* (2004) have demonstrated both atrophy of myotubes and mature muscle fibres by FOXO stimulation and a reduction in the induction of *atrogin-1* by fasting after inhibition of FOXO function. These authors suggested that FOXO factors stimulated many atrophy related genes and enhanced protein degradation, most likely through transcription of the gene for *MuRF-1*. In conditions of reduced insulin concentration or resistance to its effects AKT is dephosphorylated, resulting in activation FOXO and therefore transcription of *atrogin-1*, *MuRF-1* and other genes that promote muscle wasting. These changes reduce protein synthesis through the dephosphorylation of mTOR, GSK and S6K resulting in a reduction in cell protein and myofibre size.

Peroxisome proliferator-activated receptors.

Peroxisome proliferator-activated receptors (PPARs) belong to the steroid/thyroid/retinoid superfamily and are nuclear lipid-activable receptors that control

a number of genes in several pathways of lipid metabolism. These pathways include fatty acid transport and uptake, intracellular binding and activation, catabolism (by β-oxidation and ω-oxidation) or storage. PPARs possess the classic domain structure of other nuclear receptors (e.g. steroid or thyroid hormone receptors) (Ferré, 2004) with an NH_2-terminal region with a ligand-independent transactivation domain (AF-1), followed by a DNA-binding domain and a ligand and dimerization domain and a ligand-independent activation domain (AF-2) at the COOH-terminus. The PPARs bind to the peroxisome proliferator response element on the DNA as obligate heterodimers with the 9-*cis*-retinoic acid receptor (RXR) (Desvergne and Wahli, 1999).

Three related PPAR isotypes (PPARα, PPARβ/δ and PPARγ) have been identified in humans (Dreyer *et al*, 1992; Schmidt *et al*, 1992).

- PPARα

Data from research performed in human subjects indicate that PPARα is found predominantly in the liver, but also in heart, kidney, skeletal muscle and large intestine (Mukherjee *et al*, 1997; Auboeuf *et al*, 1997). The expression of PPARα appears to correlate with high mitochondrial and peroxisomal β-oxidation activities, such as in cardiac muscle and the cells of the kidney proximal tubule (Desvergne and Wahli, 1999).

PPARα binds unsaturated fatty acids at a higher affinity than the other isoforms, with a lower affinity for saturated fatty acids. In the liver activation of PPARα induces the expression of fatty acid transport proteins and long-chain acyl-CoA synthetase

(Schoonjans *et al*, 1995). In association with the promotion of peroxisomal β-oxidation (through effects on enzymes such as acyl-CoA oxidase) this increases the availability of fatty acids for mitochondrial β-oxidation. Within the mitochondrion both the expression of medium-chain acyl-CoA dehydrogenase (involved in β-oxidation) and the mitochondrial form of hydroxymethylglutaryl-CoA synthase (involved in ketone body synthesis) is stimulated by PPARα activation (Gulick *et al*, 1994; Rodriguez *et al*, 1994).

A rise in glucocorticoid concentration will stimulate the expression of PPARα (Lemberger *et al*, 1994), and so stress situations and fasting, which lead to a rise in the concentrations of glucocorticoids, also lead to an increase in the expression of PPARα mRNA and protein. In contrast to this finding, insulin has been demonstrated to lead to a reduction in the expression of PPARα in rat liver (Steineger *et al*, 1994).

- PPARβ/δ

Analysis of samples from human subjects has demonstrated moderate expression of PPARβ/δ in all tissues tested, often at higher levels than the other two PPARs, with increased expression in tissue of placental and colonic origin (Mukherjee *et al*, 1997; Auboeuf *et al*, 1997). Little is currently understood about the regulation of PPARβ/δ expression, but it has been suggested that PPARβ/δ may serve as a general regulator of fat oxidation (Wang *et al*, 2003) or to compensate for reduced expression of the other PPAR isoforms in the tissues where this is low.

- PPARγ

PPARγ is expressed abundantly in human adipose tissue (Mukherjee *et al*, 1997; Auboeuf *et al*, 1997), but is also expressed in several transformed human B lymphocyte and myeloid cell lines as well as in primary bone marrow stromal cells in culture (Greene *et al*, 1995). It exists in two protein isoforms (γ1 and γ2) which differ in that the γ2 form has 28 additional amino acids on the NH_2-terminal side in humans. The γ1 isoform is the dominant form in human adipose tissue (Rosen and Spiegelman, 2001).

The regulation of the PPARγ gene has been extensively studied due to its key role in adipogenesis and as a receptor for insulin-sensitizing drugs. Induction of PPARγ mRNA has been observed in vitro following the exposure of adipocytes to both insulin and corticosteroids (Vidal-Puig *et al*, 1997). The same group demonstrated that the adipose tissue of obese human subjects was found to have increased expression of PPARγ2 mRNA, as well as an increase in the ratio of PPARγ2 to PPARγ1, which was reduced following a low calorie diet. Researchers have concluded that PPARγ1 expression in adipose tissue does not correlate with BMI or fasting blood insulin concentration from a study conducted in 29 subjects with varying degrees of obesity (Auboeuf *et al*, 1994) and, based upon these findings, it has been suggested by Desvergne and Wahli (1999) that in humans PPARγ2 is involved in the control of adipocyte function, rather than PPARγ1. In a study examining PPARγ expression in skeletal muscle (Kruszynska *et al*, 1998) an increase in expression was detected in both obese non-diabetic and patients with type 2 diabetes mellitus in direct relation to their BMI and fasting blood insulin concentration. Although mutations in PPARγ2 have been associated with lower BMI and increased

insulin sensitivity in non-obese subjects (Deeb *et al*, 1998), there is no evidence of an association between this single mutation and either obesity or diabetes mellitus.

The fibrate family of hypolipidaemic drugs (e.g. clofibrate and Wy-14,163) preferentially bind to PPARα, whilst the thiazolidinediones (troglitazone, pioglitazone and rosiglitazone) although derived from clofibric acid selectively bind PPARγ (Lehmann *et al*, 1995). Insulin enhances the transcriptional activity of the PPARγ AF-1 (Werman *et al*, 1997) as well as inducing the activity of the PPARα AF-1 domain (Juge-Aubry *et al*, 1999) suggesting that insulin induces phosphorylation of PPAR via a mechanism that may involve posttranslational modification of an auxiliary factor (Desvergne and Wahli, 1999).

In skeletal muscle stimulation of PPARβ/δ by administration of the selective agonist GW501516 leads to an increase in fatty acid β-oxidation and a marked increase in PDK4 expression (Tanaka *et al*, 2003). Following the administration of high-fat diet the same researchers also noted a significant improvement in glucose tolerance and insulin sensitivity through a mechanism that is currently unclear. They suggested that PPARβ/δ may direct metabolism from the utilisation of carbohydrate towards the utilisation of fatty acids via β-oxidation.

PPARα has a stimulatory effect on human skeletal muscle PDK4 gene expression (Muoio *et al*, 2002). Through this effect it is suggested that during states of high fatty acid availability, such as diabetes and fasting, the reduction in PDC activation will increase

the sparing of glucose derived carbons from metabolism (as the increase in fatty acid oxidation capacity and ketone body synthesis seen following PPARα activation in the liver will already allow for the preservation of carbohydrate derived fuel), allowing for their use in hepatic glucose production. The oxidation of fatty acids and ketone bodies in oxidative muscle spares glucose stores during periods of reduced glucose availability (e.g. fasting) as well as limiting the requirement for hepatic gluconeogenesis from muscle protein catabolism.

The proportion of energy derived from fat oxidation at a given exercise intensity is increased following endurance training, and this is thought to be related to changes in PPAR controlled gene expression rather than changes in the intramuscular fatty acid concentration, as basal levels are similar in both trained and untrained human subjects (Hurley *et al*, 1986). The relative expression in skeletal muscle of PPARβ/δ is greater than PPARα, which in turn is greater than PPARγ (Gilde and Van Bilsen, 2003). PPAR controlled gene expression may be involved in the response of skeletal muscle fat oxidation, and fuel selection during endurance exercise, to changes in intramuscular fatty acid concentrations (Kiens, 2006). This suggestion is supported by the finding that PPARβ/δ-null mice are only able to sustain 30-40% of the running time and distance of control mice (Wang *et al*, 2004).

Aims

The aim of this thesis is to determine the role of the pyruvate dehydrogenase in the regulation of human skeletal muscle metabolism. It is suggested that PDC activation, either by DCA infusion or exercise, and PDC inhibition through high-fat feeding will influence the selection of fuel for subsequent bouts of contraction.

In order to investigate this I intend to take muscle biopsies from human volunteers during exercise following pyruvate dehydrogenase activation, by DCA infusion. At low intensity exercise the contribution of energy for contraction is predominantly from a fat-derived source (van Loon *et al*, 2001), and my hypothesis is that PDC activation using DCA will alter fuel selection towards carbohydrate derived rather than fat derived sources, with a reduction in metabolic inertia at the onset of contraction due to the more efficient provision of ATP from carbohydrate metabolism.

The secondary aim of this thesis would investigate the effect of PDC activation at rest upon the ventilatory threshold. DCA pre-treatment had previously been demonstrated to reduce the blood lactate concentration in humans at rest and during contraction (Timmons *et al*, 1998a; Howlett *et al*, 1999a) and I therefore hypothesise that if the lactate and ventilatory threshold are linked through an increase in VCO_2 occurring from increased buffering of H^+ derived from lactic acid dissociation, then DCA pre-treatment will lead to an alteration in the onset of the ventilatory threshold.

As a tertiary aim I will investigate the effect of PDC activation on human volunteers after high-fat feeding. Previous research has demonstrated that increasing the availability of fatty acids results in an upregulation of PDK and corresponding PDC inhibition (Putman *et* al, 1993; Peters *et al*, 2001), which reduces the contribution of carbohydrate oxidation to skeletal muscle energy provision at the onset of contraction. Administration of DCA, a systemic PDK inhibitor, is hypothesised to reverse the inhibition of PDC induced by high-fat feeding.

Chapter 2.

GENERAL METHODS

Human Volunteers.

Healthy men under the age of 40 years were recruited into all of the studies in this thesis by poster advertisement from the University of Nottingham student body. All the studies were approved by the ethics committee of the University of Nottingham Medical School in accordance with the declaration of Helsinki (1964), and subjects were not permitted to take part if they had participated in another study within the last 3 months. Written information was provided upon initial subject enquiries, and further verbal information was given prior to obtaining written consent to enrol in the studies. All subjects were free to withdraw from the study at any time. Each participant was required to attend a health screening visit prior to participation in any of the experimental visits. This initial visit consisted of a general health questionnaire, as well as measurements of height, weight, resting blood pressure and haematological (full blood count) and biochemical (urea and electrolyte and liver function tests) indices. These blood samples (approximately 8 millilitres) were obtained by venepuncture of the superficial veins within the antecubital fossa using a closed Vacutainer system and analysed by the Departments of Haematology and Clinical Chemistry, Queen's Medical Centre, Nottingham, UK.

After successful completion of the initial health screening visit, subjects visited the laboratory on the morning of the experiment after fasting from midnight having abstained from alcohol and strenuous exercise for at least 24 hours. A record of all procedures was kept, and medical cover was present at all times. All visits were separated by a two week period to ensure similar basal plasma and muscle concentrations between experimental visits.

Dichloroacetate Infusions.

During visits within each experiment subjects received an intravenous infusion of sodium dichloroacetate (50 $mg.kg^{-1}$, which is equal to 0.33 $mmol.kg^{-1}$) for 30 or 60 minutes. Sodium dichloroacetate was obtained from Fluorochem Limited (Derby) and, after quality control testing, was prepared by the Sterile Production Department (Queens Medical Centre, Nottingham) to a concentration of 25 $mg.ml^{-1}$. Following the antegrade insertion of an 18-gauge intravenous cannula (Venflon, Becton Dickinson, New Jersey, USA) into a forearm vein the required dose was infused over a sixty (**Chapter 3**), thirty (**Chapter 4**) or forty-five (**Chapter 5**) minute period. In the latter two experiments this infusion was then followed by thirty or forty-five minutes of supine rest. This dose has been demonstrated in previous studies within the School of Biomedical Sciences (Constantin-Teodosiu *et al*, 1999; Timmons *et al*, 1998b) to completely activate the pyruvate dehydrogenase complex in healthy human volunteers.

Blood Sampling.

On arrival in the laboratory during the experimental visits of **Chapters 4** and **5** an 18-gauge intravenous cannula was retrogradely inserted into a distal forearm or hand vein and the hand kept in a hand warming device (air temperature approximately 55 degrees C) to arterialise the venous blood (Gallen & Macdonald 1990) and an infusion of 0.9% saline chloride solution (Maco Pharma Ltd, London, UK) was used to ensure continued patency of the cannula.

During the visits of **Chapters 4** and **5** five millilitres of arterialised venous blood were obtained for immediate analysis of blood glucose and lactate concentration. During the visits of **Chapter 5** two millilitres of this blood were placed into lithium heparin containers and centrifuged for 14,000 rpm for 2 minutes. This plasma was then removed and immediately frozen in liquid nitrogen, after which it was stored at -80 degrees C for later analysis for free fatty acid (FFA) concentration.

Blood Analysis.

Blood glucose and lactate concentrations.

Glucose concentrations were determined in a small sample of blood, immediately after withdrawal from the sampling cannula, using a glucose/lactate analyser (YSI 2300 STATplus, Yellow Springs Instruments, Ohio, USA). The analyser was calibrated using glucose and lactate standards of known concentration (YSI, Yellow Springs, Ohio, USA).

Plasma non-esterified fatty acid.

Plasma non-esterified fatty acid (NEFA) concentration was determined using an enzymatic-colorimetric assay kit (NEFA C kit, WAKO Chemicals GmbH, Neuss, Germany). Five µl sample or standard (0 - 29.1 $\mu mol.l^{-1}$) were incubated in a 96-well plate for 10 min at 37 C with 100 µl of reagent A, which contained acyl-CoA synthetase (ACS), ATP, and CoASH. The acyl-CoA, formed from endogenous NEFA or oleic acid standard by the action of ACS (Reaction 2), was then oxidised to hydrogen peroxide by the addition of 200 µl of reagent B (Reaction 3), which contained acyl-CoA oxidase (ACO), and incubated for a further for 10 min at 37 C.

$$\text{NEFA} + \text{ATP} + \text{CoASH} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + PP_i \quad [2]$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACO}} \text{2, 3-trans-enoyl-CoA} + H_2O_2 \quad [3]$$

Reagent B also contained peroxidase, which allowed the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline (MEHA) with 4-aminoanti pyrine to form a purple coloured solution, the light absorption of which was measured on a spectrophotometer (Spectra Max 190, Molecular Devices Ltd, Sunnyvale, California, USA) at 550 nm. The NEFA concentration in $mmol.l^{-1}$ was calculated using the slope of the regression line from the standard curve. The regression coefficients were usually >0.9995.

Plasma ammonia.

Two millilitres of blood were mixed with EGTA and centrifuged. The resultant plasma was snap frozen in liquid nitrogen and then analysed for ammonia content according to the methods described by Neeley and Phillipson (1988).

Muscle Sampling.

Muscle biopsies were obtained (**Chapters 3** and **5**), whilst subjects rested in a supine position, from the vastus lateralis (m. quadriceps femoris) using the percutaneous needle biopsy technique (Bergström, 1962). Briefly, the skin around the area on the thigh to be sampled was shaved (if necessary) and then cleaned using an iodine solution. Thereafter, following a small incision (<1 cm) in the skin and fascia using a scalpel blade under local anaesthetic (1% lignocaine HCl, B. Braun, Melsungen, Germany), a 5 mm gauge

Bergström biopsy needle (Bignell Surgical Instruments Ltd, West Sussex, UK) was used to take a small sample of muscle (50–200 mg wet weight) which was then immediately frozen in liquid nitrogen. When more than one biopsy was taken, a distance of at least 2.5 cm was allowed between the prospective sites in order to minimise the effects of an inflammatory response on muscle metabolite concentrations (Constantin-Teodosiu et al, 1996). The sampling time between insertion of the needle, withdrawal, and plunging into liquid nitrogen lasted no more than 10 s.

Muscle Analysis.

Muscle metabolite extraction.

Approximately two-thirds of each muscle biopsy sample was subsequently freeze-dried over night (Edwards Freeze Dryer Modulyo), powdered, and stored at -80 C for metabolite extraction and analysis at a later date. The muscle powdering procedure took place at room temperature using a pestle and mortar for 20 min (per sample) under a lamp and magnifying glass with all visible blood and connective tissue being removed. The remaining muscle tissue was stored 'wet' in liquid nitrogen and used for the determination of mRNA (**Chapter 5**) and pyruvate dehydrogenase complex activity (**Chapters 3** and **5**) analysis at a later date.

The extraction of the membrane bound metabolites, contained within the powder, was performed by the addition of ice-cold 0.5M PCA, containing 1mmol.l^{-1} EDTA to each aliquot of powdered muscle. Samples were then kept on ice over a period of 10 minutes with each one, in turn, being subjected to aggressive vortexing. Extracts were then

centrifuged (for 2 minutes at 14000 rpm) and the resulting supernatants removed and neutralised with 2.2 mol.l^{-1} $KHCO_3$. Extracts were then re-centrifuged, followed by the removal and storage of their supernatants at -80 C for later analysis. The extraction dilution factor (F) for each sample was calculated as follows:

$$F = \frac{Vol_{PCA} \times (Vol_{SUP} + Vol_{KHCO3})}{Vol_{SUP} \times Wt_{MUS}}$$

Where:

Vol_{PCA} = Volume of PCA added (µl)

Vol_{SUP} = Volume of PCA supernatant (µl)

Vol_{KHCO3} = Volume of $KHCO_3$ added to the PCA extract (µl)

Wt_{MUS} = Weight of the muscle powder (mg)

Muscle ATP, PCr, creatine, glucose-6-phosphate, lactate and glycogen determination.

Muscle glucose-6-phosphate (G-6-P), ATP and PCr concentrations were determined spectrophotometrically in NADPH/NADH linked reactions (Harris *et al*. 1974) as outlined below:

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\text{G-6-PDH}} \text{P-gluconolactone} + NADPH + H^+$$

$$ATP + \text{Glucose} \xrightarrow{\text{HK}} ADP + \text{Glucose-6-phosphate} + H^+$$

$$\text{Phosphocreatine} + ADP + H^+ \xrightarrow{\text{CPK}} \text{Creatine} + ATP$$

Briefly, the absorbances at 340 nm of microcuvettes containing all components of the reaction mixture (including sample/blank/standard), other than hexokinase (HK), glucose-6-phosphate dehydrogenase (G-6-PDH) and creatine phosphokinase (CPK) were

measured giving absorbance-1 (A1). Absorbances were also measured following the addition of G-6-PDH, giving absorbance-2 (A2), following the addition of HK, giving absorbance-3 (A3), and following the addition of CPK, giving absorbance-4 (A4), to the reaction mixture. Following addition of each enzyme, cuvettes were incubated at room temperature with constant agitation on a rotating plate until the reaction had reached a plateau. Changes in absorbance measurements of samples were used to calculate G-6-P, ATP and PCr concentrations of the samples using the following calculations:

$$[\text{G-6-P}]\ \text{mmol.kg}^{-1}\ \text{dry muscle} = \frac{(V2\ (A2 - Bl2) - V1\ (A1 - Bl1)) \times F}{6.22 \times SV}$$

$$[\text{ATP}]\ \text{mmol.kg}^{-1}\ \text{dry muscle} = \frac{(V3\ (A3 - Bl3) - V2\ (A2 - Bl2)) \times F}{6.22 \times SV}$$

$$[\text{PCr}]\ \text{mmol.kg}^{-1}\ \text{dry muscle} = \frac{(V4\ (A4 - Bl4) - V3\ (A3 - Bl3)) \times F}{6.22 \times SV}$$

Where:

V1 = Initial reaction volume (µl)

V2 = V1 + volume of G-6-PDH (µl)

V3 = V2 + volume of HK added (µl)

V4 = V3 + volume of CPK added (µl)

A1 (or Bl1) = Initial absorbance of reagent + sample (or blank) in cuvette

A2 (or Bl2) = Absorbance following addition of G-6-PDH to sample (or blank)

A3 (or Bl3) = Absorbance following addition of HK to sample (or blank)

A4 (or Bl4) = Absorbance following addition of CPK to sample (or blank)

F = Extraction dilution factor

6.22 = Millimolar absorption coefficient of NADPH at 340 nm

SV = Sample Volume

Muscle creatine concentration was determined by a similar method using NADH linked reactions in accordance with the method of Harris *et al*. (1974) outlined below:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

$$\text{ADP} + \text{phosphoenolpyruvate} + H^+ \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{CPK}} \text{phosphocreatine} + \text{ADP} + H^+$$

The absorbances at 340 nm of microcuvettes containing all components of the reaction mixture (including sample/blank/standard) other than CPK were measured (A1). Following the addition of CPK and incubation until the reaction has reached its end-point (constant rate of decline in absorbance), absorbances of cuvettes were measured (A2). Changes in absorbance measurements of samples were also used to calculate creatine (Cre) concentration of the samples using the following calculation:

$$[\text{Cre}]\ \text{mmol.kg}^{-1}\ \text{dry muscle} = \frac{(V2\ (A2 - Bl2) - V1\ (A1 - Bl1))\ \text{x}\ F}{6.22\ \text{x}\ SV}$$

Muscle lactate concentration was determined by a similar method using an NADH linked reaction in accordance with the method of Harris *et al*. (1974) outlined below:

$$\text{Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + H^+$$

The absorbances at 340 nm of microcuvettes containing all components of the reaction mixture (including sample/blank/standard) other than LDH were measured (A1). Following the addition of LDH and incubation until the reaction has reached a plateau,

absorbances of cuvettes were measured (A2). Changes in absorbance measurements of samples were also used to calculate lactate concentration of the samples using the following calculation:

$$[\text{Lactate}]\ \text{mmol.kg}^{-1}\ \text{dry muscle} = \frac{(V2\ (A2 - Bl2) - V1\ (A1 - Bl1)) \times F}{6.22 \times SV}$$

Muscle glycogen content was determined by the method of Harris *et al* (1974) and utilized 0.5 – 4.0 mg of freeze dried muscle powder. Muscle glycogen concentration was determined by an assay that first involved hydrolysis of glycogen by heating at 80 C for 10 minutes in 100 – 160 µl of 0.1 $mol.l^{-1}$ NaOH, followed by neutralization with 400 – 640 µl of buffered HCl (3:1 ratio of 0.2 $mol.l^{-1}$ citric acid/0.2 $mol.l^{-1}$ Na_2HPO_4 buffer : 0.1 $mol.l^{-1}$ HCl). This mixture was then incubated at room temperature for 60 minutes in the presence of 15 – 25 µl aminoglucosidase and the supernatant stored at - 80 C for the subsequent determination of glucose content of sample, standards and blanks. The extraction dilution factor (F_{GLY}) of the sample was then calculated by the following equation:

$$F_{GLY} = \frac{Vol_{EXT} + Wt_{MUS}}{Wt_{MUS}}$$

Where:

Vol_{EXT} = Sum of all extraction reagent volumes.

