THE EFFECTS OF R(+)-LIPOIC ACID SUPPLEMENTATION ON REGULATION OF HUMAN SKELETAL MUSCLE PYRUVATE DEHYDROGENASE

by

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A Thesis submitted in partial fulfillment of the requirements for

the degree of Master of Science in Applied Health Sciences

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ABSTRACT

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This thesis investigated whole body glucose disposal and the adaptive changes in skeletal muscle carbohydrate metabolism following 28 d of supplementation with 1000 mg R(+)-lipoic acid in young sedentary males (age, 22.1 ± 0.67 yr, body mass, 78.7 ± 10.3 kg, n=9). In certain individuals, lipoic acid decreased the 180-min area under the glucose concentration and insulin concentration curve during an oral glucose tolerance test (OGTT) (n=4). In the same individuals, lipoic acid supplementation decreased pyruvate dehydrogenase kinase activity (PDK) (0.09 ± 0.024 min^{-1} vs. 0.137 ± 0.023 min^{-1}, n=4). The fasting levels of the activated form of pyruvate dehydrogenase (PDHa) were decreased following lipoic acid (0.42 ± 0.13 mmol·min^{-1}·kg^{-1} vs. 0.82 ± 0.32 mmol·min^{-1}·kg^{-1}, n=4), yet increased to a greater extent during the OGTT (1.21 ± 0.34 mmol·min^{-1}·kg^{-1} vs. 0.81 ± 0.13 mmol·min^{-1}·kg^{-1}, n=4) following lipoic acid supplementation. No changes were demonstrated in the remaining subjects (n=5). It was concluded that improved glucose clearance during an OGTT following lipoic acid supplementation is assisted by increased muscle glucose oxidation through increased PDHa activation and decreased PDK activity in certain individuals.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Sandra Peters for her guidance and support throughout the work on this thesis. She has been a tremendous source for advice for matters both in and outside of the lab. Without her encouragement and enthusiasm for research, this thesis would yet to be completed. It has been a privilege and an honour to work with her.

I am grateful to my thesis committee, Drs. George Heigenhauser and Brian Roy, who have challenged me to consider all possibilities. George's sense of humour and ability to first overwhelm, and then inspire one with research has made for an unforgettable graduate experience.

I gratefully acknowledge Grieg Inglis for his collaboration with this work. His support in the lab throughout the study was invaluable, as were our didactic discussions. Thank you also to Dr. David Gabriel for his assistance with the area under the curve calculations.

While studying at Brock University, I have had the incredible opportunity to work with wonderful individuals. I would like to thank so many students, but especially, Nikki Bradley, Laura MacPherson, Matt Mulligan, and Ashley Jones, for their friendship and never ending assistance.

The support of my family has been relentless and awe-inspiring. Their encouragement and guidance are remarkable. I am at a loss on how to adequately thank my husband, David. His unconditional love, patience and support are immeasurable....without his encouragement, I could not have completed this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

Acetyl-CoA	acetyl-coenzyme A
Acetyl-carn	acetyl carnitine
ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
CHO	carbohydrate
CoA	coenzyme A
CS	citrate syntase
DHLA	dihydrolipoic acid
E1	pyruvate dehydrogenase
E2	dihydrolipoamide transferase
E3	dihydrolipoamide dehydrogenase
E3BP	dehydrolipoamide dehydrogenase binding protein
FFA	free fatty acid
L1	lipoyl-bearing NH_2 terminal domain
L2	lipoyl-bearing interior domain
N_2	liquid nitrogen
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nictinamide adenine dinucleotide
NADPH	nictinamide adenine dinucleotide phosphate
PDH	pyruvate dehdrogenase
PDHa	active pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
TCA	tricarboxylic acid cycle
TPP	thiamine pyrophosphate

CHAPTER 1

INTRODUCTION

The pyruvate dehydrogenase complex (PDH) is a fundamental regulatory multienzyme complex. It is the rate-limiting enzyme catalyzing oxidative decarboxylation of pyruvate, the three-carbon end product of glycolysis. Thus, through PDH, acetyl CoA and NADH are generated for the tricarboxylic acid (TCA) cycle and the electron transport chain for the production of adenosine triphosphate (ATP) and the synthesis of fatty acids, cholesterol, and some amino acids. Due to its pivotal role in fuel selection and muscle metabolism, PDH is tightly regulated both allosterically and covalently. In situations where there is an abundance of fatty acids or where carbohydrate sources for energy are scarce, PDH can be down regulated, thereby decreasing carbohydrate flux. Whereas, during situations of high blood glucose, PDH can be up regulated to increase oxidation of glucose and synthesis and/or storage of fatty acids [92]. This management of PDH is primarily through two associated regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP).

One of the principle metabolic challenges of the body is to maintain normal blood glucose levels of 3.9-5.6 mmol/liter (70-110 mg/100 ml) [75]. Promptly after eating, gastric inhibitory peptide and the rise in blood glucose concentration both stimulate insulin release from pancreatic beta cells. In many ways, insulin stimulates absorptive state metabolism. Insulin facilitates the entry of glucose, free fatty acids (FFAs) and amino acids into cells of many tissues. In the absorptive state, glucose from the blood stream has several fates. While most body cells use glucose to produce ATP by completely oxidizing glucose to carbon dioxide and water through glycolysis, the TCA

1

cycle, and the electron transport chain. Glucose can also be stored as glycogen in the liver

for future use. [75, 19]. Further, glucose can be taken up by adipocytes and converted

into triglycerides for storage. In liver and adipose tissue, insulin enhances synthesis of

triglycerides [75, 19]. Insulin stimulates phosphorylation of glucose in hepatocytes and

conversion of glucose 6-phosphate to glycogen in both liver and muscle [75, 19]. Skeletal

muscle plays a fundamental role in glucose homeostasis by virtue of the vast amount of

skeletal muscle in the body and its capacity for glucose uptake and disposal. In addition

to oxidation of glucose, skeletal muscle can also store glucose for future use in the form

of glycogen [75, 19] (Figure 1). The primary focus in the present study is on glucose

disposal through skeletal muscle oxidation.

Figure 1. **Principal pathways of post absorptive glucose** (Adapted from Tortora et al. [120]). G.I. tract, Gastrointestinal tract; CO_2, Carbon dioxide; H_2O, Water; ATP, Adenosine triphosphate; FA, Fatty acids; TG, Triglycerides; G3P, Glucose-3-phosphate.
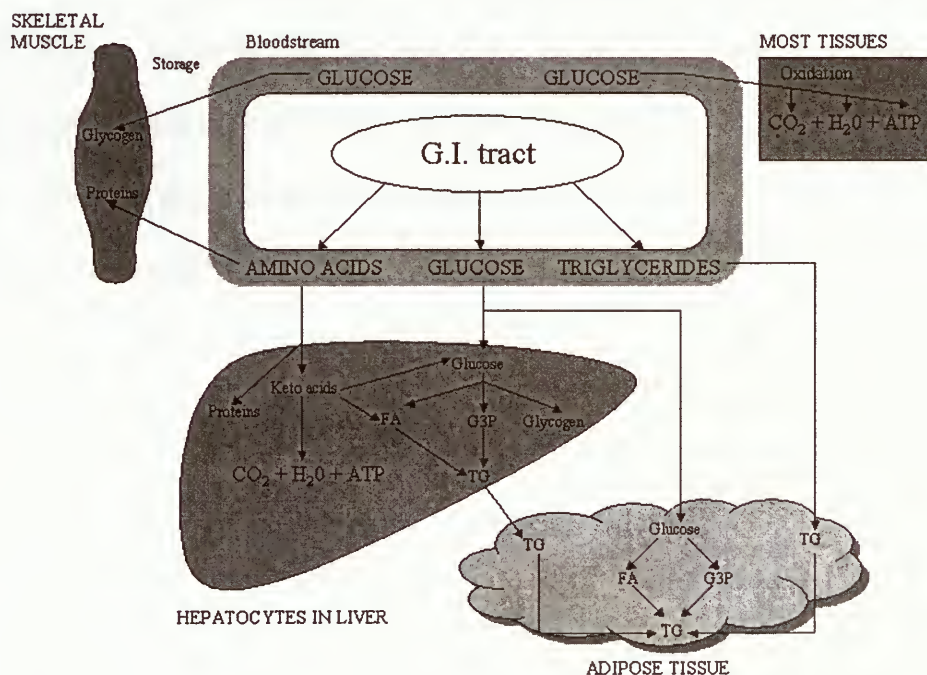
Maintaining proper blood glucose levels are a concern among various populations. It has been demonstrated that in non-insulin dependent diabetes mellitus (or type II diabetes), there are impairments in skeletal muscle insulin cell signalling, as well as an impairment of pyruvate oxidation elicited by a decrease in PDH activity via chronically increased activity of PDK demonstrated in heart muscle [125].