The concentration of glycosyl units in the glycogen digests were determined spectrophotometrically in NADH linked reactions in accordance with Harris *et al* (1974) as outlined below:

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-phosphate} + H^+$$

$$\text{Glucose-6-phosphate} + NAD^{+} \xrightarrow{\text{G-6-PDH}} \text{P-gluconolactone} + NADH + H^{+}$$

The absorbance at 366 nm of microcuvettes containing all the components of the reaction mixture (including sample/blank), other than HK and G-6-PDH were measured (A1/Bl1). Following addition of HK and G-6-PD and incubation until the reaction had reached a plateau, absorbances of cuvettes were measured (A2/Bl2). Changes in absorbance measurements of samples and blanks were used to calculate glycosyl concentrations of the samples using the following calculation:

$$[\text{Glucose}]\ \text{mmol.l}^{-1}\ \text{dry muscle} = \frac{[((A2 \times V2) - (A1 \times V1)) - ((Bl2 \times V2) - (Bl1 \times V1))] \times F_{GLY}}{3.34 \times SV}$$

Where:

3.34 = millimolar absorption coefficient of NADH at 366 nm.

After removal of visible blood and connective tissue, the freeze-dried muscle samples were powdered and analyzed for acetylcarnitine content using a modified version of the radioenzymatic method of Cederblad *et al*. (1990).

Muscle acetyl-CoA, free-CoASH, acetylcarnitine and free carnitine determination.

Acetyl-CoA, free-CoASH, acetylcarnitine and free carnitine were determined from the perchloric acid muscle extracts used for ATP, PCr and creatine according to the radioisotopic assays of Cederblad *et al* (1990). Briefly, the determination of acetyl-CoA was composed of two isotopic reactions. Firstly fresh [^{14}C]-oxaloacetate was produced from [^{14}C]-aspartate by aspartate aminotransferase in accordance with Cooper *et al* (1986). The second reaction condenses the sample's acetyl-CoA with the [^{14}C]-oxaloacetate forming [^{14}C]-citrate in the presence of citrate synthase as outlined below:

L-[^{14}C]-aspartate + 2-oxoglutarate $\longleftrightarrow$ [^{14}C]-oxaloacetate + L-glutamate

[^{14}C]-oxaloacetate + acetyl-CoA $\longleftrightarrow$ [^{14}C]-citrate + CoASH

Any remaining [^{14}C]-oxaloacetate was transaminated back to [^{14}C]-aspartate enabling the [^{14}C]-citrate to be recovered, by the addition of 1 ml of Dowex (H^+) resin (18 g/30 ml reagent grade water) removing the negatively charged [14C]-aspartate from the supernatant. Sample radioactivity was determined within a 0.5 ml aliquot of the supernatant, to which 5 ml of scintillation fluid was added, with each sample being counted over 5 minutes (1214 Rackbetar, β-liquid scintillation counter, LKB Wallac). An acetyl-CoA standard curve (0 – 125 pmol; $r > 0.9999$) was run in duplicate within each batch of analysis.

To determine the concentration of acetylcarnitine, the perchloric acid muscle extractions used for ATP, PCr and creatine were further diluted (10:100) with reagent grade water. Briefly, acetylcarnitine's acetyl group was transferred to CoASH, in a reaction catalysed by carnitine acetyltransferase, as outlined below:

Acetylcarnitine + CoASH $\longleftrightarrow$ acetyl-CoA + carnitine

The resulting acetyl-CoA was then determined, in the common step, as described previously. An acetylcarnitine standard curve (0 – 125 pmol; $r > 0.9999$) was run in duplicate within each batch of analysis. Each sample was run once.

Free carnitine was assessed by using the carnitine acetyltransferase reaction and [^{14}C]-acetyl-CoA. The labelled acetyl group was transacetylated onto the endogenous carnitine forming [^{14}C]-acetylcarnitine, as outlined below:

[^{14}C]-acetyl-CoA + carnitine $\longleftrightarrow$ [^{14}C]-acetylcarnitine + CoASH

An L-carnitine standard curve (0 – 3600 pmol; r > 0.9999) was run in duplicate with each batch of analysis. Each sample was run once. The reaction was stopped by passing the reaction brew through a 5 cm column of Dowex (Cl^-) resin (1 x 8-100, Mesh 200 – 400) which retains the excess [^{14}C]-acetyl-CoA and allows the Dowex to be regenerated following a salting out process.

Muscle metabolite correction for total creatine concentration.

All samples, whenever possible, were analysed for the same metabolite on the same day using the same batch of enzyme and made up reagents. Samples were analysed in a blind manner, where the order of the samples was unknown until the later checking of records. This was done to prevent any user bias. All metabolites that cannot move freely between the intracellular and extracellular spaces (ATP, phosphocreatine, glucose-6-phosphate, creatine and acetylcarnitine) were corrected for the average total creatine concentration (PCr + creatine) within the study population. By this means it is possible to compensate for any admixture of connective tissue and other non-muscular elements within each individual muscle biopsy sample (Hultman and Sjöholm, 1983).

Pyruvate dehydrogenase complex activation status.

The PDC activation status was assessed using the method described by Constantin-Teodosiu *et al* (1991). This analysis optimised ~4 mg chipped wet samples, stored under liquid nitrogen, prepared previously. Briefly, the assay involves the measurement of PDC that exists in its active moiety (PDCa) at the time of extraction. This assay is possible for PDC as activation *in vivo* is as a result of covalent modification, which

survives the extraction procedure (Hansford, 1994). Following homogenisation and extraction the amount of PDC in its active state was conformationally locked by the addition of DCA and NaF to the buffer, to inhibit the intrinsic kinase (PDK) and phosphatase (PDP) respectively. The rate this preparation converted 1 mM of added pyruvate into acetyl-CoA, over the course of a 3 minute period, was then investigated. The resultant acetyl-CoA was determined as described previously. All samples were run in duplicate and blanks performed by substituting water for pyruvate in the reaction brew, each day. PDCa was expressed as mmol acetyl-CoA.min^{-1}.(mg protein)$^{-1}$, but as protein content has been seen to hardly vary between samples (Constantin-Teodosiu, personal communication) the rate was expressed as a specific activity with units of mmol acetyl-CoA.min^{-1}.(kg wet muscle)$^{-1}$ at 37 C. Each sample was run once and had a CV of 5.0%.

Real time PCR.

Total RNA was isolated from snap frozen muscle using RNA plus (Qbiogene) according to the manufacturer's protocol. First strand cDNA was then synthesised from 1 μg RNA sample using random primers (Promega) and PowerScript Reverse Transcriptase (BD Biosciences).

Taqman PCR was carried out using an ABI prism 7000 sequence detector (Applied Biosystems, USA), with 2 μl of cDNA, 18 μM of each primer, 5 μM probe, and Universal Taqman 2x PCR Mastermix (Eurogentec) in a 25 μl final volume. Each sample was run in triplicate, in duplex reactions. Cyclophilin A labelled with the fluorescent dye VIC was used as internal control, while all genes of interest were labelled with the fluorescent reporter FAM. The thermal cycling conditions used were: 2 min at 50 C, 10

min at 95 C, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min. Ct values of the target gene were normalized to Ct values of the internal control cyclophilin A, and the final results were calculated according to the $2^{-\Delta\Delta Ct}$ method. The group of vehicle treated rats was used as calibrators with a value of 1.

Statistical Analysis.

All data within this thesis are reported as means ± SEM calculated using Microsoft's Excel 2003 spreadsheet package. Comparisons between treatments, for both absolute concentrations and changes from rest, were carried out using two-way (time and treatment) analysis of variance (ANOVA) with repeated measures, unless otherwise stated in the text, by use of the specialised statistical package SPSS (Version 12.0 for Microsoft Windows). When a significant F value was found, accepted at or below the 5% level, an LSD post-hoc analysis was performed to locate differences. Pearson correlation coefficients were used for describing linear relationships and linear regression was used in the determination of the concentration of carnitine, CoASH and their acetylated forms.

Calculation of ATP production from substrate level phosphorylation.

The amount of ATP produced from non-oxygen dependent routes; better termed substrate level phosphorylation (SLP) was calculated from changes in the concentration of muscle PCr, lactate and ATP using the following equation:

$$SLP\ (mmol.(kg\ dry\ muscle)^{-1}) = (\Delta[PCr]) + (1.5 \times \Delta[muscle\ lactate]) + (2 \times \Delta[ATP])$$

Where:

Δ = Difference in concentration of stated metabolite between two biopsy sampling points.

It was taken that the lactate formed was derived solely from glycogen and hence the net yield of ATP derived from glycolysis here would be 3 per glucosyl unit. Therefore, when calculated from lactate the yield becomes 1.5 since each glucosyl unit yields 2 molecules of lactate. Due to the stochiometric rise in [IMP] concentration when muscle ATP concentration falls the net yield of 'ATP-equivalents' involving the myokinase reaction is the equivalent of 2 ATPs for every 1 mmol.(kg dry muscle)$^{-1}$ change in concentration (Spriet *et al* 1987).

Coefficients of Variation for analytical measurements.

The co-efficients of variation (CV) for the measurement of substrate and metabolite concentrations within blood and muscle samples are expressed as percentages and presented in Table 2.1. The CV % was determined by repeated measurements of the same sample ten times and was calculated as follows:

$$\text{CV \%} = \frac{\text{Standard deviation of repeated measurement}}{\text{Mean of repeated measurement}} \times 100$$

Tissue	Measurement	*n*	CV %
Blood	Glucose	10	1.2
	Lactate	10	1.0
Muscle	G-6-P	10	0.5
	ATP	10	0.6
	PCr	10	0.8
	Creatine	10	1.0
	Lactate	10	0.9
	Glycogen	10	1.3
	Acetyl-CoA	10	3.9
	Free-CoASH	10	3.9
	Acetylcarnitine	10	3.9
	Free Carnitine	10	3.9
	PDCa	10	5.0

Table 2.1 Coefficient of variances for analytical measurements. Number of trials (*n*)

Chapter 3.

THE CONTRIBUTION OF PYRUVATE DEHYDROGENASE COMPLEX ACTIVATION TO OXIDATIVE ENERGY RELEASE DURING LOW INTENSITY EXERCISE

Introduction.

The lag in oxidative ATP delivery that occurs at the onset of steady-state exercise, characterised by a rapid hydrolysis of muscle phosphocreatine (PCr) and accumulation of lactate, has been attributed by some to an inertia in mitochondrial ATP production (Yoshida *et al*, 1995; Grassi *et al*, 1998a, 1998b), rather than the classically accepted theory of a delay in muscle oxygen delivery (Margaria *et al*, 1965). Moreover, it appears that this 'metabolic inertia' resides at the level of the skeletal muscle pyruvate dehydrogenase complex (PDC).

The PDC is covalently regulated by two competing enzymes, a Ca^{2+}-dependent phosphatase, which dephosphorylates pyruvate dehydrogenase (PDH) and transforms the enzyme to the active form, and a kinase, which catalyses the ATP-dependent phosphorylation of PDH and inactivates the enzyme complex (Wieland, 1983). The outcome of this antagonism determines the amount of PDC existing in a non-phosphorylated, active form (PDCa). The flux through the PDC reaction (its catalytic activity), is dependent on the amount of PDCa and the availability of pyruvate, free coenzyme A (CoASH) and NAD^+. It appears that, whilst pyruvate production is important to the magnitude of flux through the PDC reaction *in vivo*, it is of little importance to the magnitude of PDCa; increased muscle Ca^{2+} availability, as a result of muscle contraction, and insulin are most likely the principal activators of PDC transformation *in vivo* (Mandarino *et al*, 1987; Constantin-Teodosiu *et al*, 1991a, 2004; Putman *et al*, 1995). As a result of the PDC reaction, pyruvate and CoASH are oxidized to acetyl-CoA, while NAD^+ is reduced to NADH. The acetyl group formed is either further oxidized in the

tricarboxylic acid (TCA) cycle or, when the availability of acetyl-CoA becomes greater than its rate of entry into TCA cycle, is sequestered by carnitine to form acetylcarnitine (Childress *et al*, 1966; Constantin-Teodosiu *et al*, 1991a).

It has been demonstrated in ischemic contracting canine and human skeletal muscle (Timmons *et al*, 1997, 1998a; Roberts *et al*, 2002a), and in humans during bicycling exercise under normoxic conditions (Howlett *et al*, 1999a), that pharmacological activation of the PDC, using dichloroacetate (DCA; a systemic PDC kinase inhibitor), resulted in a stockpiling of acetyl groups (i.e. acetyl-CoA and acetylcarnitine) at rest and reduced PCr hydrolysis and lactate accumulation compared to control at the onset (i.e. within the first min) of subsequent muscular contraction. This research suggested that there is a period during the rest-to-work transition when acetyl group availability does not meet the energy demands of contraction due to a lag in PDC activation, resulting in non-oxidative ATP production. Indeed, Roberts *et al* (2002a) demonstrated in canine gracilis muscle (where 5 muscle biopsies were taken over the first minute of contraction) that a lag in acetyl group provision (an 'acetyl-group deficit') occurred during the initial 20 s of contraction, which resulted from, and was mirrored by, a lag in PDC activation. Thus, these investigations collectively established the activation of the PDC as a rate-limiting step in the rate of rise in mitochondrial ATP production in skeletal muscle at the onset of contraction, which in turn will dictate the magnitude of non-oxidative ATP production, and thereby the rate of fatigue development during contraction.

The above studies investigated the metabolic inertia at the level of PDC at the onset of moderate to high intensity muscle contraction. It has been demonstrated that at the onset of very high intensity bicycling exercise (Howlett *et al*, 1999b; Savasi *et al*, 2002) or one-legged knee-extensor exercise (Bangsbo *et al*, 2002) in humans during normoxic conditions, DCA administration does not alter non-oxidative ATP provision or muscle O_2 uptake during the rest-to-work transition compared to control. This would suggest that there is an upper workload intensity above which acetyl group delivery is no longer limiting towards TCA cycle demand, most likely due to the rapid, Ca^{2+}-induced activation of PDC at this exercise intensity (Putman *et al*, 1995; Howlett *et al*, 1998). Timmons *et al* (2004) hypothesised, and demonstrated, that when the initial rate of PCr degradation at the onset of very high intensity exercise exceeded approximately 1 $mmol \cdot (kg\ dm)^{-1} \cdot s^{-1}$, the priming of oxidative metabolism with DCA was not readily measurable, most probably because the exercise intensity employed did not rely on a large enough contribution from mitochondrial ATP production and, therefore, was unlikely to be influence by acetyl group availability. In contrast to these findings, it is suggested that during low intensity exercise the magnitude of metabolic inertia will be less, as the rate of ATP demand will be lower and will be better matched by the rate of PDC activation and flux at the onset of exercise. In line with this hypothesis, it was demonstrated that increasing muscle acetyl group availability independently of PDCa, by intravenously infusing sodium acetate, had no effect on muscle PCr degradation or lactate accumulation, compared to control, in healthy human volunteers bicycling at ~65% VO_2max (Evans *et al*, 2001). However, although both the increase in PDCa and the stockpiling of acetyl groups in resting muscle contribute to reducing inertia in

mitochondrial ATP production at the onset of subsequent contraction, the former is quantitatively more important (Roberts *et al*, 2001) and, to date, has not been investigated during low intensity exercise under normoxic conditions.

The aim of the present study was, therefore, to investigate whether metabolic inertia at the level of PDC would be overcome at the onset of exercise <65% VO_2max in human skeletal muscle, by pre-treatment with DCA. Furthermore, it is not known whether the reported effect of DCA on PDCa persists over more than one bout of exercise, and, therefore, an additional aim of the present study was to investigate the effect of DCA on PDC activation during a second bout of exercise following a period of resting recovery.

Methods.

Subjects

Nine healthy, non-smoking, non-vegetarian young men (age 27 ± 2 yr, body mass 77.4 ± 3.6 kg, body mass index 24.0 ± 0.8 $kg \cdot m^{-2}$) participated in the present study. Upon entry to the study each participant performed a continuous, incremental exercise test to exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Lode, Groningen, The Netherlands) to determine their maximal rate of oxygen consumption (VO_2max), measured using an on-line analysis system (SensorMedics, Anaheim, CA, USA), which was confirmed 3 days later. The mean VO_2max for the group was 43.0 ± 1.9 $ml \cdot min^{-1} \cdot kg^{-1}$.

Experimental protocol

Each subject reported to the laboratory at 0900h on two occasions, separated by a 2-week washout period, and voided their bladder. All subjects had abstained from alcohol and strenuous exercise for the previous 48 h. On arrival, subjects were asked to rest in a supine position on a bed while a cannula was placed in an antecubital vein in the non-dominant forearm. At t=0, dichloroacetate (DCA; Fluorochem Ltd., Derby, UK), or the equivalent volume of 0.9% saline (CON; Baxter Healthcare, Northampton, UK), was intravenously infused at a rate of 50 $mg \cdot kg^{-1} \cdot h^{-1}$ for 1 h. Thereafter, the cannula was removed and subjects commenced two-legged cycling exercise on an electrically braked cycle ergometer (Lode Excalibur, Lode, Groningen, The Netherlands) for 6 min at a predetermined workload equivalent to 60% VO_2max (195 ± 12 W), whilst maintaining a pedalling frequency of ~70 rpm. Following a 15 min resting period, subjects performed

another 6 min bout of bicycling exercise at 60% VO_2max. Perceived exertion, assessed using the Borg Ratings of Perceived Exertion scale (Borg, 1970), and heart rate were recorded every minute during the exercise bouts.

Sample collection and analysis

Muscle biopsy samples were obtained from the vastus lateralis muscle immediately before and after each bout of exercise using the percutaneous needle biopsy technique (Bergstrom, 1975), and were snap frozen in liquid nitrogen less than 5s after removal from the limb. These biopsies were taken as the major changes in skeletal muscle PDC activation occur rapidly, but changes in ATP and PCr are constant throughout exercise from 6 minutes onwards (Karlsson and Saltin, 1970). One portion of the sample was subsequently freeze-dried and stored at -80 degrees C, and the remainder was stored "wet" in liquid nitrogen.

Statistical analysis and calculations

A two-way ANOVA (time and treatment effects; GraphPad Prism 4.02, GraphPad Software Inc, CA) was performed to locate differences in muscle PDCa and muscle carnitine, acetylcarnitine, glycogen, lactate, PCr, ATP and glucose-6-phosphate content. When a significant main effect was detected, data were further analysed with Student's paired *t* tests using the Bonferroni correction. Statistical significance was declared at $P<0.05$, and all the values presented in text, Tables, and Figures are means ± standard error of the mean (SEM). ATP production derived from substrate level phosphorylation

was calculated from ΔPCr + (1.5 x Δlactate) + (2 x ΔATP), where Δ is the difference between pre and post exercise concentrations.

Results.

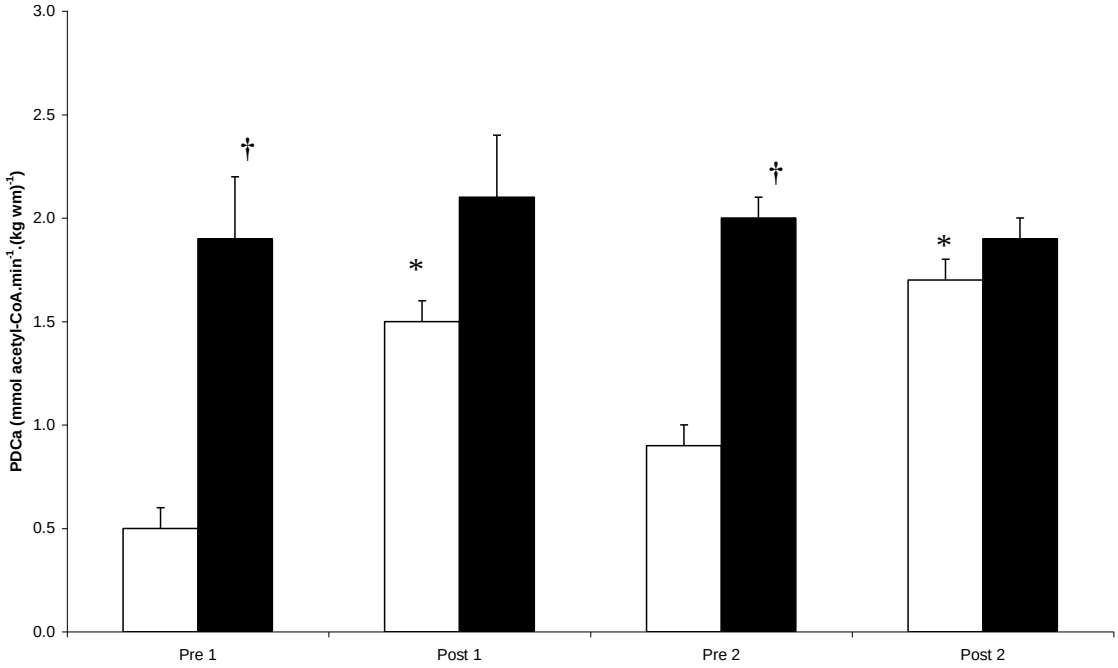
Heart rate and perceived exertion

There was no difference between either group with respect to heart rate and perceived exertion at the end of the two bouts of exercise. Borg scale values were the same (14 ± 1) at the end of both exercise bouts in both visits. Heart rate was 153 ± 5 vs. 153 ± 7 and 161 ± 5 vs. 159 ± 6 beats·min^{-1} for control and dichloroacetate pre-treated groups at the end of the first and second exercise bouts, respectively.