In addition, patients suffering from type II diabetes have also exhibited depressed serum levels of lipoic acid, and exogenous treatment with lipoic acid in both animal and human models of diabetes have proven beneficial as improvements in blood glucose uptake have been demonstrated [31, 49, 52, 50, 51]. Lipoic acid is naturally found covalently attached to a lysyl residue of dihydrolipoamide transacetylase or E2, a component of the PDH complex and is an essential co-factor of 2-oxoacid dehydrogenase multienzyme complexes including PDH. As well, lipoic acid functions as a cofactor in various enzymatic reactions where it can be fully incorporated into the PDH complex, altering its function and activity [59, 14, 4]. The exact link between lipoic acid and oxidative glucose disposal in human skeletal muscle and PDH has not yet been studied. Therefore, the purpose of this thesis is to investigate the effect of lipoic acid supplementation on PDH and PDK activity in healthy human subjects.

CHAPTER 2

LITERATURE REVIEW

In this chapter, the literature reviewed related to (a) conventional regulation of pyruvate dehydrogenase, (b) the characteristics of lipoic acid, (c) experimental animal and clinical studies using lipoic acid, and (d) theories regarding possible mechanisms of action of lipoic acid will be discussed as they play an important role in providing background for the present study.

THE PYRUVATE DEHYDROGENASE COMPLEX

Overview

The PDH multienzyme complex is fundamental for the regulation of carbohydrate metabolism and production of substrate for fatty acid synthesis in several mammalian tissues. [18, 21]. In skeletal muscle, PDH is located in the mitochondrial matrix and is associated with the inner membrane [21]. It catalyzes the irreversible oxidative decarboxylation of pyruvate through the action of its four catalytic components: pyruvate dehydrogenase/decarboxylase (E1), dihydrolipoamide transacetylase (E2), dihydrolipoamide dehydrogenase binding protein (E3BP), and dihydrolipoamide dehydrogenase (E3) [6, 39, 21]. As such, it regulates the carbohydrate flux into the TCA cycle in the presence of thiamine pyrophosphate (TPP), magnesium (Mg^{2+}), coenzyme A (CoA), and NAD^+ [85] through the following reaction [6, 39, 21]:

$$Pyruvate + CoA + NAD^+ \rightarrow Acetyl\ CoA + CO_2 + NADH$$

4
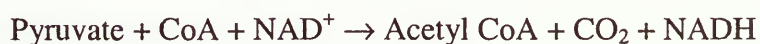
Due to its vital role in glucose homeostasis, the flux through PDH is closely regulated to meet the specific needs of different tissues during the fed and fasted states. Thus, the activity and subsequent flux through the PDH complex is achieved via a phosphorylation-dephosphorylation cycle though two inherent regulatory enzymes, PDK and PDP [85]. PDH is inactivated by phosphorylation of serine residues on the E1 catalytic subunit while dephosphorylation by the endogenous PDP activates the complex [6, 21, 53, 64, 65]. Therefore, the relative reciprocal activities of the two regulatory enzymes will determine the level of activity of the PDH complex and thus the level of carbohydrate oxidation at any time [128, 115] (Figure 2).

Figure 2. **Role and regulation of the PDH complex.** (From Sugden & Holness, [112]) PDK1-4, PDH kinases 1-4; PDP1-2, PDH phosphatases 1-2.
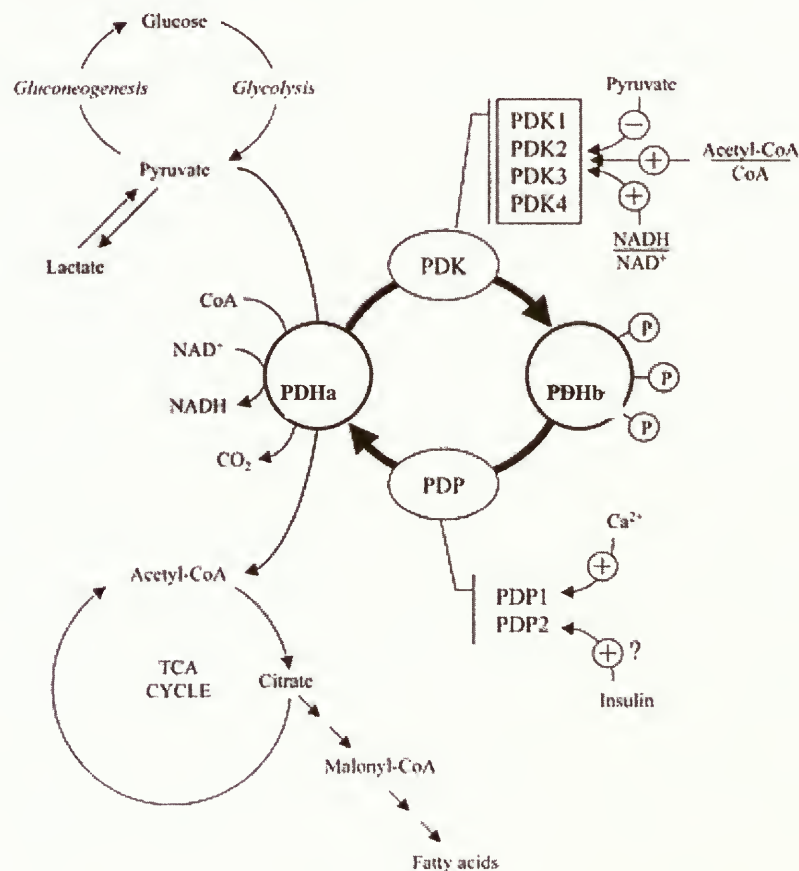
PDH structure and function

The organization of the complex, as outlined in Figure 3, is arranged to enhance metabolic efficiency. The complex allows for a clustering or concentration of the catalysts whereby the intermediate produced by one enzyme could interact with the next sequential enzyme or cofactor rapidly. A random allocation of each structurally independent subunit could hinder and thus impede activity [95].

The E1 component is a tetramer ($\alpha_2\beta_2$) containing two thiamine pyrophosphate (TPP) binding sites, and specific tryptophan and lysine residues, located at or near the TPP-binding site [85]. It was initially postulated that the function of the two non-identical subunits may be that the α subunits served a regulatory role while the β subunits were primarily catalytic or that both subunits had a catalytic function [3]. However, further investigations revealed that the α subunit of E1 alone is responsible for the TPP-dependent decarboxylation of pyruvic acid resulting in the release of carbon dioxide [21] representing the first, and irreversible rate-limiting step in the PDH catalyzed series of reactions [6, 83, 18, 64]. The α E1 subunit is also the only part of the complex to undergo phosphorylation and inactivation by the specific kinase.