PDC activation status

Skeletal muscle PDCa before and after the two bouts of exercise in the CON and DCA visits is presented below (figure 3.1). As previously demonstrated, muscle PDCa increased following the first bout of exercise within the control group from a basal value of 0.5 ± 0.1 to 1.5 ± 0.1 mmol acetyl-CoA·min^{-1}·(kg wet muscle)$^{-1}$ ($p<0.01$). Thereafter, muscle PDCa returned to basal following the 15 min resting recovery period (0.9 ± 0.1 mmol acetyl-CoA·min^{-1}·(kg wm)$^{-1}$), which then increased significantly following the second bout of exercise (1.7 ± 0.1 mmol acetyl-CoA·min^{-1}·(kg wm)$^{-1}$; $p<0.01$).

Pre-treatment with intravenous dichloroacetate resulted in a 4-fold greater resting muscle PDCa compared to the control group (1.9 ± 0.3 mmol acetyl-CoA·min^{-1}·(kg wm)$^{-1}$; $p<0.01$). Following the first bout of exercise PDCa remained elevated in DCA compared to the control group following the first bout of exercise (2.1 ± 0.3 mmol acetyl-CoA·min^{-1}·(kg wm)$^{-1}$). This increase in PDCa was also detected before and after the second bout



<u>Figure 3.1</u> Muscle PDC activation. Muscle PDCa before (pre) and after (post) two 6 min bouts of cycling exercise at 60% VO_2max, separated by 15 min rest, following 1 h of intravenous 0.9 % saline (CON; white blocks) or dichloroacetate (DCA; black blocks) infusion. $^{*}p<0.01$ vs. corresponding pre-exercise value. †$p<0.05$ DCA vs. CON.

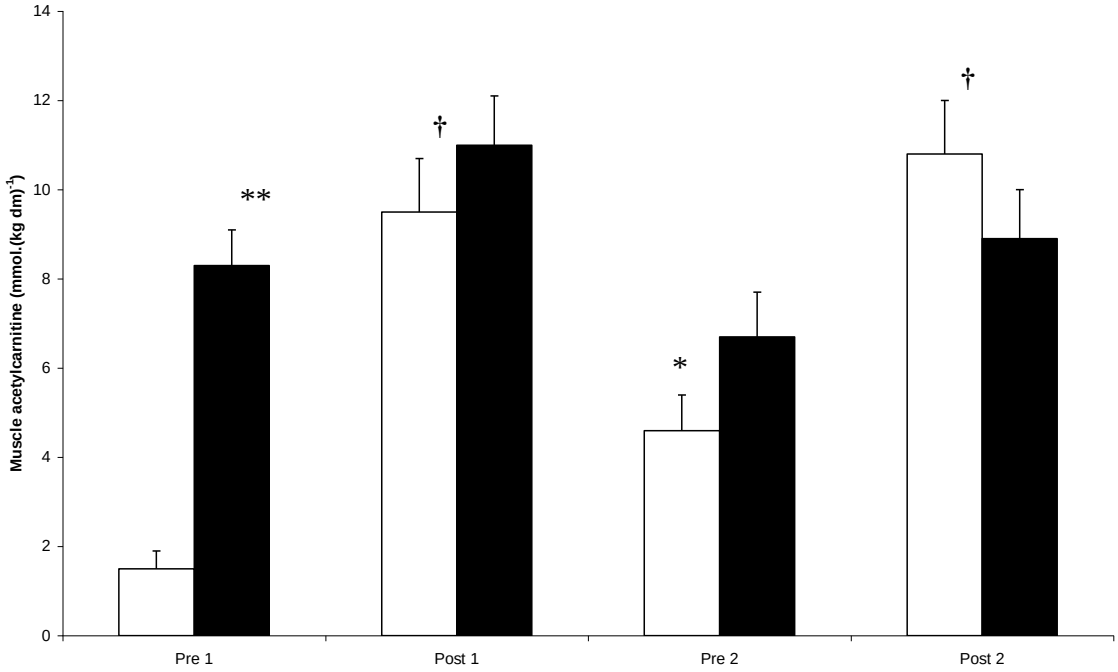
of exercise when compared to the control group (2.0 ± 0.1 and 1.9 ± 0.1 mmol acetyl-CoA·min^{-1}·(kg wm)$^{-1}$, respectively; $p<0.05$).

Muscle metabolites

Figure 3.2 shows skeletal muscle acetylcarnitine content before and after the two bouts of exercise in both visits. Muscle acetylcarnitine content increased following the first bout of exercise in the control group from 1.5 ± 0.4 to 9.5 ± 1.2 mmol·(kg dry muscle)$^{-1}$ ($p<0.01$). Following the 15 min resting recovery period muscle acetylcarnitine content remained elevated above basal (4.6 ± 0.8 mmol·(kg dm)$^{-1}$; $p<0.05$), and then increased further compared to basal following the second bout of exercise (10.8 ± 1.2 mmol·(kg dm)$^{-1}$; $p<0.01$).

Pre-treatment with dichloroacetate resulted in a 5.5-fold greater resting muscle acetylcarnitine content when compared to the control group (8.3 ± 0.8 mmol·(kg dm)$^{-1}$; $p<0.01$). When compared to the basal value within the control group, muscle acetylcarnitine content remained elevated in DCA following the first bout of exercise (11.0 ± 1.1 mmol·(kg dm)$^{-1}$; $p<0.01$) and before and after the second bout of exercise (6.7 ± 1.0 and 8.9 ± 1.1 mmol·(kg dm)$^{-1}$, respectively; $p<0.01$).

Other skeletal muscle metabolite contents before and after the two bouts of exercise in the both visits are presented in Table 3.1. Muscle ATP and glucose-6-phosphate content remained constant throughout all time points in both visits in both the control and dichloroacetate pre-treated groups. Muscle phosphocreatine content decreased from



<u>Figure 3.2</u> Muscle acetylcarnitine content. Muscle acetylcarnitine content before (pre) and after (post) two 6 min bouts of cycling exercise at 60% VO_2max, separated by 15 min rest, following 1 h of intravenous 0.9% saline (CON; white blocks) or dichloroacetate (DCA; black blocks) infusion. $^{*}p<0.05$ vs. corresponding pre-exercise 1 value, $^{\dagger}p<0.05$ vs. corresponding pre-exercise value. **$p<0.05$ DCA vs. CON.

Table 3.1 Muscle metabolites.

	Exercise bout 1				Exercise bout 2			
	CON		DCA		CON		DCA	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
ATP	25.4 ± 1.6	24.5 ± 1.6	26.2 ± 1.0	23.5 ± 1.3	22.5 ± 2.2	23.8 ± 1.8	23.8 ± 2.1	22.2 ± 1.9
PCr	71.0 ± 2.1	63.0 ± 4.0	74.4 ± 3.1	57.0 ± 4.0†	71.5 ± 4.1	65.6 ± 3.0	73.3 ± 7.0	63.7 ± 4.6
Lactate	5.3 ± 0.9	35.7 ± 6.7†	3.7 ± 0.8	33.8 ± 7.2†	11.7 ± 2.0*	31.9 ± 5.7†	12.0 ± 3.0*	30.0 ± 6.9†
Glycogen	321 ± 33	266 ± 35	320 ± 40	222 ± 30	273 ± 33	185 ± 33	216 ± 22*	197 ± 22
Glucose-6-P	4.0 ± 0.3	5.1 ± 0.4	4.0 ± 0.6	4.7 ± 0.5	3.1 ± 0.4	4.7 ± 0.3	4.3 ± 0.5	6.2 ± 0.7
Carnitine	13.1 ±1.6	6.3 ± 0.5†	7.5 ± 0.8**	4.2 ± 0.6† **	10.7 ± 0.8	5.6 ± 0.6†	7.7 ± 1.1**	5.6 ± 1.1

Values are means ± SEM expressed as mmol.(kg dm)$^{-1}$. $^{*}p<0.05$ vs. corresponding pre-exercise 1 value, $^{\dagger}p<0.05$ vs. corresponding pre-exercise value. $^{**}p<0.05$ DCA vs. CON.

basal to a similar degree following the first exercise bout in control ($p<0.05$) and DCA pre-treated ($p<0.01$) groups. Following this PCr content returned to basal following the 15 min resting recovery period and decreased from basal following the second bout of exercise in both visits ($p<0.05$). There was no difference between groups at any point with regard to muscle phosphocreatine content.

Muscle lactate increased above basal to the same extent in both control and DCA pre-treated groups following the first exercise bout ($p<0.01$) and remained elevated above basal following the 15 min resting recovery period and the second bout of exercise ($p<0.01$). There was no difference in muscle lactate content between groups at any time point in this study.

Following the first exercise bout, muscle glycogen content decreased below basal in control and DCA pre-treated groups ($p<0.05$). It remained at that content following the 15 min resting recovery period ($p<0.05$), and decreased further following the second bout of exercise in both groups ($p<0.01$). There was no difference in muscle glycogen content between groups detected at any time point in this study.

Muscle carnitine content decreased following the first bout of exercise in the control group ($p<0.01$), and remained below basal following the 15 min recovery period ($p<0.05$). This then decreased further compared to basal following the second bout of exercise ($p<0.01$). Intravenous DCA infusion resulted in around a 2-fold lower resting muscle carnitine content compared to controls ($p<0.01$). Furthermore, muscle carnitine content remained lower in the DCA pre-treated group compared to the basal

control value following the first bout of exercise and before and after the second bout of exercise ($p<0.01$).

The calculated contribution from ATP production derived from substrate level phosphorylation was the same for both groups following the two bouts of exercise (55.5 ± 11.9 vs. 68.0 ± 12.3 and 33.6 ± 8.6 vs. 39.9 ± 16.6 $mmol \cdot (kg\ dm)^{-1}$, respectively, $p>0.05$). However, ATP production from substrate level phosphorylation was less during the second bout of exercise in both groups (time effect $p<0.05$).

Discussion.

The primary aim of the present study was to investigate whether a metabolic inertia, at the level of the PDC, could be overcome by DCA pre-treatment at the onset of low intensity bicycling exercise in human skeletal muscle under normoxic conditions. In this respect, the most significant finding of the present study was that, despite a 4-fold increase in resting muscle PDCa (Fig. 3.1) and a 5.5-fold increase in resting muscle acetylcarnitine content (Fig. 3.2), there was no significant effect of DCA infusion on muscle PCr degradation or lactate accumulation during 6 min of exercise at 60% VO_2max compared to control (Table 3.1). Indeed, there was no effect of DCA pre-treatment on calculated substrate level phosphorylation (SLP) during exercise compared to control (Fig. 3.3), which is in contrast to that routinely observed during moderate to high intensity exercise. This finding could suggest that the magnitude of metabolic inertia at the level of PDC is minimal at the onset of low intensity exercise, as the rate of ATP demand will be lower and could therefore be better matched by the rate of PDC activation and flux. It should be noted, however, that the effect of PDC activation, by DCA pre-treatment, on non-oxidative ATP production at the onset of low intensity exercise (<60% VO_2max) has been previously investigated (Timmons *et al*, 1998b; Parolin *et al*, 2000). In contrast to the present study, both of these studies demonstrated a metabolic inertia (i.e. increased muscle PCr degradation and lactate accumulation after 1, 8, and 15 min of contraction) at the level of the PDC at the onset of low intensity exercise. However, in the study by Timmons *et al* (1998b) single leg knee extensions were performed, and in the study of Parolin *et al* (2000) bicycling exercise was performed under hypoxic conditions whilst breathing 11% O_2. Both of these exercise conditions resulted in a considerably greater PCr degradation during control compared to the present study, and hence, the potential for a reduction in non-

oxidative ATP production with DCA pre-treatment. Thus, this is the first study to investigate the effect of DCA on PDCa and non-oxidative ATP production during low intensity dynamic exercise under normoxic conditions.

The magnitude of PDCa is central to the control of acetyl-CoA delivery to the TCA cycle and carbohydrate oxidation in contracting skeletal muscle, and, as such, PDCa increases at the onset of exercise, from its relatively inactive or low state at rest, in parallel with exercise intensity up to an intensity of ≈90% VO_2max, when the rate of carbohydrate oxidation becomes maximal (Constantin-Teodosiu *et al*, 1991a; Howlett *et al*, 1998). The premise that the intensity of exercise influences the magnitude of the delay in PDC activation at the onset of contraction, particularly under normoxic conditions, is also supported by studies involving exercise with an intensity greater than 90% VO_2max (Howlett *et al*, 1999b; Savasi *et al*, 2002; Bangsbo *et al*, 2002). Similarly, if exercise intensity is low enough not to rely on a significant contribution from non-oxidative ATP production at the onset of exercise, then one would expect not to see an effect of DCA pre-treatment. Indeed, PCr stores were only reduced by around 10% in CON at the exercise intensity employed in the present study. Thus, as previously suggested by Roberts *et al* (2002a), it would appear that a metabolic inertia will exist at the onset of contraction over a predictable range of exercise intensities under normoxic conditions (between ≈65 and 90% VO_2max), above and below which increasing PDCa and resting acetyl group availability via DCA pre-treatment will be ineffective at reducing non-oxidative ATP production and improving contractile function.

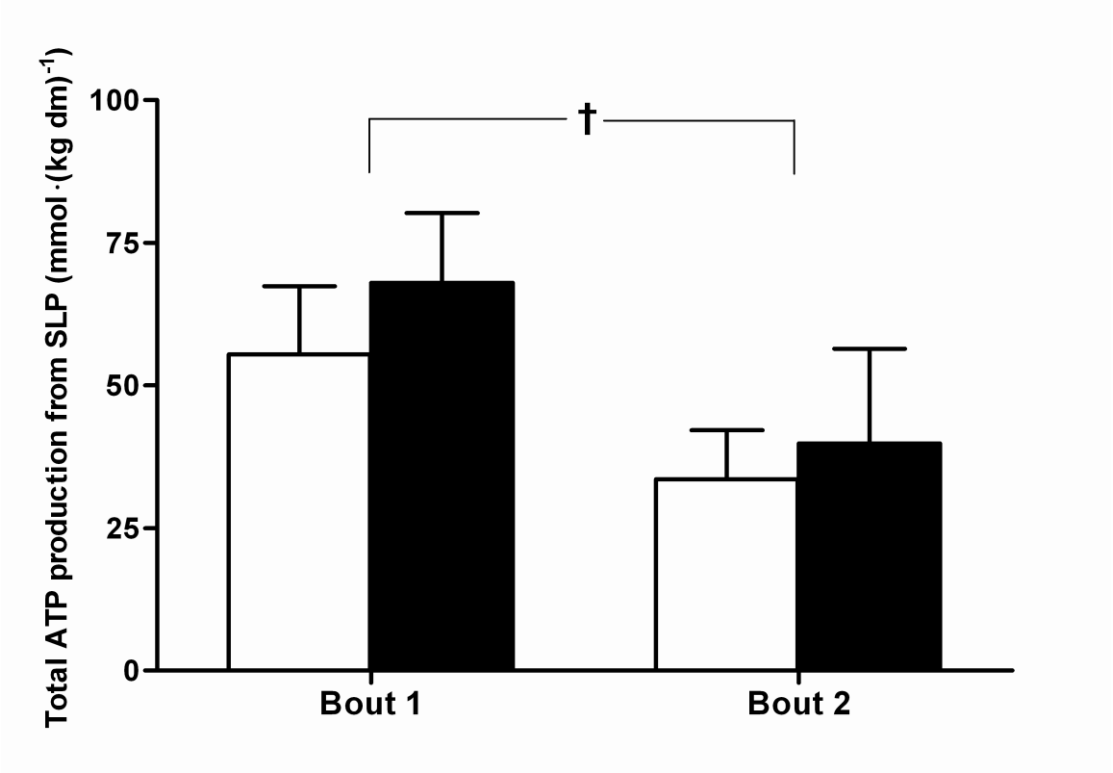


Figure 3.3 ATP production from substrate level phosphorylation.

Calculated total ATP production derived from substrate level phosphorylation after two 6 min bouts of cycling exercise at 60% VO_2max, separated by 15 min rest, following 1 h of intravenous saline (CON; white blocks) or dichloroacetate (DCA; black blocks) infusion. †$p<0.05$, exercise bout 2 significantly lower than exercise bout 1.

In accordance with previous human studies (Constantin-Teodosiu *et al*, 1999; Gibala *et al*, 1999), the infusion of 50 $mg \cdot kg^{-1}$ DCA prior to the first exercise bout resulted in a significant increase in PDC activation. Consequently, there was a substantial accumulation of muscle acetylcarnitine, and most probably acetyl-CoA, in DCA at rest, thus providing readily available substrate for oxidative ATP production at the onset of exercise (muscle acetyl-CoA content is directly related to acetylcarnitine content, albeit at a different order of magnitude; Carlin *et al*, 1990; Constantin-Teodosiu *et al*, 1991a). Previous studies that have calculated the contribution of oxidative flux to ATP production at the onset of exercise, by measuring the relative accumulation of acetyl groups in the form of acetylcarnitine, have demonstrated that the difference in acetylcarnitine accumulation during exercise between DCA and control corresponds to the difference in calculated SLP (assuming 1 mol of acetylcarnitine is oxidised to 12 mol of ATP equivalents; Timmons *et al*, 1997; Roberts *et al*, 2002b). However, despite approximately a 5 $mmol \cdot (kg\ dm)^{-1}$ greater accumulation of muscle acetylcarnitine during both bouts of exercise in CON of the present study (equating to around 60 $mmol \cdot (kg\ dm)^{-1}$ of ATP), there were no differences in SLP between the groups during either exercise bout, suggesting that oxidative ATP production was the same (assuming work output was the same). A possible explanation for the discrepancy between acetylcarnitine accumulation and SLP during exercise in the present study, compared to the previously mentioned studies, could be that the contribution from fat to oxidative ATP production was greater in CON compared to DCA, particularly as the contribution from fat oxidation during CON will be greater at 60% VO_2max than at higher exercise intensities (van Loon *et al*, 2001). Indeed, the acetylation of the carnitine pool at rest in DCA was such that free carnitine availability could have been limiting to fat oxidation during

the exercise bout (van Loon *et al*, 2001; Stephens *et al*, 2006), thus increasing the reliance on carbohydrate oxidation. Studies *in vitro* have demonstrated that when the concentration of TCA cycle intermediates is low, as observed at the onset of contraction (Gibala *et al*, 1998), carnitine reduces the rate of acetyl-CoA oxidation by the TCA cycle (Childress *et al*, 1966). This effect would have been ameliorated in the DCA group as carnitine availability was reduced at rest as a result of DCA pre-treatment (Table 3.1). Whatever the mechanism(s), it appears that DCA switched fuel use towards carbohydrate oxidation during exercise in the present study, particularly as muscle glycogenolysis was 2-fold greater in DCA compared to CON during the first exercise bout (Table 3.1). However, because the workload during exercise was of insufficient intensity to require a significant provision of acetyl groups for entry into the TCA cycle from carbohydrate oxidation in CON, then an increase in carbohydrate oxidation and PDCa with DCA pre-treatment would not be expected to alter the requirement for SLP to meet the demand for ATP production. It is also worth noting when carbohydrate oxidation is increased that up to ≈12% less oxygen utilisation is required for the same ATP production than when using fat as a fuel, which may explain why some investigations have failed to demonstrate a measurable change in pulmonary VO_2 kinetics at the onset of during high intensity exercise with DCA pre-treatment (Jones *et al*, 2004).

A second aim of the present study was to investigate the effect of DCA on PDC activation during a second bout of exercise. PDCa increased in CON following the first bout of exercise at 60% VO_2max to a similar degree as reported previously (Constantin-Teodosiu *et al*, 1991a), and remained elevated in DCA (Fig. 3.1). A novel finding from the present study was that, whereas PDCa returned towards basal

following the 15 min resting period in CON, PDCa remained elevated in DCA (Fig. 3.1). Furthermore, PDCa remained elevated in DCA following the second bout of exercise (Fig. 3.1). Available data from previous studies that have administered up to 100 $mg \cdot kg^{-1}$ DCA, and measured skeletal muscle PDCa in healthy human volunteers, demonstrates that DCA maintains maximal PDC activation for up to 15 min of contraction (Gibala *et al*, 1999; Parolin *et al*, 2000). In the present study total exercise time was 12 min, and there was a 15 min rest interval between the two exercise bouts. Thus, the present study demonstrates, for the first time, that a 1 h intravenous infusion of 50 $mg \cdot kg^{-1}$ DCA can sustain maximal PDC activity for more than one bout of exercise over a period of at least 30 min. This finding could be important if DCA is to be used as a metabolic tool to investigate the regulation of PDCa or therapeutically in the treatment of repeated episodes of muscle fatigue and pain and exercise intolerance in certain cardiovascular diseases (e.g. peripheral vascular disease).

Another interesting finding during the second bout of exercise in the present study was that SLP was 40% less in both CON and DCA (Fig. 3.3). Previous research within the School of Biomedical Sciences (Roberts *et al*, 2005) has shown that administration of sodium acetate, which markedly increased the availability of acetyl-CoA and acetylcarnitine in resting canine skeletal muscle independent of PDC activation, decreased the contribution from SLP by 40% during the first minute of subsequent muscle contraction (when the PDC was largely inactive) compared to control. Similarly, Campbell-O'Sullivan *et al* (2002) also demonstrated that performing a bout of high intensity exercise (75% VO_2max) 3 min following the performance of short duration low intensity exercise (55% VO_2max), which increased muscle acetylcarnitine content 2-fold greater than at rest but did not affect PDCa, was

associated with a 40% reduction in SLP and an acceleration of VO_2 on-kinetics in healthy male volunteers. However, in the present study the lower SLP during the second bout of exercise in CON is unlikely be due to the higher acetylcarnitine content (Fig. 3.2) accumulated during the first bout of exercise as this data indicated that acetyl group availability was not limiting to oxidative ATP production during the first bout of exercise. Furthermore, there was no difference in muscle acetylcarnitine content (Fig. 3.2) and PDCa (Fig. 3.1) before the onset of exercise between the first and second exercise bouts in DCA. Taken together, this would suggest that during low intensity exercise factors other than a metabolic inertia at the level of PDC may limit oxidative ATP production. Such factors are not discernable from the present study, but perhaps highlight the well reported benefits of "warming up" before exercise, which have classically been attributed to an exercise induced elevation of muscle temperature and/or the augmentation of local muscle blood flow.