Phosphorylation can take places on one of three serine residues (designated as sites 1, 2, and 3) on the α E1 subunit identified in both rat and human skeletal muscle. While phosphorlyation of site 1 is correlated with initial and vital inactivation (60-70%) of E1, further inactivation is continued through further phosphorylation of sites 2 and 3 [85, 116]. The E2 subunit is required for phosphorylation of the two subsequent sites whereas phosphorylation of site 1 can occur in E1 alone [83]. It has been suggested that phosphorylation of sites 2 and 3 may play a role in attenuating the dephosphorylation of

site 1 and hence activation of the PDH complex [85]. However, the exact nature and role of each site is still under debate. The role of the β component of the E1 subunit has not been as well defined, although it has been hypothesized to be responsible for catalyzing the transfer of the resultant acetyl group from the initial reaction to a lipoyl moiety on the E2 subunit of the complex [83, 21].

The E2 catalytic subunit acts as the structural core of the complex providing the framework for assembly of the complex and integrating the sequential reactions of PDH. E2 functions to catalyze the transfer of acetyl groups from S-acetylhydrolipoate to CoA providing E3 with fully reduced lipoyl moieties as an electron source [6]. E2 is comprised of four globular domains consisting of an amino terminal lipoyl domain (L1), an inner lipoyl domain, (L2), and E1 binding domain, and a core-forming, transacetylase-catalyzing inner domain at the carboxyl terminal end [85, 96]. The core consists of 60 copies of E2 polypeptide chains arranged in an icosachedral dodecahedron E2 [6] acting as the foundation to which 20-30 hetrotetramers ($\alpha_2\beta_2$) of E1 and 6-12 homodimers of dihydrolipoamide dehydrogenase (E3) are bound. The core also contains 12 monomers of dihydrolipoamide dehydrogenase-binding protein (E3BP) formerly known as protein X [32]. Research has illustrated similarities between E3BP and E2 as it consists of three linker-connected domains in which the distinct inner domain of E3BP binds to E2. The exact role of the E3BP however, is still not fully apparent [39]. The two lipoyl-bearing regions associated with the core, an interior domain (L2) and an NH_2 terminal end (L1) are easily reduced and acetylated, thereby providing substrate for the overall PDH reaction [96, 6, 16, 97]. The lipoyl domains also serve as the base for the 1-2 homodimers of the PDK and 2-3 heterodimers of PDP [83].

The cofactor lipoic acid is also covalently attached to the E2 component and has been speculated to serve as a swinging arm in the transfer of electrons and acetyl groups between catalytic sites [59]. It has been postulated that in addition to its role in the catalytic reactions, lipoic acid has an important role in the regulation of the PDH complex [14].

In mammalian PDH, the E3 subunit is a homodimer where each polypeptide tightly but noncovalently binds a molecule of flavin adenine dinucleotide (FAD) [39]. E3 can be further divided into four structural domains: a FAD binding domain, an NAD^+ binding domain, a central domain and an interface domain [84]. The two active sites participate in the electron transfer from dihydrolipoamide to NAD^+ [84]. As such, E3 is responsible for catalyzing the oxidation of dihydrolipoic acid to lipoic acid, where there is a subsequent reduction of NAD^+ to NADH [6].

Figure 3: **Overall reaction sequence of the PDH complex** (Adapted from Behal et al. [6]).
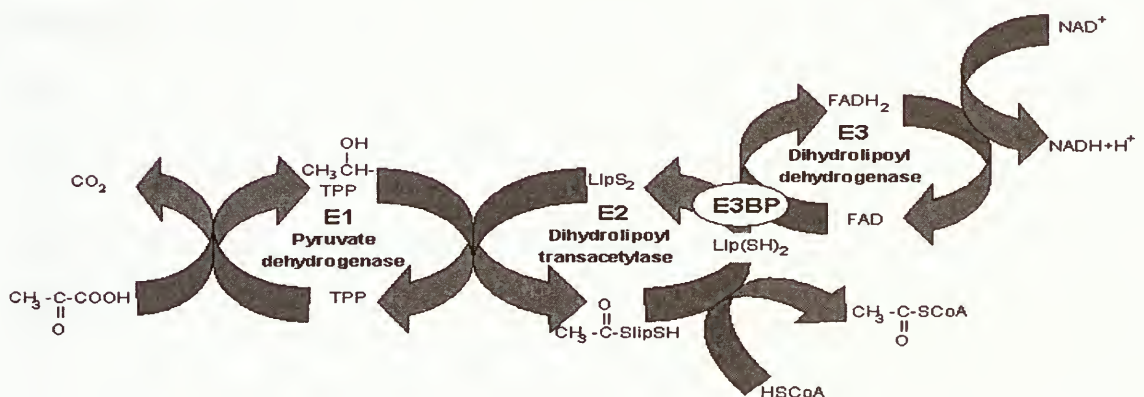
Acute regulation of PDH

Intramitochondrial effector regulation

The PDH complex is tightly regulated to ensure the optimal reciprocal relationship between metabolism of glucose and fatty acids or ketone bodies. Acute regulation of PDH activity refers to adaptations taking place within seconds, minutes and hours, whereas regulation exerted at a transcriptional level occurring over days and weeks is considered long term or adaptive regulation.

While tissue-specific differences exist, there are generally three different types of regulatory mechanisms. The first form, also the simplest, is achieved through end-product inhibition by acetyl CoA [6, 18]. The second form involves covalent modification of the complex demonstrated through a phosphorylation/dephosphorylation cycle. Fundamentally, the PDH complex can be deactivated through phosphorylation of one of three sites on the E1 α subunit, whereas PDH can be stimulated and thus reactivated through dephosphorylation of one of the two isoforms of PDP [6, 21, 53, 45, 64, 65, 112]. The third form of control is through feedback by nucleotides or energy charge [6].

As discussed previously, PDK and PDP are fundamentally responsible for the management of the cycle where metabolite effectors act intrinsically and, hormones and nutritional state exert their influence extrinsically. Generally, PDK and PDH activity respond to increases in the intramitochondrial ratios of $NADH/NAD^+$, ATP/ADP and acetyl CoA/CoA by increased phosphorylation and subsequent decreased PDHa activity [89] (Figure 2).

Specifically, PDK is tightly bound to the PDH core through the lipoyl domains of E2 [65, 127] where it monitors changes in the reduced and acetylated states of the lipoic

9

acid ligand and adjusts its activity accordingly. As such, the binding of PDK to the core increases activity [109].

There have been several genes of PDK identified in the PDK family. Of the 25 investigated, 4 of these are expressed in mammalian cells [109]. The PDK1 isoenzyme is present mostly in heart in both rats and humans where it displays a moderately high specific activity compared to the other isoforms [34]. PDK2 has the lowest specific activity of the isoforms and is found in most tissues; including liver, heart, skeletal muscle, with lower amounts in spleen and lung [11]. PDK3 has the highest specific activity of all 4 isoforms and is expressed mostly in the testis and lung in rats in very small amounts in human skeletal muscle. Finally, PDK4 is generally limited to heart and skeletal muscle [34, 99, 11].

Initially, it was thought that low levels of pyruvate had a stimulating effect on PDK by raising the nonphosphorylated fraction of E1 [65, 39]. However, in vivo work has demonstrated that pyruvate allosterically inhibits the kinase reaction [6, 65, 112]. It also has been demonstrated that several PDKs are activated by binding to E2 [127]. PDK2 is the isoform most sensitive to pyruvate inhibition whereas PDK4 is the least sensitive to pyruvate inhibition [11, 113, 112, 125].

While PDK is sensitive to inhibition by ADP, sensitivity of PDK to ADP can be increased in the presence of K^+ and NH_4^+ due to a lowered inhibitory constant (K_i) for ADP [6]. The K_m values of the PDK isoforms for ADP were generally similar between PDK1, PDK3, and PDK4 (50-65 µl), and lower for PDK2 (10 µl) [11]. Further, PDK1 displayed the highest K_i for ADP (370 µM), whereas PDK2, PDK3 and PDK4 were approximately 3 times lower (80-120 µM) [11]. The cofactor TPP also inhibits

phosphorylation and PDK activity by binding to the catalytic site of E1 to promote a conformational change, causing one of the hydroxyl groups on the α subunit to become less accessible to PDK. Alternatively, feedback products NADH and acetyl CoA stimulate PDK, particularly PDK2 activity through the reduction and acetylation of the lipoyl moieties in the complex [89, 84, 11]. PDK1-3 demonstrate a similar activation response with an increase in NADH concentration (1.1-1.3 fold increase), whereas PDK4 activity approximately doubles in the presence of increased NADH levels [11].