In conclusion, although PDCa and acetylcarnitine content was increased in muscle at rest, DCA had no effect on muscle PCr hydrolysis or lactate accumulation during exercise in the present study, supporting the suggestion that the magnitude of metabolic inertia at the level of PDC is minimal in the first six minutes of low intensity exercise. Furthermore, the effects of DCA on PDCa can persist during recovery between two bouts of exercise, which could have important implications if DCA is to be used therapeutically.

Chapter 4.

CHANGES IN THE VENTILATORY THRESHOLD IN EXERCISE DURING CONDITIONS OF NORMAL AND REDUCED BLOOD LACTATE CONCENTRATION

Introduction.

The ventilatory threshold can be defined as "the point at which there is a non-linear increase in ventilatory rate in relation to oxygen consumption" (Williams, 1994) and has been suggested and used as a method for indicating exercise performance in healthy and disease states within both an adult and paediatric population (Seiler *et al*, 2007; Roels *et* al, 2005; Thomas *et al*, 2007; Ohuchi *et al*, 1996). It is believed to occur at least in part due to a fall in the blood pH, arising from, amongst other things, an increase in the muscle production of lactic acid that at physiological pH values completely dissociates to form hydrogen and lactate ions.

The lactate threshold, sometimes referred to as the 'anaerobic' threshold, was defined as "the first sudden and sustained increased in blood lactate above the near-resting concentrations" (Wasserman *et* al, 1973) and was thought to occur at the point at which demand for ATP to maintain contraction exhausted the ability of oxygen dependent mechanisms to supply it. Increased non-oxygen dependent ATP regeneration, most notably by glycolysis, leads to increased generation of lactate which is then released to the circulation. From this reasoning it was therefore assumed that the lactate threshold indicated the ability of an individual's skeletal muscle to generate energy aerobically, which was greater in trained individuals, and could therefore be used as a predictor of aerobic exercise performance (Bentley *et al*, 2007).

The release of lactic acid into the circulation, as well as directly determining the lactate threshold, leads to an increased production of CO_2, through the buffering of the hydrogen ions by bicarbonate. There is therefore an increase in VCO_2, which leads to

an increase in the rate of ventilation in order to exhale the excess CO_2. The rate of ventilation during exercise may also be influenced by an increase in the mitochondrial NADH/NAD^+ ratio (*i.e.*, increase in the redox potential). The increase in mitochondrial NADH above that of NAD^+ is transmitted to the cytosol, where it activates lactate dehydrogenase (LDH) and leads to lactate formation.

There is growing evidence that the accumulation of lactate at the onset of skeletal muscle contraction occurs due to inertia in mitochondrial ATP production (Yoshida *et al*, 1995; Grassi *et al*, 1998a; Grassi *et al*, 1998b), rather than a consequence of the oxygen deficit (Margaria *et al*, 1965). At an exercise intensity below 90% VO_2max this inertia has been suggested to reside at the level of the pyruvate dehydrogenase complex in humans during bicycle exercise in normoxic conditions (Howlett *et al*, 1999), which may indicate that the rise in lactate concentration may instead reflect a "spill-over" or by-product of pyruvate production as the rate of glycolysis increases with exercise intensity.

Katz and Sahlin (1990) discussed the link between oxygen dependency and lactate formation in their review. They demonstrated that muscle oxygen uptake (VO_2) is unchanged when measured at the mouth during exercise when the muscle's response to lactate is altered, either by previous aerobic training or the presence of hypoxia. They surmised that a reduction in oxygen present within the muscle resulted in an increased concentration of NADH, ADP and inorganic phosphate (Pi). This induced an increased rate of glycolysis, increasing the cytosolic concentration of NADH, which in turn activated LDH. Whilst they believed that other factors would influence the formation of lactate within the muscle during sub-maximal exercise, the reduction

of oxygen availability was believed to be of major importance to the rate of formation of lactate.

Roberts *et al* (2005), following the work of Timmons *et al* (1998; 1997; 1996) however demonstrated in an isolated canine muscle model with fixed oxygen availability that the generation of lactate in the rest to work transition was instead dependent upon activation of the pyruvate dehydrogenase complex (PDC). They suggested that the previously described oxygen deficit is in a large part dependent upon acetyl-CoA availability and that by ensuring near maximal activation of the PDC, with corresponding acetylation of the muscle carnitine and CoASH pools, they were able to reduce the formation of metabolic by-products (such as lactate ions, hydrogen ions and inorganic phosphate).

During exercise in which there is a high ATP turnover rate and low phosphocreatine availability there is an increase in the activation of AMP deaminase (Katz *et al*, 1986) leading to production of ammonia ions ($NH4^+$) from adenine nucleotide catabolism. The reamination of IMP during contraction is thought to be slow (Katz *et al*, 1986), and recovery of adenine nucleotides is believed to occur during the recovery period. Research in human volunteers undergoing intense dynamic single legged exercise demonstrated that the total net ammonia formed corresponded with the total accumulation of muscle inosine 5-monophosphate, suggesting that the ammonia produced during this exercise was derived from AMP deamination (Graham *et al*, 1990). Therefore any delay in oxygen dependent mitochondrial ATP production, and associated increase in flux through AMP deaminase, would be associated with a rise in plasma ammonia levels.

Dichloroacetate (DCA) is a potent systemic inhibitor of pyruvate dehydrogenase kinase, an intrinsic enzyme of the pyruvate dehydrogenase complex which phosphorylates and inactivates the complex. Pre-treatment with DCA has been shown to increase PDC activation at rest to levels seen during intense exercise (Timmons *et al*, 1998) with a subsequent reduction in phosphocreatine breakdown (Parolin *et al*, 2000). Previous research has demonstrated that pre-treatment with DCA in human volunteers undergoing both high (Rossiter *et al*, 2003) and moderate (Jones *et al*, 2004) intensity exercise had no effect upon VO_2 kinetics, despite a significant lactate lowering effect.

After pre-treatment with 35 $mg.kg^{-1}$ dichloroacetate, Carraro *et al* (1989) demonstrated a lower blood lactate concentration during exercise in healthy human volunteers at workloads less than 80% of maximal oxygen uptake and during the recovery period from exercise. They noted that DCA pre-treatment neither altered the time taken to reach exhaustion (defined in this study as the inability to maintain pedal speed at a rate of 60 rpm on the cycle ergometer) nor the blood lactate concentration at exhaustion. However whilst the concept of the anaerobic threshold was addressed within this paper, there was no measurement of the effects of DCA upon this threshold.

The aim of the present study was to assess whether activation of the pyruvate dehydrogenase complex using DCA, with a corresponding reduction in blood lactate concentration, would influence the onset of the ventilatory threshold during continuous incremental exercise commencing at 50% VO_2max. It also aims to

demonstrate that pre-treatment with DCA, and the corresponding reduction in phosphocreatine breakdown during contraction, would lead to a reduction in the delay of mitochondrial (oxygen dependent) ATP production at the ventilatory threshold and therefore a reduced ammonia efflux from the muscle.

Methods.

Subjects

Eight healthy recreationally active male volunteers were recruited to take part in this study. The mean (range) age, body mass index and peak oxygen uptake (VO_2max) were 29.2 (24-40) years, 25.5 (21.6–33.5) $kg.m^{-2}$ and 48.9 (31.4-62.3) $ml.kg^{-1}.min^{-1}$ respectively. Following a thorough explanation of the experimental protocol, written informed consent for entry into the study was obtained from all subjects prior to enrolment. At least one week prior to the first study visit each subject underwent estimation of their maximal oxygen uptake (VO_2max) using an on-line analysis system (SensorMedics, Anaheim, CA, USA) during continuous incremental exercise to exhaustion on a cycle ergometer. This study was approved by the University of Nottingham Ethics Committee.

Experimental procedure

Monosodium dichloroacetate (DCA) was purchased from Fluorochem Limited (Derby, UK) and prepared on the day of the study for sterile infusion at a concentration of 25 $mg.ml^{-1}$ by the sterile production unit at the Queens Medical Centre, Nottingham. Subjects rested in a supine position and a retrograde 21 gauge cannula was inserted into a forearm vein to allow blood samples to be taken. This hand was placed within a warmer (maintained at an air temperature of approximately 55 degrees C) to arterialise the venous drainage of the hand (Gallen and Macdonald, 1990). An antegrade 21 gauge cannula was also inserted into a separate forearm vein for infusion. Each subject received, in a randomised crossover fashion, 50 $mg.kg^{-1}$ body mass of dichloroacetate or an equivalent volume of saline (CON, 0.9% NaCl solution; Baxter Healthcare, Northampton, UK) over a 30 minute period, followed by

30 minutes of supine rest. The subject then commenced incremental exercise on a cycle ergometer (Lode Excalibur, Lode, Groningen, The Netherlands) commencing at 50% of VO_2 max, increasing by 25 watt increments every four minutes with measurements of heart rate (averaged over the preceding minute) recorded for the last minute of every increment. Throughout exercise exhaust gases were collected for breath by breath online gas analysis (SensorMedics, Anaheim, CA, USA). Exercise was ceased at the next incremental increase in intensity after the subject was judged to have reached the ventilatory threshold (detected by an inflection point in VE/VO_2 without concomitant rise in VE/VCO_2 (Caiozzo *et al*, 1982)).

Sample collection and analysis

Three millilitres of arterialised-venous blood were taken at the end of every increment for immediate analysis of blood glucose and lactate (Yellow Springs Instrument lactate analyser, Columbus, OH). The remaining blood was mixed with EGTA and centrifuged. The resultant plasma was snap frozen in liquid nitrogen and then analysed for ammonia content according to the methods described by Neeley and Phillipson (1988). The subjects remained seated for 20 minutes following the cessation of exercise whilst further measurement of expired gases and blood metabolites were taken. After a washout period (minimum duration of seven days) the subjects returned to the laboratory and repeated the same protocol with the alternate infusion.

Statistical analysis and calculations

End points measured included the comparison between treatment groups of respiratory variables and blood metabolites over time. The data were analysed using

two-way analysis of variance for repeated measures (ANOVA) with two within subject factors (time and trial). When the ANOVA test results in a significant *F* ratio ($P<0.05$), the location of the difference was identified using Scheffe's test.

Results.

Respiratory/Ventilatory data.

The results for the control group demonstrate a steady increase in ventilation (VE) throughout exercise (Figure 4.1), with maximum first derivative of the time/ventilation curve detected between 17 and 18 minutes of exercise. Measurements of oxygen consumption (VO_2) showed a steady increase for each minute throughout exercise once a steady state had been achieved. Measurements of CO_2 production (VCO_2) showed a steady increase throughout exercise, with a similar inflection point in the time/VCO_2 curve detected as with ventilation.

Following DCA pre-treatment a similar steady increase in ventilation was seen during incremental exercise. There was also an inflection point in the time/ventilation curve detected between 17 and 18 minutes of exercise. After achieving a steady state, the DCA pre-treated group also showed a steady increase in VO_2 throughout the period of exercise. VCO_2 increased steadily throughout exercise, with a similar inflection point in the time/VCO_2 curve detected as with ventilation.

Comparison of the ventilatory data between the two groups therefore revealed no significant difference between the two populations at any time during the study. Nor was there any difference in the area under the curve for either population with respect to VE/VCO_2 (638.31±34.75 vs. 640.19±41.36).

Blood metabolites.

Lactate (Figure 4.2)

Throughout the infusion period, there was no change in blood lactate concentration in the control population. A steady increase was noted throughout exercise, with a marked increase during the last increment, within which the subjects reached their ventilatory threshold. During the recovery period the blood lactate concentration returned towards normal, but at the end of the recovery period the blood lactate concentration was still significantly different from the resting value. Infusion of DCA led to a significant fall in the resting lactate concentration as demonstrated previously (Roberts *et al*, 2005). The changes noted during exercise were similar to those seen in the control group, with no difference detected between the two groups. Whilst there was no difference between populations comparing absolute blood lactate concentration during exercise, the area under the curve (Figure 4.3), representing the amount of lactate produced during this period was significantly lower during exercise in the DCA group (50.46 ±5.2 vs. 44.28 ±5.0, $p<0.01$).

Glucose (Table 4.1)

Throughout the infusion period there was no change in blood glucose concentration in the control population. A slight decrease was noted during exercise, with a corresponding increase during the rest period. Infusion of DCA led to a fall in the resting blood glucose concentration (4.60 vs. 4.36, $p=0.051$). Within the DCA pre-treated population no fall in blood glucose concentration was noted during exercise, but at the completion of exertion there was a similar rise in glucose concentration to the control population.

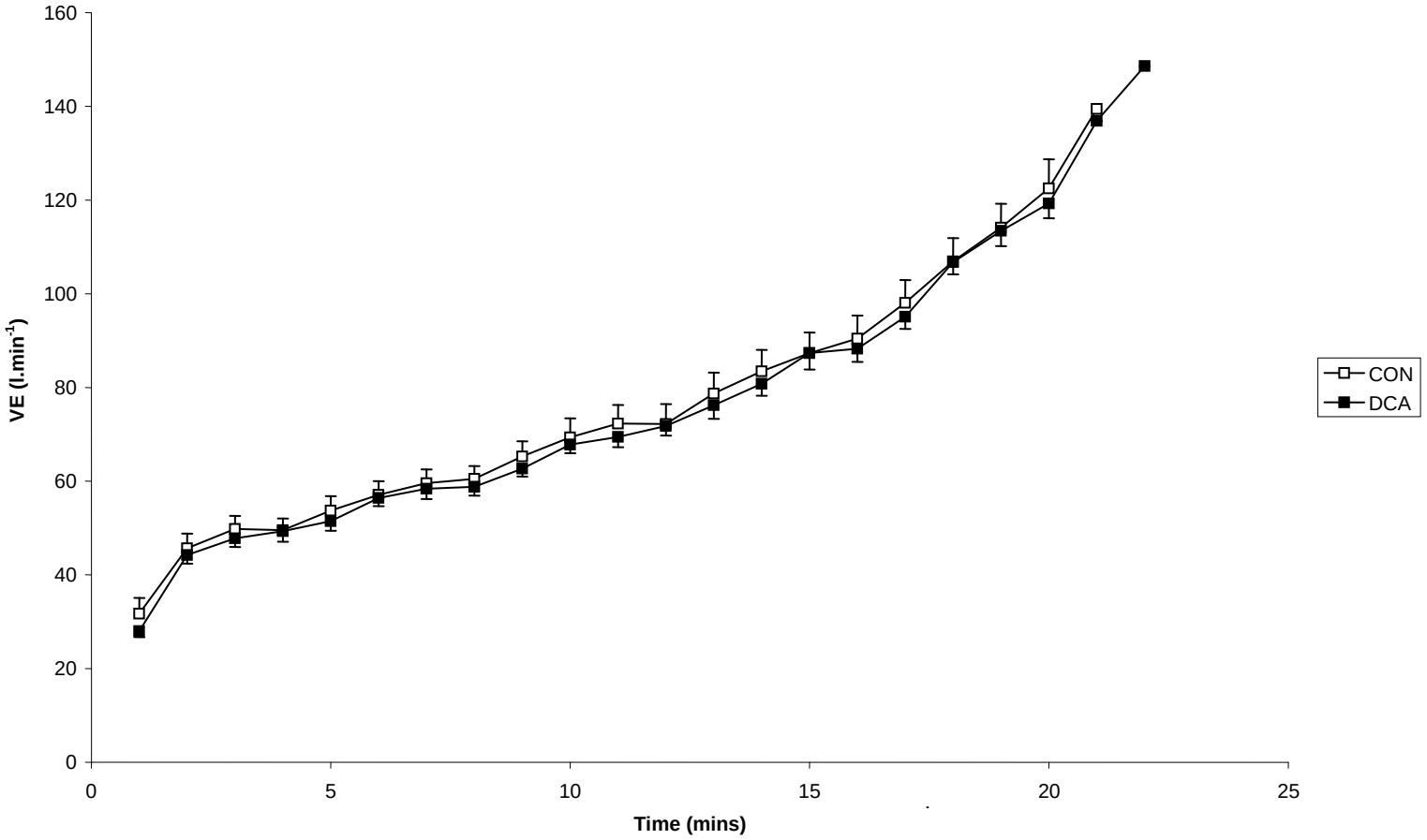


Figure 4.1 Ventilation. Ventilation during incremental cycling exercise following intravenous 0.9% saline (CON, open boxes) or dichloroacetate pre-treatment (DCA, closed boxes).

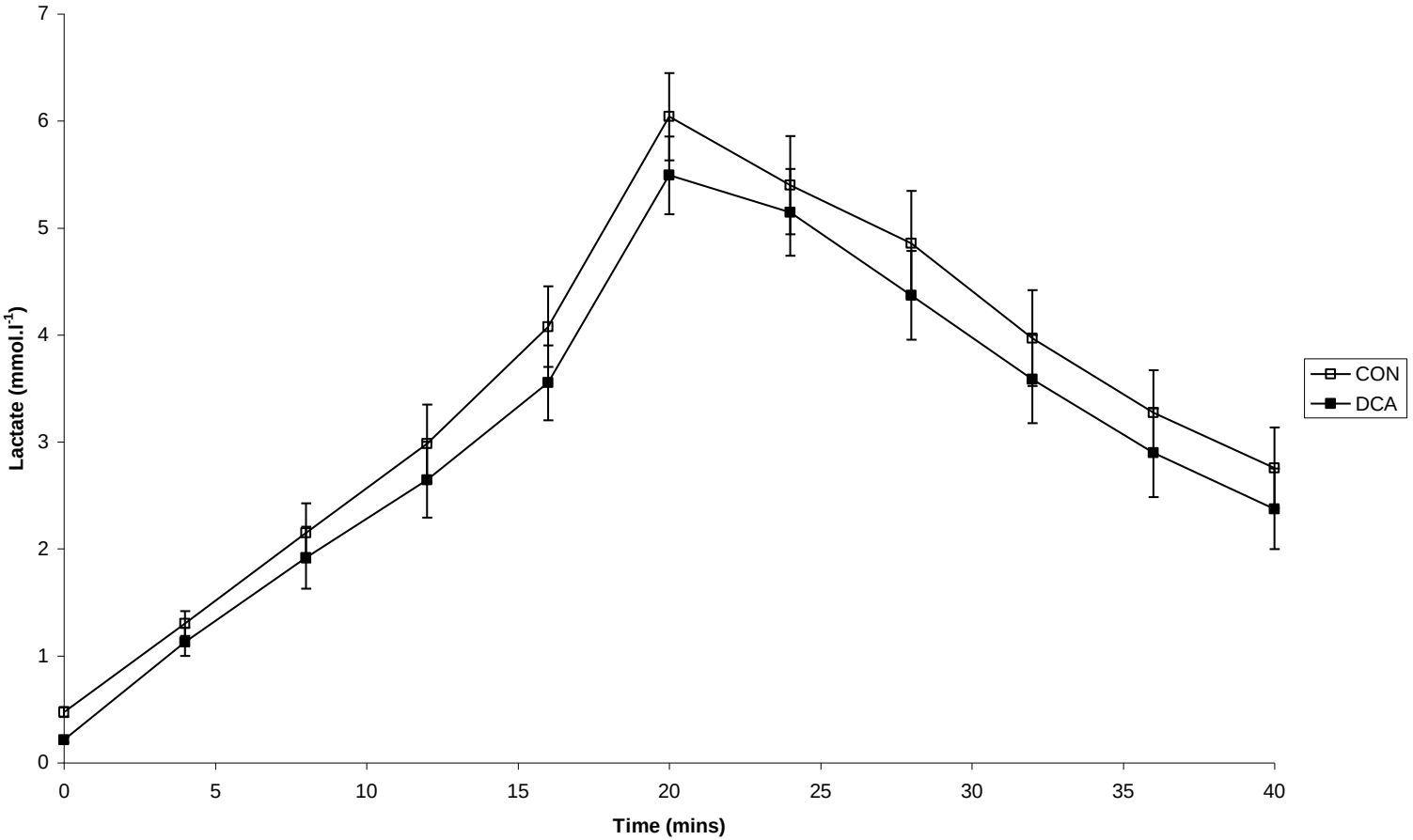


Figure 4.2 Blood lactate concentration. Blood lactate concentration during incremental exercise and recovery following intravenous 0.9% saline (CON, open boxes) or dichloroacetate pre-treatment (DCA, closed boxes).

Ammonia (Table 4.1)

There was no change in plasma ammonia concentration throughout the infusion period in the control population. Exercise led to a steady increase in concentration until 16 minutes, where there was a larger increase for the final increment of exercise. At the completion of exercise there was a steady decline in the ammonia concentration to resting levels. Infusion of DCA led to a reduction in the resting concentration of ammonia (83.17 vs. 63.00, $p=0.04$ at -30 minutes) but this was not present at the onset of exercise (74.67 vs. 59.00, $p>0.05$). A steady increase in ammonia concentration was found during exercise in the DCA pre-treated population, with the final increment being associated with a larger rise in ammonia concentration than the previous increments. As in the control population at the completion of exercise the ammonia concentration returned to its resting value. During the exercise and recovery period there was no significant difference in plasma ammonia concentration. The area under the curve, which represents the net balance of apparent total plasma ammonia production, release from the muscle and uptake and clearance by the liver, was not significantly different between groups at any time during exercise or recovery (Exercise 1822.4 ± 157.9 vs. 1544.8 ± 272.1, $p>0.05$; Recovery 1856.8 ± 201.2 vs. 1454.4 ± 356.7, $p>0.05$).