In terms of regulation via acetyl CoA, it has been noted that one characteristic unique to PDK2 is the manner in which it responds to acetyl CoA. Exposure to acetyl CoA and a high $NADH/NAD^+$ ratio increase PDK2 activity to more than 3 fold over the control [11]. Such activation of PDK activity followed by inhibition of PDH may be observed when oxidation of fatty acids or ketone bodies presents an overabundance of these metabolites.

The regulation of PDP appears to be more straightforward. While polyamines such as spermine have been shown to enhance PDP activity, micromolar increases in Ca^{2+} increases the phosphatase reaction indirectly by facilitating the binding of the phosphatase to the transacetylase core, thereby enhancing dephosphorylation by lowering the K_m for the PDP substrate (phosphorylated PDH) and Mg^{2+} ions. However this is only known to occur through the PDP1 isoform [6, 45, 112, 88]. While both PDK and PDP are Mg^{2+} dependent, it has been demonstrated that PDP requires 10 fold greater concentration of Mg^{2+} for optimal activity [64]. From this, it has been suggested that the level of Mg^{2+} available is dependent on the ATP/ADP ratio [64].

Extrinsic control

Less is known regarding the extrinsic control of PDH. There is some evidence demonstrating that hormones elicit acute effects. Several studies have examined the role of insulin on PDH regulation. It has been demonstrated that in adipose tissue, liver and kidney, insulin has a beneficial effect on PDP, stimulating PDH activity ten fold by increasing the sensitivity of the phosphatase to Mg^{2+} [20]. Further, using hindlimb rat preparations, Huang et al. [1998] demonstrated that PDP2 may be the isoform mediating insulin regulation, since Ca^{2+} ions do not regulate PDP2 activity in rat liver [45]. However the precise signalling mechanism, from the insulin receptor on the cell surface to the PDH complex located in the intramitochondrial membrane remains unknown. Mechanisms such as alteration of the phosphorylation state of the intracellular proteins, adaptations, inactivation of second-messenger, and the production of small mediator molecules have been proposed [6]. There is strong evidence to support the notion that increased intramitochondrial Ca^{2+} exerts a stimulatory effect on PDH activity in response to force production, epinephrine or other positive inotrophic agents in the heart. Additionally, angiotensin can have a stimulatory effect [68, 85].

Contrary to what would be expected, elevations of thyroid hormones, triiodothyronine (T3) and thyroxine (T4) have demonstrated inhibitatory effects of PDH activity. Characteristically, hyperthyroidism results in a greater carbohydrate and fatty acid fuel availability and utilization through the stimulation of Na^+/K^+ ATPases in all tissues, which is further accompanied by an increase in glucose transporters (GLUT4) expression in skeletal muscle [13, 114]. Therefore it would be expected that there would be an accompanying increase in PDH activity. However, hypothyroidism causes

12

increased PDK4 protein in heart and muscle, and increased PDK2 protein in fast twitch

muscle [114]. Therefore, Sugden et al., hypothesized that hyperthyroidism effectively

uncouples glycolytic flux and pyruvate oxidation via decreased PDH activity, thereby re-

directing glucose carbon to lactate and alanine to be released from muscle as

gluconeogenic precursors [114].