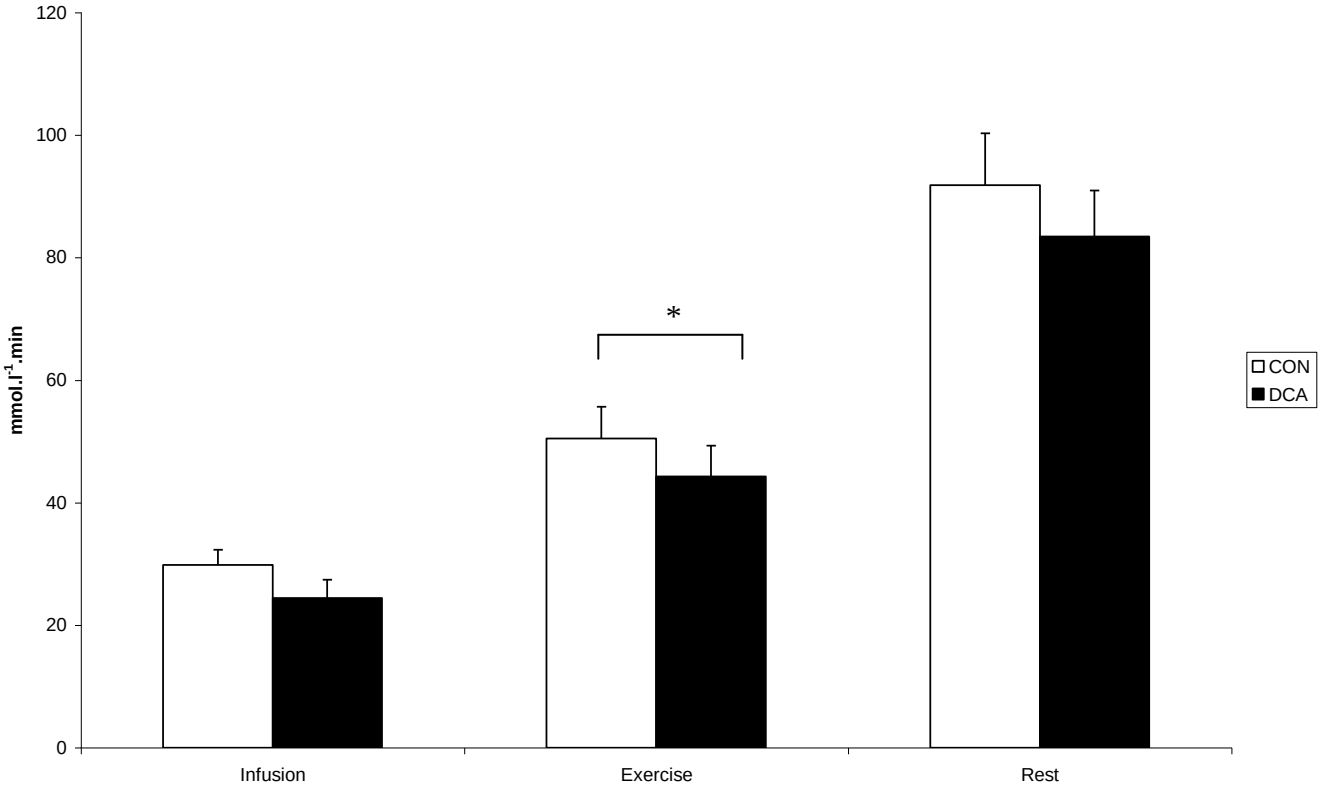


Figure 4.3 Net lactate production. Net lactate production during infusion (60 mins), incremental exercise (20 mins) and recovery (20 mins) following intravenous 0.9% saline (CON, white blocks) or dichloroacetate pre-treatment (DCA, black blocks). * $p<0.01$ DCA vs. CON.

Table 4.1 Blood metabolites. Metabolites measured during incremental cycling exercise following intravenous 0.9% saline (CON) or dichloroacetate pre-treatment (DCA). * p=0.04 (DCA vs. CON)

Time (minutes)		**-60**	**-30**	**0**	**4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	**36**	**40**
Glucose ($mmol.l^{-1}$)	**CON**	4.73	4.67	4.76	4.44	4.53	4.39	4.47	4.57	5.10	5.19	5.09	5.06	4.93
	DCA	4.60	4.47	4.36	4.36	4.31	4.33	4.39	4.36	5.06	4.93	4.81	4.74	4.73
Ammonia ($\mu mol.l^{-1}$)	**CON**	74.17	83.17*	74.67	80.33	82.33	94.00	99.33	129.17	116.17	94.67	81.83	72.83	66.17
	DCA	63.40	63.00*	59.00	63.50	67.83	73.50	82.17	104.83	90.17	72.83	62.50	52.00	45.83

Discussion.

The primary aim of this study was to investigate if reducing the blood lactate concentration would influence the onset of the ventilatory threshold in healthy human volunteers during continuous incremental bicycling exercise from 50%VO_2max to exhaustion. With respect to this the most significant finding was that, despite a significant reduction in blood lactate concentration (Figure 4.2) following DCA pre-treatment, there was no reduction in either the time taken to reach the ventilatory threshold (Figure 4.1) or the lactate concentration measured at and after the ventilatory threshold. This finding supports the study by Carraro *et al* (1989) that the infusion of DCA did not alter the time to exhaustion, nor did it alter the lactate concentration at that time, despite a reduction of resting blood lactate concentration and a trend towards lower blood lactate concentrations during the exercise and recovery periods of the experimental visit. It also supports the findings of both Rossiter *et al* (2003) and Jones *et al* (2004) that pre-treatment with DCA has no effect on VO_2 kinetics, although this is the first study to demonstrate that effect during continuous incremental cycling exercise.

The link between lactate and the ventilatory threshold has been cast into doubt by the finding that the ventilatory threshold can be detected prior to the lactate threshold (Davis and Gass, 1981), as well as research performed in subjects who do not produce lactate, who also have a non-linear increase in ventilation during exercise (Paterson *et* al, 1990). The data support the suggestion that there is another factor responsible for the non-linear increase in ventilation seen during exercise, and further research has suggested potassium as the factor that may be responsible. Within the same population that does not produce

lactate (McArdles syndrome) plasma potassium levels seem to match the ventilatory changes better than lactate within both the exercise and recovery phases of this study. However McLoughlin *et al* (1994) found results to the contrary, and the role of potassium in influencing ventilatory changes is not yet clear.

The lactate lowering effect of DCA pre-treatment is thought to be due to increased flux through the pyruvate dehydrogenase complex, which also leads to a reduction in phosphocreatine hydrolysis during submaximal exercise. An increase in the activation state of PDC has been suggested to result in direction of more of cellular pyruvate production towards Acetyl-CoA production and from there onwards to acetylcarnitine, so as to keep the CoA available for other mitochondrial processes. The sparing of the indicators of anaerobic metabolism after DCA infusion has been suggested to occur due to the ready availability of acetyl groups for entry into the TCA cycle. This reduces the lag present during the change in mitochondrial metabolism during the rest to work transition, and therefore better couples oxygen dependent ATP production to the demands of contraction. With a reduction in the requirement for ATP generation by anaerobic mechanisms, the deleterious by-products of this process are reduced and muscle function therefore improved. The findings do not support a reduction in ATP generation due to anaerobic methods, as the total ammonia efflux seen after DCA pre-treatment was not significantly different to that measured in the control population.

One suggestion is that this is likely due to the minimal effect of DCA infusion at the initial low intensity workload as noted in the previous chapter (**Chapter 3)**. This has

demonstrated no effect of DCA pre-treatment in healthy human volunteers at an exercise workload of below 65% VO_2max, and it may be that as the activation of PDC at this workload within the control group is not rate limiting to the provision of ATP for skeletal muscle contraction, the benefits seen at higher exercise intensities are met in the control population by the normal increases in PDC activation and the contribution of fatty acid oxidation during increasing intensity of exercise.

Although there was no significant difference in the blood lactate concentrations during exercise between groups, pre-treatment with DCA did lead to a reduction in the net lactate production during exercise (Figure 4.3). This is likely to reflect a reduction in the formation of lactate within the contracting skeletal muscle, due to better matching of the oxidative ATP production and skeletal muscle demand in this group as previously demonstrated in both canine and human studies (Roberts *et al*, 2005; Timmons *et al*, 1998a) during the rest to work transition.

Previous research (Graham *et al*, 1990) has shown the measured total ammonia formation to be equivalent to muscle inosine 5'-monophosphate (IMP) accumulation, which results from the further catabolism of adenine nucleotides during contraction. The same authors demonstrated that reamination of IMP occurs predominantly during recovery. If ATP provision and utilisation for contraction is well matched then the further breakdown of ADP does not occur and therefore little ammonia is generated during contraction. However if ATP generation and utilisation are poorly matched (for example at the onset of exercise in the presence of metabolic inertia) then further breakdown of adenine

nucleotides will result in an increase in the formation of IMP and therefore an increase in ammonia formation. The above results demonstrate no difference between groups with regard to plasma ammonia concentration at any time during the study, and if the assumption of Graham *et al* (1990) is correct then this may further suggest that the inertia present at the onset of low-intensity (50% VO_2max) exercise is minimal.

This is the first study to investigate the effect of lowering blood lactate concentration on the ventilatory threshold during incremental exercise. In conclusion, the primary finding of this study was that a reduction in resting blood lactate concentration, using the PDK inhibitor dichloroacetate, did not influence the onset of the ventilatory threshold. This study also demonstrated that during continuous incremental exercise commencing at 50% VO_2max, pre-treatment with DCA did not reduce plasma ammonia concentration.

Chapter 5.

THE POSSIBLE MOLECULAR MECHANISM RESPONSIBLE FOR DIETARY FAT MEDIATED INHIBITION OF PYRUVATE DEHYDROGENASE COMPLEX ACTIVATION IN HUMAN SKELETAL MUSCLE, WHICH IS NOT REVERSED BY ACUTE CONTRACTION

Introduction

A reduction in dietary carbohydrate intake, or increase in dietary fat intake, for several days has been demonstrated to reduce the rate of flux through the PDC during muscle contraction (Putman *et al,* 1993) which has been attributed to a blunting of PDC activation or a direct inhibition of PDC flux (Putman *et al,* 1993). This impairment of carbohydrate metabolism has been implicated in causing the metabolic syndrome, which consists of central obesity, glucose intolerance, hypertriglyceridaemia, hypertension and low HDL-cholesterolaemia (Gorter *et al*, 2004).

The pyruvate dehydrogenase complex is the rate limiting step in the entry of glucose derived pyruvate into the TCA cycle, and as such plays an important role in the control of use of carbohydrate as the source of oxidative energy for skeletal muscle contraction. As previously discussed, the PDC is regulated by a negative feedback mechanism in the short term, that is to say that the products of its reaction, i.e. NADH and Acetyl-CoA inhibit the partial reactions catalysed by the components of the enzymatic complex E1 and E3. Over the longer term, PDC activity is controlled by the competing actions of pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP)(Wieland *et al,* 1983) determining the amount of non-phosphorylated (active) pyruvate dehydrogenase complex (PDCa).

During muscle contraction in humans following a normal diet, increases in intracellular calcium and pyruvate appear to be the principal activators of PDC by activation of PDP and inhibition of PDK reactions (Constantin-Teodosiu *et al,* 2004). Dichloroacetate

mimics the structure of pyruvate and is able to fully activate PDC in resting skeletal muscle by PDK inhibition. This has been demonstrated to increase muscle carbohydrate oxidation during subsequent muscle contraction and has been used extensively in experiments examining PDC flux in skeletal muscle with normal or reduced blood flow (Timmons *et al*, 1998). As yet, there has been no study assessing if increasing PDC activation through the infusion of DCA will reduce the inhibitory effects of high-fat diet mediated insulin resistance on carbohydrate oxidation during exercise.

This study therefore aims to determine if DCA administration will reduce the stimulatory effects of high fat feeding on muscle PPARα and PKD4 expression in humans, therefore stimulating skeletal muscle carbohydrate oxidation during exercise in healthy human volunteers by increasing the flux through PDC reaction.

Methods.

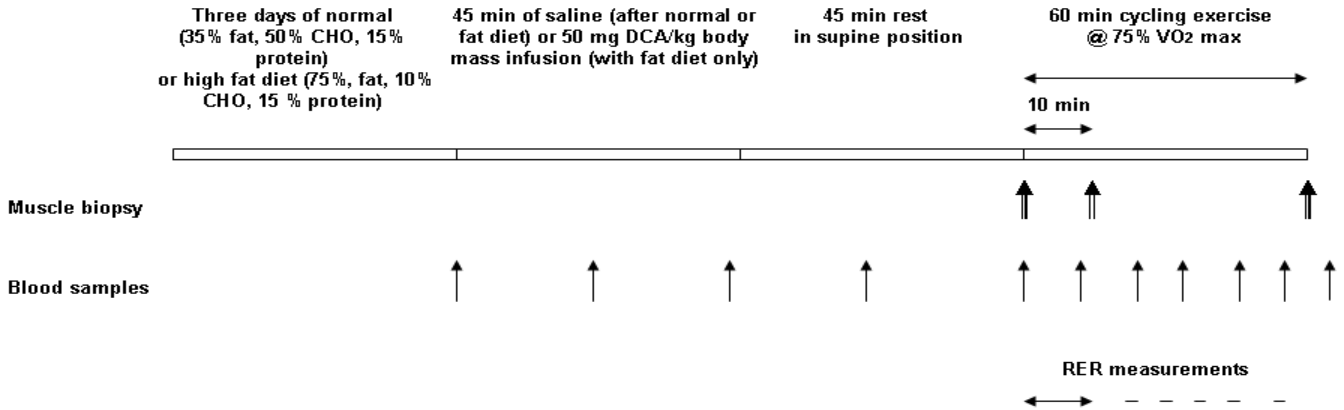
Subjects

Six healthy, non-smoking, non-vegetarian young men participated in the present study. Upon entry to the study each participant performed a continuous, incremental exercise test to exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Lode, Groningen, The Netherlands) to determine their maximal rate of oxygen consumption (VO_2max), measured using an on-line analysis system (SensorMedics, Anaheim, CA, USA), which was confirmed three days later. Each subject then completed a familiarisation visit consisting of 60 min of cycling at the required intensity (75% VO_2max) to ensure the accuracy of this calculation.

Experimental procedure.

Each subject then underwent three further laboratory visits (see Fig. 5.1), which were randomised. One visit was preceded by individuals consuming a prescribed "normal" diet for three days (55% carbohydrate, 30% fat, 15% protein; Control visit (CON)). The remaining two visits involved subjects ingesting a low carbohydrate/high fat diet (10 % carbohydrate, 75% fat 15% protein) for three days. Both diets were isocaloric. Three days prior to each visit subjects were issued with their diet for these days. The subjects were assumed to be compliant with their diet, however this was not checked. On arrival at the laboratory, a cannula was inserted retrogradely into the dorsum of the hand, using local anaesthetic (1% lignocaine), for the sampling of venous blood. An initial arterialised blood sample was taken for the measurement of free fatty acids and glucose. The subject then received a randomised infusion of either 0.9% saline (control visit and

low carbohydrate/high-fat visit (FD)) or dichloroacetate (50 $mg \cdot kg^{-1}$ body weight; second low carbohydrate/high fat visit (FD-DCA), Fluorochem Ltd., Derby, UK) over a period of 45 minutes. This was then followed by 45 minutes of supine rest. Throughout this infusion and subsequent rest period, arterialised blood samples were collected at the time points indicated in Fig. 5.1. During the 45 minutes of rest following the infusion protocols, three muscle biopsy sites were prepared prior to commencing exercise, using local anaesthetic (1% lignocaine), according to established protocol. An initial biopsy was taken immediately prior to exercise using a Bergström needle (Bergström, 1975). The subject then commenced cycling at 75% VO_2max, with continuous expired gas collection occurring for the initial 10 minutes of exercise for measurement of VO_2 and VCO_2. After 10 minutes, the subject briefly stopped cycling as a second muscle biopsy was obtained from the vastus lateralis, whilst the subject remained seated (and supported) on the cycle ergometer. This biopsy was required as the major changes in skeletal muscle PDC activation occur rapidly, but changes in ATP and PCr are constant throughout exercise from 3 minutes onwards (Karlsson and Saltin, 1970). The subject then continued cycling until 60 minutes had passed, when a final muscle biopsy was taken. Throughout exercise, blood samples were taken for assessment of glucose, lactate and free fatty acid concentrations, as well as VO_2 and VCO_2 estimates every ten minutes (over two minutes). After a seven-day washout period, the subject returned for their second visit and after a further seven-day washout their final visit. Thus, subjects attended three experimental visits in total. Each involved 3 muscle biopsy samples and 11 blood samples and was preceded by 3 days of normal dietary intake (Control visit; CON) or low carbohydrate/high fat intake (fat visit (FD) and fat + DCA visit (FD-DCA)).



<u>Figure 5.1</u> Visit protocol.

Sample analysis

Three millilitres of arterialised-venous blood was taken at the end of every increment for immediate analysis of blood glucose and lactate (Yellow Springs Instrument lactate analyser, Columbus, OH). Two millilitres of this blood were collected into lithium heparin containers, and after centrifugation, the plasma was removed and immediately frozen in liquid nitrogen. These samples were then stored at –80 C and analyzed at a later date for free fatty acid (FFA) concentration, using an enzymatic-colorimetric assay kit (NEFA C kit; Wako Chemicals, Neuss, Germany).

Snap frozen vastus lateralis muscle was subsequently divided into two parts whilst under liquid nitrogen. One part was freeze-dried, dissected free from visible connective tissue and blood, and powdered. Five to 10 mg of this muscle powder was then extracted with 0.5 M perchloric acid containing 1 mM EDTA, and, after centrifugation, the supernatant was neutralised with 2.2 M $KHCO_3$. Free carnitine and acetylcarnitine were measured in the neutralised extract by enzymatic assays that made use of a radioisotopic substrate, as previously described (Cederblad *et al*, 1990). Muscle ATP, PCr, creatine and lactate concentrations were determined fluorometrically using a modification of the method of Harris *et al* (1974). Total RNA was isolated from snap frozen soleus muscle using RNA plus (Qbiogene) according to the manufacturer's protocol. First strand cDNA was then synthesised from 1 μg RNA sample using random primers (Promega) and PowerScript Reverse Transcriptase (BD Biosciences). Taqman PCR was carried out using an ABI prism 7000 sequence detector (Applied Biosystems, USA), with 2 μl of cDNA, 18 μM of each primer, 5 μM probe, and Universal Taqman 2x PCR Mastermix (Eurogentec) in a 25

μl final volume. Each sample was run in triplicates, in duplex reactions. Cyclophilin A labelled with the fluorescent dye VIC was used as internal control, while all genes of interest were labelled with the fluorescent reporter FAM. The thermal cycling conditions used were: 2 min at 50^{o}C, 10 min at 95^{o}C, followed by 40 cycles at 95^{o}C for 15 sec and 60^{o}C for 1 min. Ct values of the target gene were normalized to Ct values of the internal control cyclophilin A, and the final results were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis and calculations

All data are expressed as mean ± SEM. To investigate the treatment effect repeated measurements of analysis of variance (ANOVA) was applied. When a significant F-ratio was obtained, a LSD post-hoc test was applied to locate specific differences. Significance was set at the $P<0.05$ level of confidence.

Results.

Respiratory parameters

All subjects were able to exercise at the required intensity for 60 minutes throughout the study. Whole body VO_2 was lower, although not to a significant degree, in CON group when compared to the high fat diets at the same exercise intensity (Fig. 5.2). There was an initial increase in respiratory exchange ratio (RER) at the onset of exercise in all groups which had reached a steady state by 10 minutes of exercise, and despite the control group displaying a trend towards higher values there was no significant difference throughout all visits (Fig. 5.3).

Blood metabolites

Blood lactate concentrations prior to exercise were similar in all three treatment groups (Fig. 5.4). Following infusion of DCA there was a significant reduction in blood lactate concentration. Upon exercise, blood lactate concentrations increased in all groups. However, the blood lactate concentrations during exercise in the FD-DCA diet group were lower than in the CON and FD groups (Fig 5.4).

Plasma metabolites

Plasma FFA concentrations prior to exercise were elevated following three days of high fat diet (Figure 5.5). Infusion of DCA led to a non-significant reduction of plasma FFA concentration towards a similar concentration as the CON diet group, but this was not preserved after 45 minutes of supine rest. During the initial 20 minutes of exercise there was a decrease in plasma FFA concentrations in all groups. From this point until the completion of exercise there was an increase in plasma FFA concentrations in all groups.

However the magnitude of FFA concentration rise in the FD and FD-DCA diet groups was greater than in the CON group, so that by the completion of exercise there was a significant difference in concentration between CON and the other groups.

Muscle metabolites

Resting muscle lactate concentration was significantly lower in the FD-DCA diet group compared to CON and FD groups (7.4 ±1.0 vs. 8.8 ± 0.9 and 9.8 ± 1.5 mmol.kg^{-1} dm; $p<0.05$; Fig 5.6). The concentration of muscle lactate after 10 minutes of contraction was significantly higher in the CON diet group compared to the FD and FD-DCA groups (47.8 ± 8.2 vs. 35.5 ± 4.0 and 38.8 ± 5.8 mmol.kg^{-1} dm, respectively; $p<0.05$). At the completion of exercise there was no significant difference in the muscle lactate concentration.

Resting muscle glycogen concentrations were significantly higher in the CON diet group compared with FD and FD-DCA groups (550 ± 31 vs. 428 ± 46 and 368 ± 24 mmol.kg^{-1} dm, respectively; $p<0.05$). The decline in muscle glycogen during the whole 60 minutes, split for the first 10 minutes and the last 50 minutes of exercise at 75% VO_2max are presented in Fig. 5.7. The rate of muscle glycogenolysis in the FD-DCA group was significantly lower compared with the CON group. This difference was entirely accounted for by the significantly lower muscle glycogen that occurred outside of the first 10 minutes of exercise in this group.

Muscle PDC activation (PDCa) and the muscle acetylcarnitine concentrations are presented in Figs. 5.8 and 5.9 respectively. Ingestion of a high fat diet for three days reduced the PDCa in the FD group compared with CON group, but this did not achieve

statistical significance. However, due to the effect of DCA infusion prior to exercise, PDCa increased more than 4-fold in the FD-DCA group compared with the CON group.

Upon contraction, muscle PDCa significantly increased above the resting values in all three groups. However, the magnitude of PDC activation during the first 10 minutes of exercise was significantly blunted in the FD compared with the other two groups. While further gain in PDCa was observed in the CON group after 60 minutes of exercise, the PDCa remained unchanged in the FD group. The values of PDCa in the FD-DCA group remained elevated throughout the entire experiment.

Mean resting muscle acetylcarnitine concentration in the CON group was significantly lower than in the FD and FD-DCA groups due to the effects of high fat diet and DCA infusion prior to exercise in these two groups (2.0 ± 0.5, 3.6 ± 0.5 and 10.8 ± 0.5 $mmol.kg^{-1}$ dm, respectively; $p<0.05$; Fig. 5.9). However, the rate of muscle acetylcarnitine accumulation in the FD-DCA group was significantly lower than in the CON and FD groups following 60 minutes of exercise. These differences were accounted for by the changes that had occurred during the first 10 min of exercise.

Gene Expression

The 3-day high fat diet resulted in a 3-fold raise in PDK4 mRNA at rest (Fig. 5.10), which was blunted after DCA treatment. During the first 10 minutes of exercise there was an increase in PDK4 expression in the control and FD-DCA groups followed by a reduction in gene expression after 60 minutes of cycling. Following high-fat diet, but without DCA treatment there was a reduction in PDK4 expression noted at both 10 and 60 minutes. The increase in PDK4 expression measured at rest in the fat-diet fed group

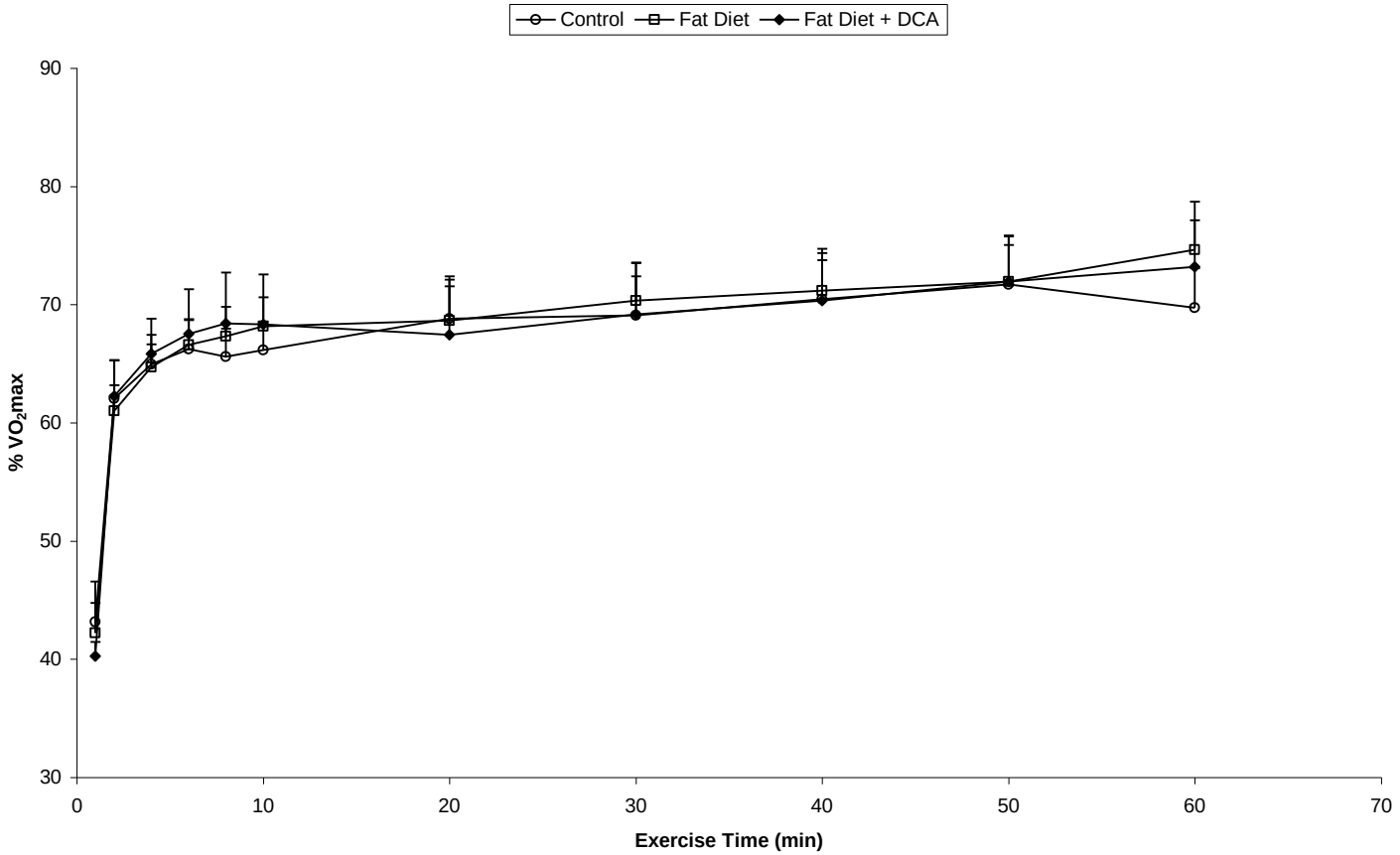


Figure 5.2 Percentage of maximum VO_2. Measured oxygen uptake expressed as a percentage of maximum during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest.

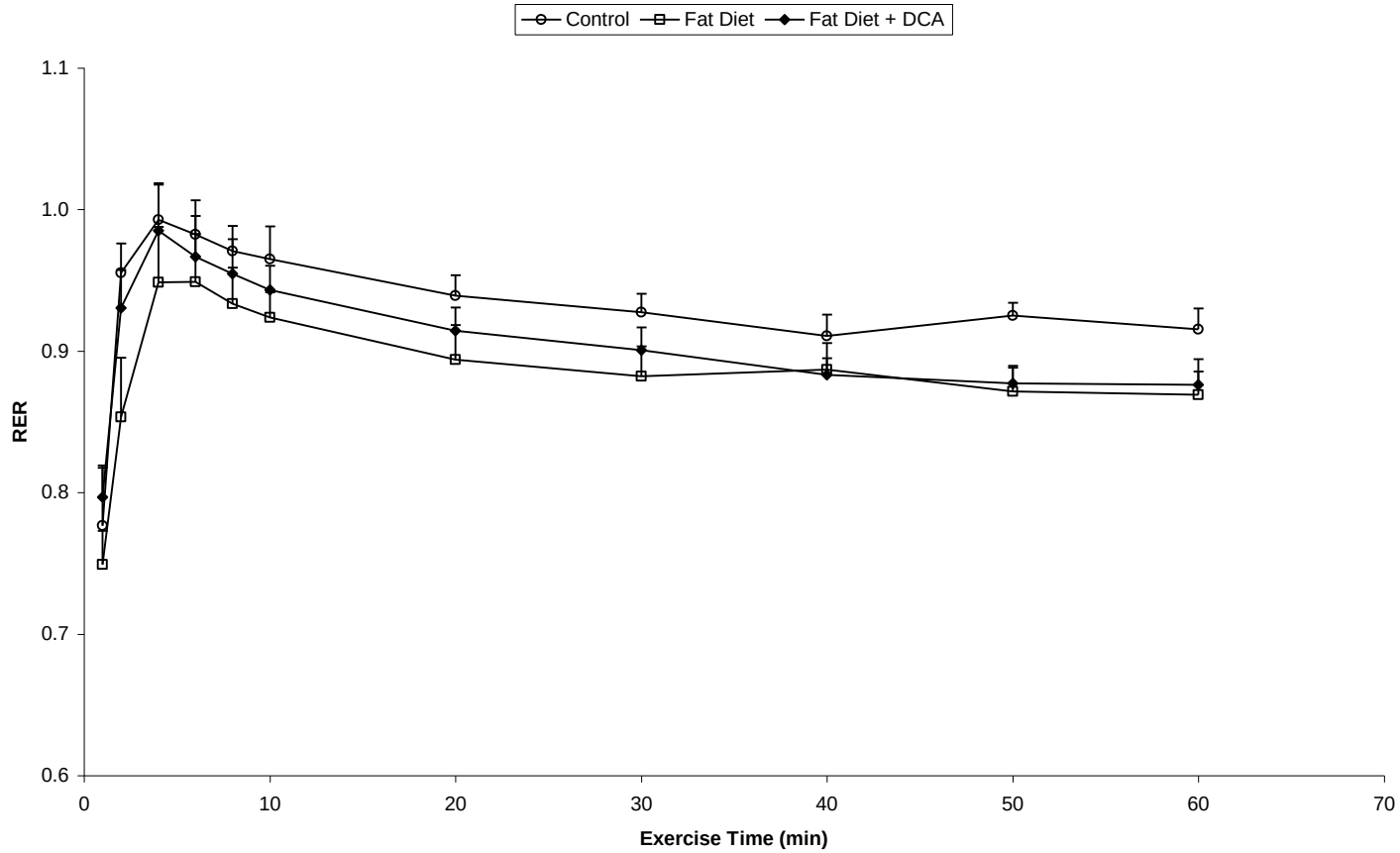


Figure 5.3 Respiratory Exchange Ratio. Calculated RER during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest.

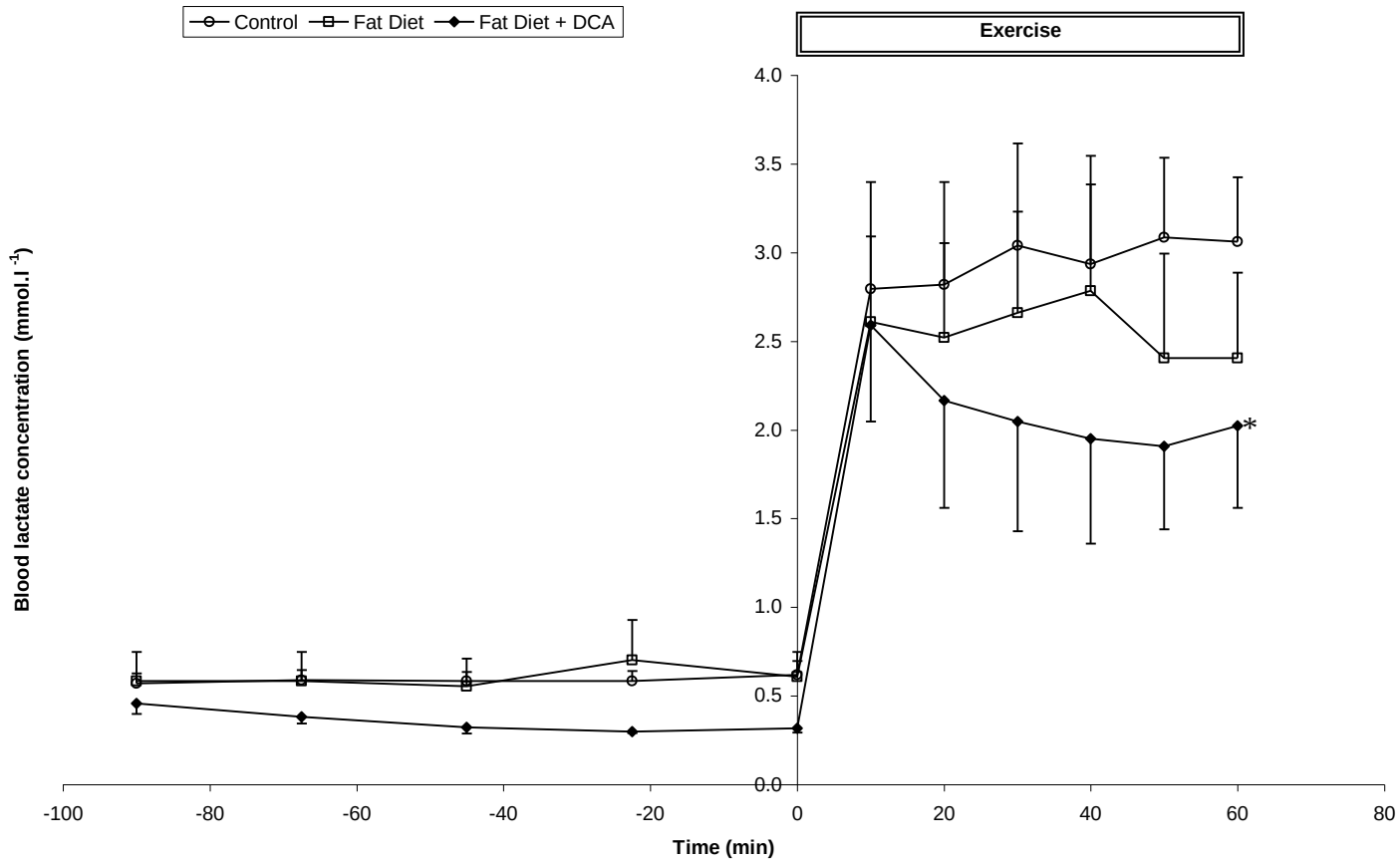
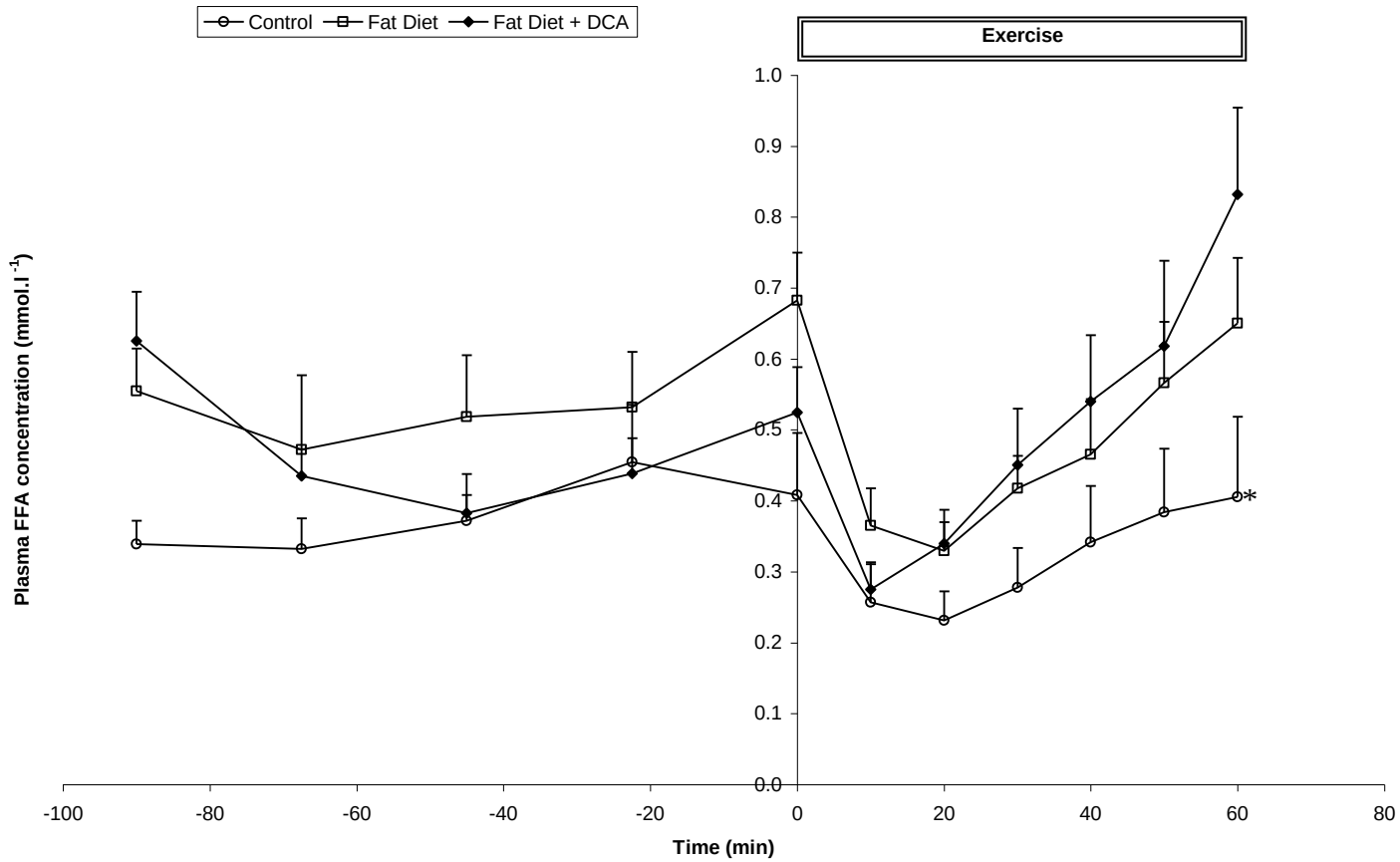


Figure 5.4 Blood lactate concentration. Blood lactate concentration measured during 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest, followed by 60 minutes of cycling at 75% VO_2max after 3 days of high fat or normal diet. * $p<0.05$ FD-DCA vs. CON and FD.



<u>Figure 5.5</u> Plasma free fatty acid concentration. Plasma free fatty acid concentration measured during 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest, followed by 60 minutes of cycling at 75% VO_2max after 3 days of high fat or normal diet. *p<0.05 CON vs. FD and FD-DCA.

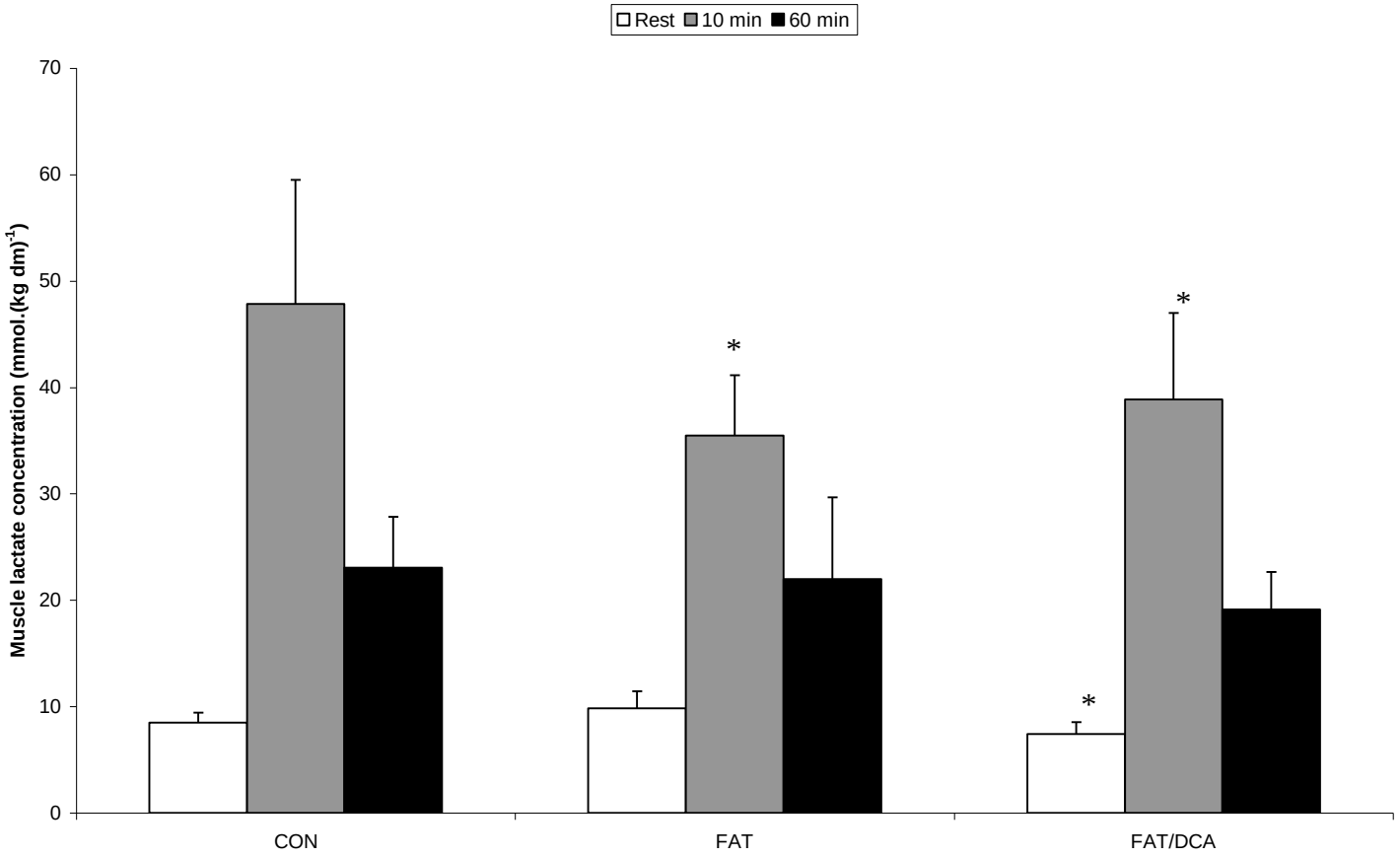


Figure 5.6 Muscle lactate concentration. Muscle lactate concentration measured during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest. * $p<0.05$ vs. corresponding control time point.

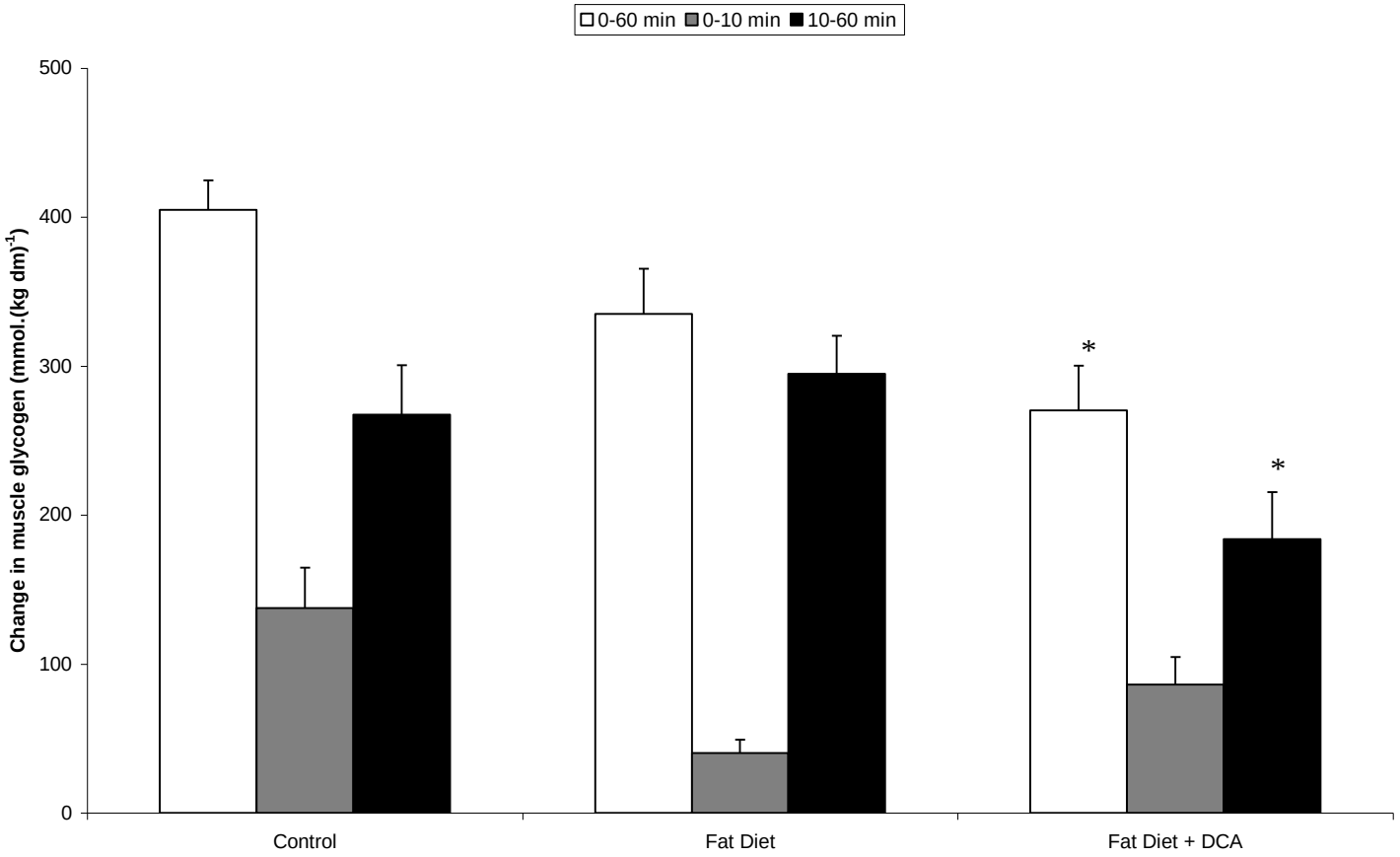


Figure 5.7 Changes in muscle glycogen concentration. Changes in muscle glycogen concentration during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest. * $p < 0.05$ vs. CON and FD.

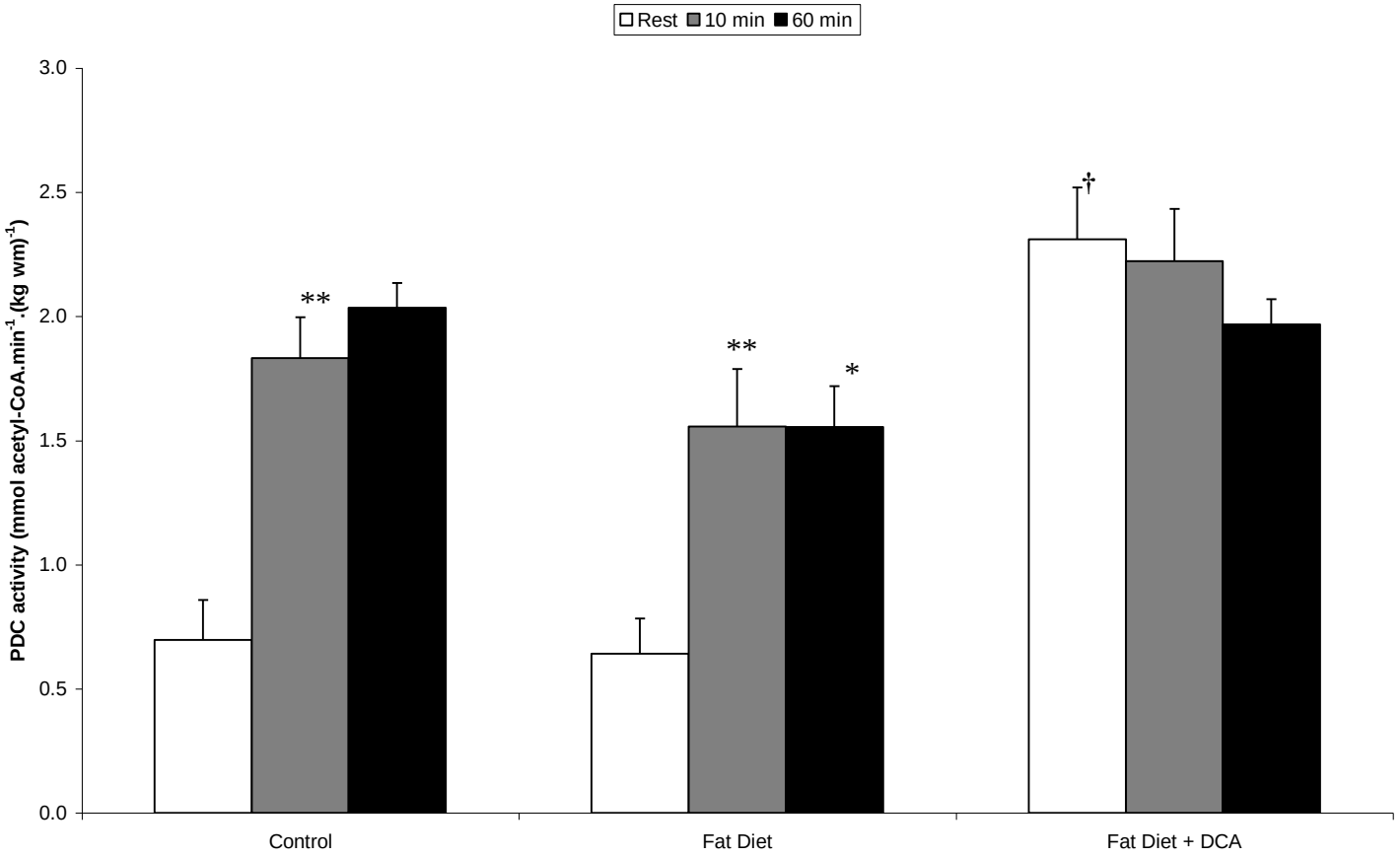


Figure 5.8 PDC activation. PDC activity prior to and during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest. * $p<0.05$ vs. CON, † $p<0.05$ vs. FD and CON, **$p<0.05$ vs. corresponding rest value.

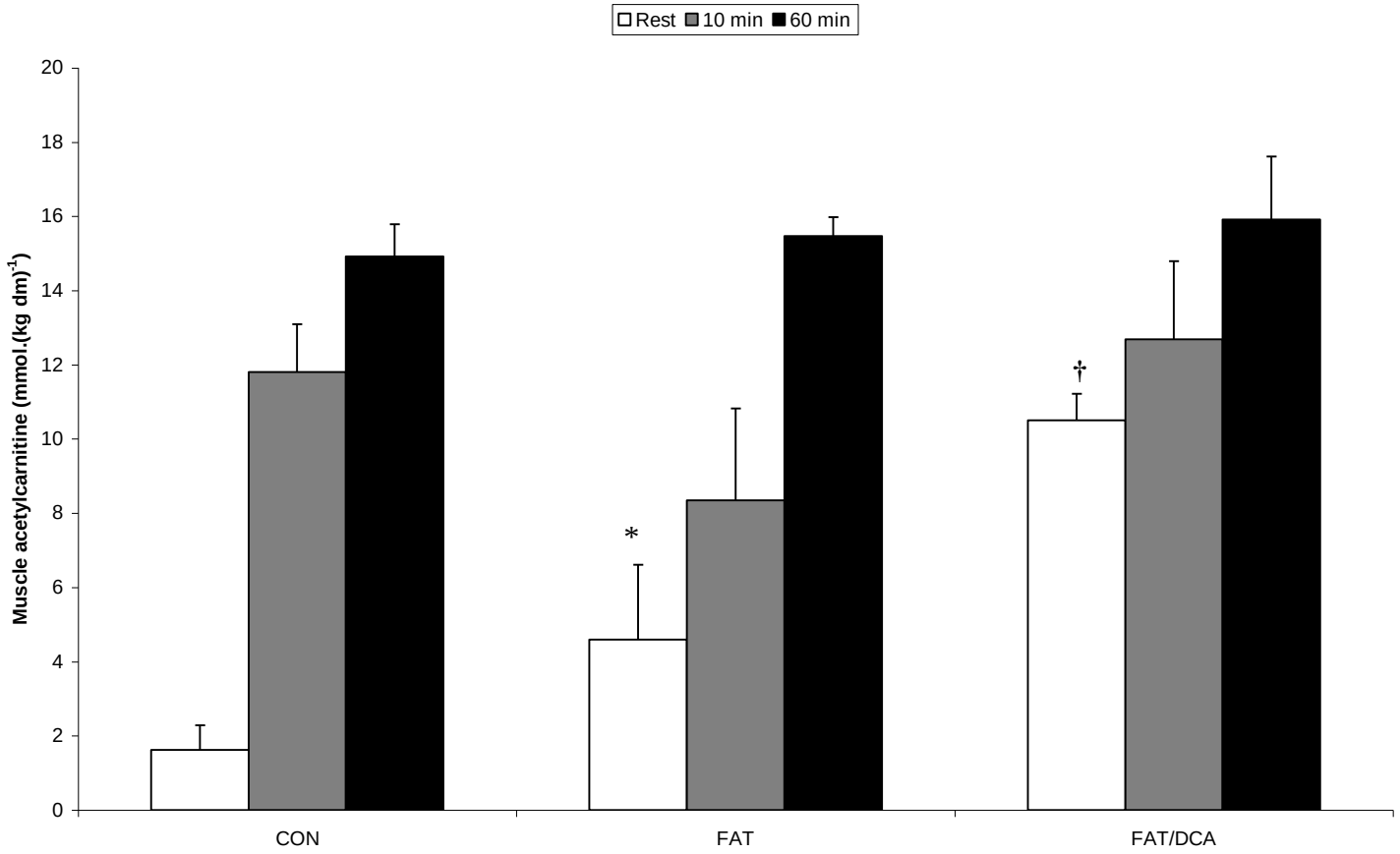
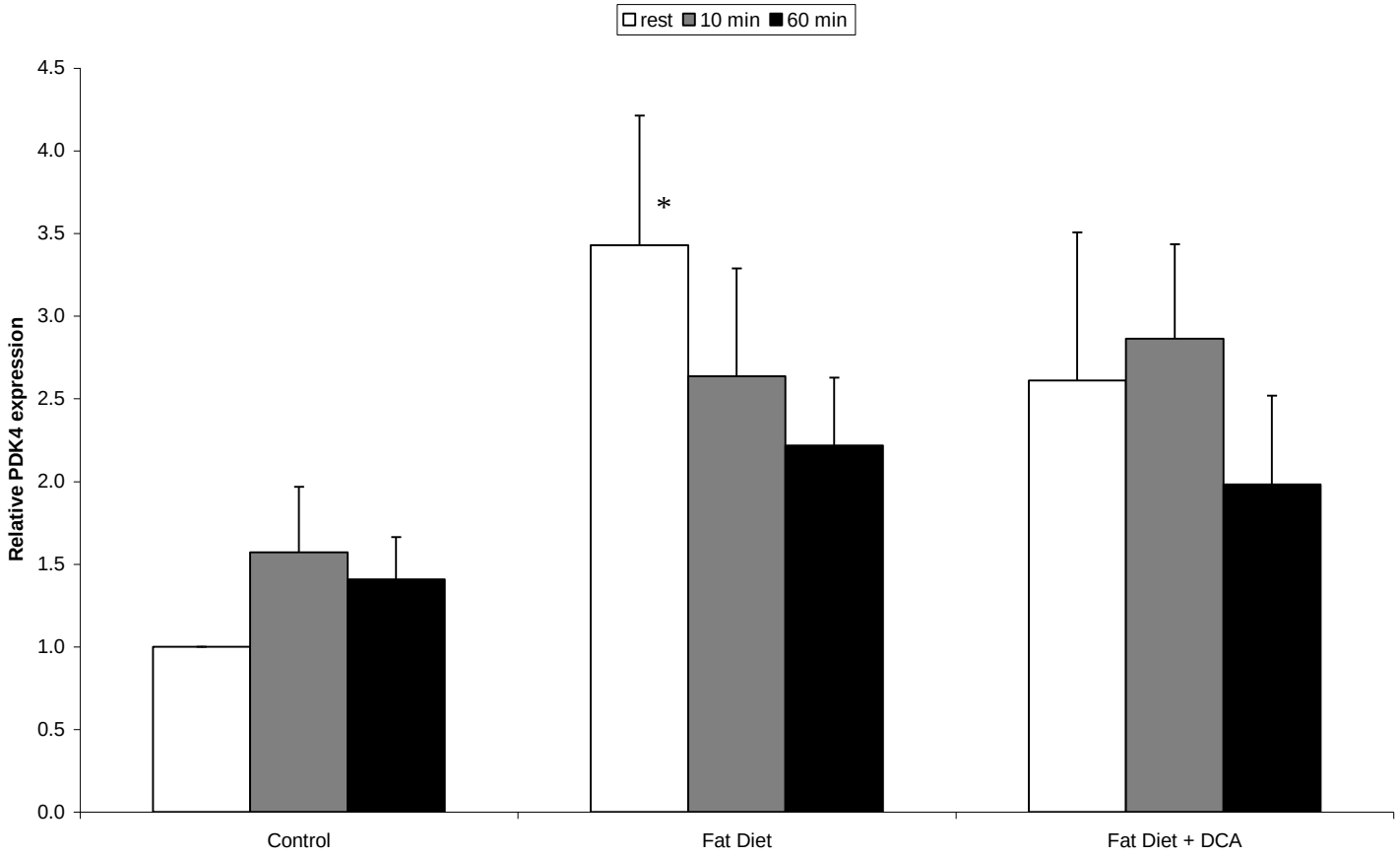


Figure 5.9 Muscle acetylcarnitine concentration. Muscle acetylcarnitine concentration during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes supine rest. * $p<0.05$ vs. CON, † $p<0.05$ vs. FD and CON.

may have been mediated by the raise in PPAR α and γ (1.5-fold) transcription (Figs 5.11 A and B). 3 days of high-fat feeding also increased the expression of FOXO1 (2-fold) after 10 minutes of exercise when compared to the control group (Fig 5.12), an effect that persisted at the completion of the exercise bout. Exercise activated PDC in CON and FD, but this activation was blunted in the latter probably due to the sustained raise in PDK4 and FOXO1 mRNAs. Furthermore, Pearson correlations between the expression of PDK4 mRNA and FOXO and PPARs mRNA expression revealed that the highest significant relationship existed between PDK4 and FOXO1 mRNA expressions ($r=0.71$, $p<0.0001$, Fig 5.13A) followed by the relationship between PDK4 and PPARα ($r=0.44$, $p=0.007$, Fig 5.13B). There was however no significant difference between the r values compared above ($p=0.31$).



<u>Figure 5.10</u> Muscle PDK4 expression. Muscle PDK4 expression during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes of supine rest.

* $p < 0.05$ vs. CON.

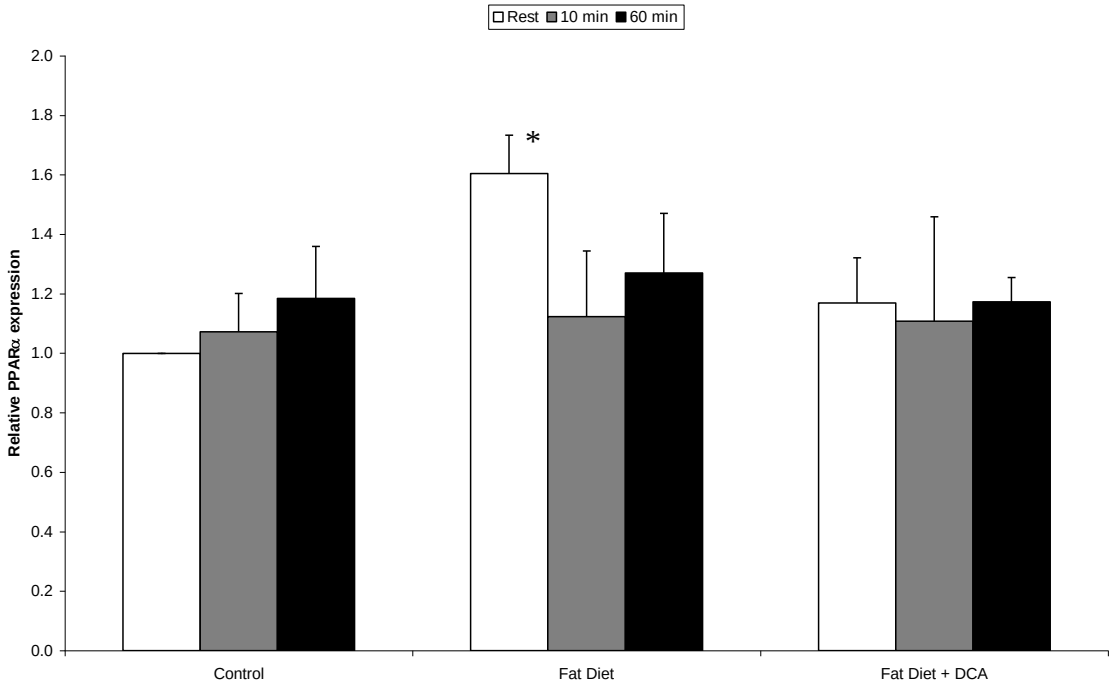


Figure 5.11A Muscle PPARα expression. *p < 0.05 vs. CON.

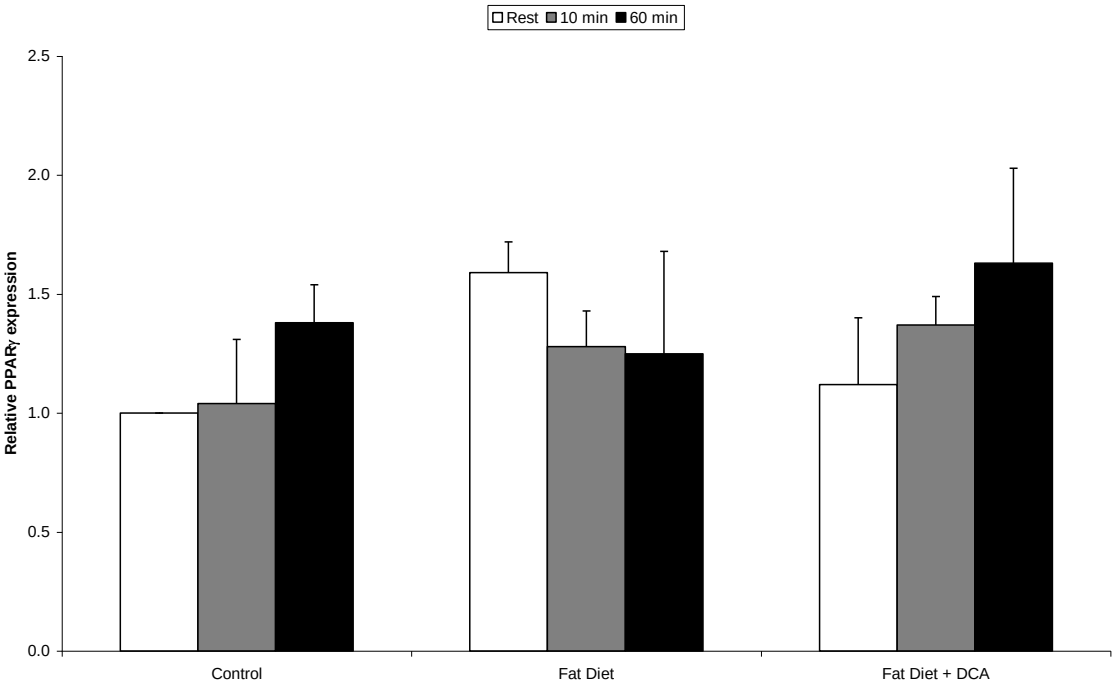


Figure 5.11B Muscle PPARγ expression.

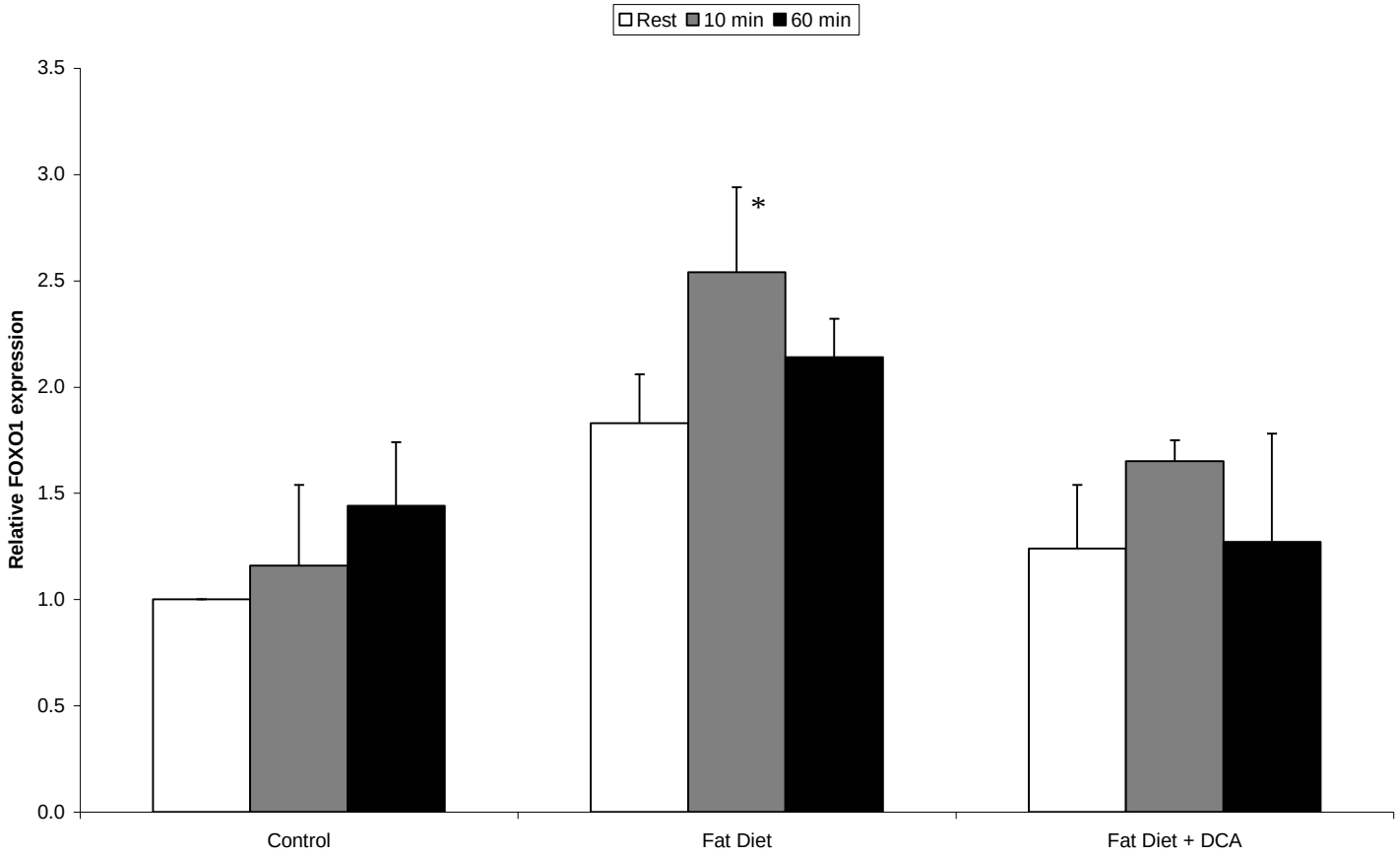


Figure 5.12 Muscle FOXO1 expression. Muscle FOXO1 expression during 60 minutes of cycling at 75% VO_2max following 3 days of high fat or normal diet, and 45 minutes of either intravenous saline or dichloroacetate infusion followed by 45 minutes of supine rest. *$p < 0.05$ vs. CON.

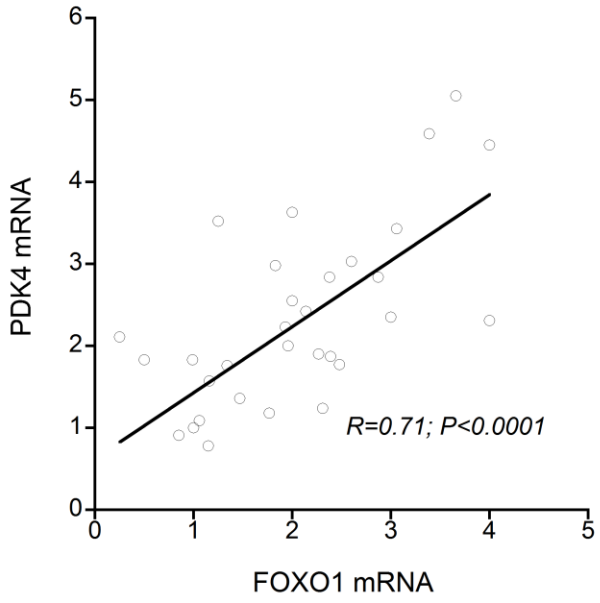


Figure 5.13A Correlation between FOXO1 mRNA and PKD4 mRNA expression.

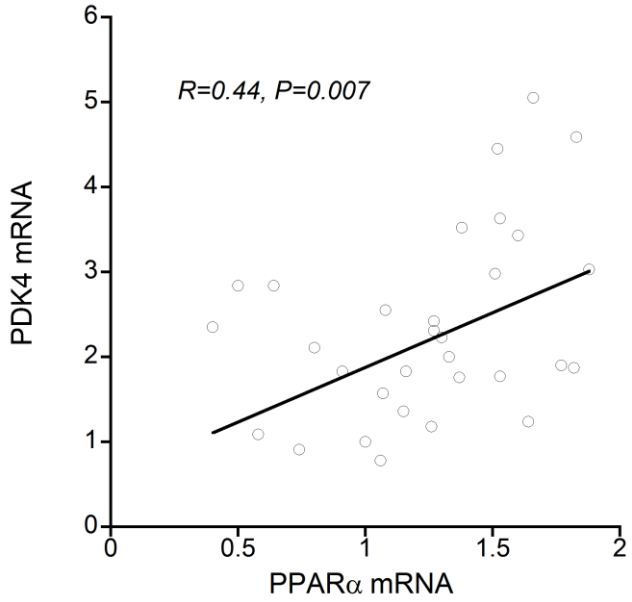


Figure 5.13B Correlation between PPARα mRNA and PDK4 mRNA expression.

Discussion.

The primary aim of this study was to determine if DCA administration would reverse the stimulation of PPARα and PDK4 expression seen during exercise after high-fat feeding. With respect to this aim, the results demonstrate that administration of DCA reversed the reduction in skeletal muscle PDC activity at the onset of exercise (Figure 5.8) following three days of high fat diet. This effect was preserved during exercise, again reversing the blunted response of PDC activation following high fat feeding. It is suggested that the dietary fat mediated reduction in PDC activation, and thereby CHO oxidation, during exercise may have been mediated by FOXO1 and PDK4 transcription, and this signalling was not blunted by contraction-mediated Ca^{2+} release.

Infusion of DCA, as in **Chapter 4**, led to a reduction in the resting blood lactate concentration. In contrast to the previous chapter, during exercise at 75%VO_2max the lactate lowering effect was preserved during exercise. This study supports previous research (Timmons *et al*, 1998a) that demonstrates the lactate lowering effect of systemic PDC activation in humans. Three days of high-fat diet elevated plasma FFA concentrations, and DCA infusion did not significantly alter the plasma fatty acid concentration during rest or exercise.

In support of previous research (Roberts *et al*, 2002a; Timmons *et al*, 1997; 1998a; Howlett *et al*, 1999a) demonstrating that DCA infusion is of benefit in overcoming metabolic inertia at the level of the PDC at the onset of contraction, the 'acetyl group deficit' proposed by Roberts *et al* (2002a), the finding that DCA pre-treatment led to a

significant increase in muscle acetylcarnitine concentration in association with a reduction in muscle lactate concentration and a reduction in muscle glycogen breakdown during exercise is of note.

These results demonstrate a stronger correlation between FOXO1 mRNA and PDK4 mRNA expression, than between PPARα mRNA and PDK 4 mRNA expression (Figure 5.10A and B). It is therefore suggested that FOXO1 transcription factor may be more important to controlling expression of PDK4 than the expression of PPARs in human skeletal muscle. It also appears that an acute bout of muscle contraction seems unable to revert the high fat diet FOXO1-mediated PDK4 inhibition on PDC activation.

Peters *et al* (2001) demonstrated in humans fed a high fat/low carbohydrate diet that the upregulation of muscle PDK4 gene expression was mediated by direct binding of FOXO1 to the promoter of the PDK4 gene. It was suggested that FOXO1 would therefore have an important role in the regulation of whole body energy metabolism through its action on PDK4 mRNA expression and the expression of PDK protein (Furuyama *et al,* 2003). These results have also demonstrated an up-regulation of FOXO1 mRNA expression following three days of high-fat diet, with a corresponding reduction in PDC activity. This may suggest that FOXO is acting as a fuel substrate sensor in skeletal muscle. FOXO is known to tightly control the regulation of both muscle-specific RING finger protein 1 (MuRF-1) and muscle atrophy F-box protein (MAFbx; also known as atrogin-1) (Sandri *et al*, 2004; Stitt *et al,* 2004) and it may be through these pathways that it has its effects upon muscle energy metabolism, given that Koyama *et al* (2007) have recently

suggested that MuRF-1 acts as a connector of muscle energy metabolism and protein synthesis.

Increase in fatty acid oxidation, induced by increased fat availability (Costill *et al*, 1977) or starvation (Wu *et al*, 1999), has been demonstrated to result in the activation of muscle PDK4. This leads to phosphorylation and therefore inactivation of the PDC, which is the rate limiting step in muscle carbohydrate oxidation. In starvation, the mechanism by which this PDC inhibition occurs has been attributed to either an increase in fatty acid mediated activation of the transcription factor FOXO1 which leads to direct binding of this to the promoter region of the PDK4 gene (Furuyama *et al*, 2003), or through free fatty acid activation of the PPARα receptor (Wu *et al*, 2001) which results in an increase in the expression of PDK4 mRNA. Research by Constantin-Teodosiu *et al* (2007) has recently demonstrated an increase in muscle PDK2 and PDK4 mRNA expression after administration of the PPARδ agonist GW610742 (100 $mg.kg^{-1}$) when compared to a control group, and this led to a doubling of the increase of PDK4 protein expression. A significant increase in β-hydroxy acyl CoA dehydrogenase activity was also noted within the same group, indicating a switch of metabolism towards lipid utilisation, and corresponding switch away from carbohydrate use, following treatment with GW610742.

St. Amand *et al* (2000) demonstrated in healthy human volunteers that the resting reduction in PDC activation induced by 6 days of low carbohydrate diet was reversed, in part, by the availability of pyruvate during cycling exercise at 65% VO_2max. Given that DCA is thought to act upon PDK through its similarities to pyruvate (Halestrap, 1975),

these results would support the suggestion that PDC activity in skeletal muscle is closely regulated by intramuscular pyruvate concentrations, as seen in cardiac muscle.

In conclusion, it is suggested that PDC inhibition, by any mechanism, may alter skeletal muscle selection of fuel for contraction towards fatty acid metabolism especially when fatty acid availability is increased. This effect may explain some of the reported reduction of muscle contractile function seen following administration of PPAR agonists (Carley *et al*, 2003). The reduction in PDC activation seen after high fat feeding has been reversed following pre-treatment with the PDK inhibitor DCA. This inhibition would seem to be due to an increase in predominantly FOXO1 promoted but also PPARα stimulated PDK4 expression.

Chapter 6.

GENERAL DISCUSSION

Overview.

Much research into the function and regulation of the pyruvate dehydrogenase complex in skeletal muscle has been performed within the School of Biomedical Sciences, Nottingham (Timmons *et al* 1998a; b; 1996; Roberts *et al* 2005; 2001). The present work follows on from this by further clarifying the physiological role of PDC with regard to its role in regulation of human skeletal muscle fuel metabolism. Alteration of fuel selection at the onset of low intensity cycling exercise (<60% VO_2max), using DCA infusion, was explored during repeated bouts of exercise. Alteration of fuel selection at the onset of moderate intensity cycling exercise (75% VO_2max), using high-fat/low-carbohydrate feeding, and its response to PDC activation was explored. The link between lactate and the ventilatory threshold was explored using the PDK inhibitor dichloroacetate to reduce resting blood lactate concentration prior to incremental cycling exercise.

Principal findings

i. Pre-treatment with DCA prior to low intensity (<60% VO_2max) exercise does not overcome the minimal metabolic inertia present (**Chapter 3**).

ii. The activation of PDC by dichloroacetate can persist during recovery between two bouts of exercise (**Chapter 3**).

iii. A reduction in the resting blood lactate concentration prior to incremental exercise does not change the blood lactate concentration at or after the ventilatory threshold (**Chapter 4**).

iv. DCA administration is able to reverse the PDC inhibition seen during exercise after high fat feeding (**Chapter 5**).

Metabolic inertia at the onset of low intensity exercise.

The work presented in **Chapter 3** has, for the first time, demonstrated that the administration of DCA prior to exercise at 60%VO_2max does not alter the amount of PCr breakdown or lactate accumulation during the following exercise bout. Prior to this work PDC activation prior to exercise at intensities below 60%VO_2max has demonstrated that PDC activation overcame the metabolic inertia (increased PCr breakdown and lactate accumulation) residing at the level of PDC (Parolin *et al*, 2000; Timmons *et al*, 1998b). However the methods used in those studies result in an increased PCr breakdown when compared to bicycling in normoxic conditions, which would therefore increase the provision of non-oxygen dependent ATP regeneration. DCA infusion, and the corresponding pooling of acetyl groups, would therefore be of benefit under these conditions.

Researchers have demonstrated an upper threshold of exercise intensities at which DCA pre-treatment is of no benefit in overcoming the metabolic inertia present within skeletal muscle (Howlett *et al*, 1999b; Savasi *et al*, 2002; Bangsbo *et al*, 2002), which seems to correspond to the rate of maximal carbohydrate oxidation measured during exercise at similar intensities (Constantin-Teodosiu *et al*, 1991a; Howlett *et al*, 1998). As the contribution from carbohydrate oxidation at low intensity exercise is minimal, I have sought to demonstrate the suggestion of Roberts *et al* (2002a) that there also exists a lower threshold of exercise intensity below which the degree of metabolic inertia is not significantly limiting to oxidative ATP provision at the onset of contraction.

The work in **Chapter 3** suggests the existence of this threshold through the finding that although the infusion of DCA led to activation of PDC (4-fold increase in PDC activation; $p<0.01$) with a corresponding increase in muscle acetylcarnitine content (5.5 fold increase; $p<0.01$) there was no reduction in PCr breakdown or lactate accumulation during either of the exercise bouts (within the limits of its statistical power). Based upon the results of this chapter I would therefore suggest that in normoxic conditions at and below 60 %VO_2max, the rate of activation of PDC is not rate limiting to ATP provision for contraction. It is likely that exercise induced PDC generation, as well as contributions from fatty acid oxidation, are able to match ATP provision with the muscle's metabolic demands.

At this exercise intensity fat derived fuel is preferentially used to provide energy for contraction (van Loon *et al*, 2001) and the increased use of carnitine stores to buffer the large amount of acetyl groups generated by the near-maximal activation of PDC at rest may have been limiting to fatty acid oxidation during exercise (Stephens *et al*, 2006). At this exercise intensity increasing PDC flux may not only reduce the amount of free carnitine available for generation of energy from fat oxidation but also increase the use of carbohydrate derived ATP for contraction where such rapid provision of energy is not required.

The increase in PDC activation seen in **Chapter 5** led to an increase in muscle acetylcarnitine concentration at rest ($p<0.05$), as observed in **Chapter 3** and previous studies (Timmons *et al*, 1998a; Roberts *et al*, 2002a). At this exercise intensity

(75%VO_2max) DCA pre-treatment was successful in overcoming metabolic inertia present at the onset of contraction, as demonstrated by the significant reduction in muscle glycogen breakdown following DCA pre-treatment.

Following the work presented in **Chapter 3**, and in support of previous studies on skeletal muscle metabolic inertia, during cycling exercise at an intensity of 75% VO_2max there was a reduction in metabolic inertia at the onset of contraction following DCA infusion. Collectively these findings support the existence of metabolic inertia at the onset of skeletal muscle contraction, but suggest the presence of a lower threshold of exercise intensity below which pharmacological activation of PDC is not of benefit during the subsequent period of exercise.

Persistence of the effect of dichloroacetate infusion upon PDC.

Previous research has demonstrated a maximal effect of DCA infusion (in doses up to 100mg.kg^{-1}) on the PDC in human skeletal muscle for up to 15 minutes of contraction (Gibala *et al*, 1999; Parolin *et al*, 2000). As well as its toxicity in chronic use (Stacpoole *et al*, 1979), this effect is also limiting to its suggested use as both a lactate lowering therapy in mitochondrial metabolic disorders and as a PDC activator in the therapy of conditions associated with premature skeletal muscle fatigue (such as PVD).

However in **Chapter 3** the results demonstrate persistence of PDC activation above basal as well as persistence of the enlarged acetylcarnitine pool available for contraction prior to the second bout of exercise, demonstrating that a one hour infusion of DCA (50 mg.kg^{-}

[1]) will activate PDC for more than a single bout of exercise over a period of at least 30 minutes. With interest into the protective metabolic effects of PDC activation during models of injury and ischaemia-reperfusion (Martin *et al*, 2004; Platz *et al*, 2007; Wilson *et al*, 2003) and the possibility of the PDC as a target for therapy in PVD or other conditions where premature skeletal muscle fatigue is present, this finding is of importance (see below).

Dichloroacetate and the ventilatory threshold.

If the ventilatory threshold is dependent on blood lactate concentration, then an alteration in the blood lactate concentration at the point of inflection in ventilation would be expected. The lactate lowering effect of dichloroacetate infusion was used to explore the relationship between the lactate and ventilatory thresholds further during healthy human volunteers during incremental cycling exercise commencing at 50% VO_2max. The findings of **Chapter 4** demonstrate no alteration of the blood lactate concentration at and after the ventilatory threshold, despite a significant reduction in the net production of lactate during exercise ($p<0.01$).

This chapter provides further evidence that the relationship between blood lactate concentration and the onset of the ventilatory threshold may not be as suggested by Wasserman *et al* (1973), in that accumulation of lactic acid within the muscles leads to an increase in the blood lactate concentration and a fall in pH. Wasserman *et* al suggested that this reduction in blood pH is buffered, in part, by bicarbonate and the resultant

production of carbon dioxide increases VCO_2. The ventilatory threshold therefore occurs due to the increased rate of ventilation required to exhale the extra CO_2 produced.

However work by other researchers (McLellan and Gass, 1989; Busse *et al*, 1992) demonstrated in glycogen-depleted individuals, in whom lactate and the partial pressure of carbon dioxide are reduced, an increased or constant rate of ventilation when compared to control conditions suggesting that the ventilatory threshold is unlikely to be due to the simple action of peripheral chemoreceptors (Myers and Ashley, 1997).

DCA infusion after high fat feeding.

Reducing carbohydrate intake for several days inhibits PDC activation during exercise due to stimulatory effects upon PDK (Putman *et al*, 1993; Peters *et al*, 2001). The molecular signalling pathways thought to account for this inhibition involve PPARs and FOXO, with their upregulation being linked to an increase in the expression of PDK4 mRNA and protein in skeletal muscle (Wu *et al*, 2001). DCA infusion is known to inhibit the activation of all the PDK isoforms (Whitehouse and Randle, 1973) but no researchers have investigated if the pharmacological activation of PDC was able to reverse the diet induced inhibition.

The work presented in **Chapter 5** demonstrates, for the first time, that pharmacological activation of PDC by dichloroacetate is able to reverse the inhibition of PDC activation by three days of high-fat/low-carbohydrate feeding. PDC activation was reduced by high-fat feeding ($p<0.05$) but the infusion of DCA led to a 4-fold increase in resting PDC

activation ($p<0.01$) which remained elevated throughout exercise when compared to the fat-diet population. This reduction in PDK activation occurred without a significant change in plasma free fatty acid concentration and persisted through contraction. The results also demonstrated that both FOXO1 and PPARα expression are correlated with PDK4 mRNA expression, and there may be a trend towards a stronger correlation between FOXO1 and PDK4 expression than between PPARα and PDK4 expression.

Future Work.

The work presented above suggests further questions for future research to address. The findings of this thesis highlight both the PDC as an important regulator of skeletal muscle fuel metabolism, and the use of dichloroacetate as a metabolic tool for the examination of PDC activation in human subjects. A discussion of future avenues of research in this field follows:-

Dichloroacetate-induced metabolic protection in ischaemia-reperfusion.

Although the toxicity of dichloroacetate limits its possible therapeutic use in the treatment of chronic ischaemia-reperfusion, as seen in patients with intermittent claudication, the results presented in **Chapter 3** indicate that the benefit in this group may not be as great as initially anticipated. It is known within patients with more severe claudication that there exists a form of metabolic myopathy (Brass and Hiatt, 2000) which in a previous study responded to three days of carbohydrate supplementation (Barker *et al*, 2004). Whether dichloroacetate administration, and its corresponding increase in skeletal muscle carbohydrate oxidation, is of benefit to patients with PVD has not yet been investigated.

In cases where there is an acute episode of ischaemia-reperfusion without the likelihood of requirement for further therapy, such as during embolectomy following acute arterial occlusion or cross-clamping of the abdominal aorta prior to open surgical repair, the toxicity of chronic dichloroacetate administration can be avoided. The metabolic

protection has already been demonstrated to be of benefit in an animal model (Wilson *et al*, 2003), and in the case of elective surgery associated with increases in metabolic stress (e.g. during orthostatic liver transplantation (Shangraw *et al*, 2008)) it would seem as though dichloroacetate infusion prior to induction of anaesthesia is likely to be of benefit.

FOXO as a fuel sensor?

Within the discussion of **Chapter 5** the suggestion that FOXO may have acted as a fuel substrate sensor for skeletal muscle metabolism was made. Nakashima and Yakabe (2007) have demonstrated that activation of AMP kinase results in an increase in the expression of FOXO mRNA and protein in skeletal muscles. AMP kinase has been established as a metabolic 'master switch' regulating glucose and lipid metabolism and it may be that the changes in FOXO mRNA expression are a reflection of this effect. With the finding of Barthel *et al* (2005) that FOXO proteins are involved in the mediation of insulin's effects on metabolism, it is apparent that FOXO may play more of a role than is currently appreciated in the regulation of skeletal muscle fuel metabolism.

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