Long term (Adaptive) regulation of PDH

While the acute effects can be transient and occur within moments, they can take

place in addition to the longer-term adaptations. Therefore, the adaptive regulation of

PDH can be independent of intramitochondrial effectors. Thus there are several layers of

regulation that need to be examined. The most studied conditions surrounding long term

PDH regulation are those where there is a decrease in carbohydrate utilization and a

reciprocal increased reliance on free fatty acid (FFA) metabolism and increased beta-

oxidation. Such conditions include, diabetes, starvation, and high fat diets where

increased PDK activity causes a functional shift in PDH from the active form to the

inactive form by means of phosphorylation, [72, 126]. An increased reliance on FFA

utilization and beta oxidation elicits an increase in both the acetyl CoA/CoA and

$NADH/NAD^+$ ratios, which is responsible for executing the appropriate acute effects as

discussed previously in addition to the chronic increases in PDK activity. This form of

PDH regulation however, is demonstrated through an increase in PDK activity

independent of mitochondrial effectors. While a decrease in PDH activity during

starvation and a high fat diet may be considered beneficial as it conserves the available

three carbon compounds for gluconeogenesis, inhibition of the complex exacerbates

insulin resistance and diabetes by inappropriately sparing glucose and gluconeogenic substrates from complete oxidation despite high levels of oxidizable glucose and other substrates in the blood [125].

The long-term consequences of the various perturbations also induce changes at the nuclear level, however, each perturbation may induce changes on the different isoforms of PDK. Little is known about PDK1 and 3 due to their low concentrations in human skeletal muscle. However, in a study that examined the response of PDK isoforms in rat skeletal muscle, no significant changes were noted in PDK1 protein or mRNA content following fasting [87].

A majority of the research however, has focused on PDK2 and 4, as they are the most abundant isoforms present in human skeletal muscle. Studies in rats have reported that PDK2 expression is unresponsive to streptozotocin-induced diabetes [11, 126] and fasting [87, 113, 126]. However it has been suggested that the role of PDK2 may be to mediate PDH activity during long-term perturbations. For example, Majer et al. [70] using the Pima Indians population, who are pre-disposed to insulin resistance and diabetes, examined the correlation between insulin sensitivity and PDK mRNA in skeletal muscle. It was illustrated that PDK2 mRNA and to a lesser extent PDK4 mRNA were increased in individuals who exhibited symptoms of the onset of type II diabetes [70]. More recently, Leblanc et al. [63] demonstrated that PDK2 protein increases with exercise training [63].

Finally, human PDK4 was characterized in Pima Indians using the gene region linked with insulin resistance and type II diabetes [99]. Wu et al. [125] demonstrated that starvation and diabetes markedly increased the abundance of PDK4 mRNA, protein

content and PDK activity in rat heart and skeletal muscle, accounting for the accompanying increase in phosphorylation of the E1 subunit [125, 110]. Further, it has been demonstrated that PDK4 expression is sensitive to fasting [87,113, 126], high fat feeding [43], hyperthyroidism [114], and streptozotocin-induced diabetes [126], where such perturbation elicit an increase in PDK4 mRNA levels.

Lipoic acid overview

Lipoic acid, sometimes referred as thiotic acid, was initially discovered in 1937 by Snell et al. [105]. It was demonstrated that certain bacteria required a compound from potato extract for growth. In 1951, Reed and his co-workers isolated this compound and further characterized lipoic acid (chemical name: 1,2-dithiolane-3-pentanoic acid) [93, 94] (Figure 4). The name lipoic acid was derived from the fact that the compound is highly soluble in fat solvents, is acidic, and is involved through oxidative decarboxylation of pyruvate in the formation of acetate which is a precursor of fatty acids [93]. Initially lipoic acid was regarded as a vitamin and an essential dietary component. However it was subsequently discovered it could be synthesized by animals and plants, thereby challenging this notion [94].

Present in most kinds of prokaryotic and eukaryotic cells as the naturally occurring enantiomer (R+), lipoic acid is linked to lysine residues of the 2-oxo acid dehydrogenase mutlienzyme complexes where it acts as a cofactor [4]. During this process, lipoic acid is accepted as a substrate and is reduced to dihydrolipoic acid (DHLA) at the expense of cellular-reducing equivalents such as NADH and NADPH

15

[103] (Figure 4). Because of this ability of lipoic acid, it is regarded as a "metabolic antioxidant" [103, 37].

Figure 4: **Lipoic acid and DHLA.** (adapted from Packer et al. [80]).
DHLA, dihydrolipoic acid

Lipoic acid

DHLA
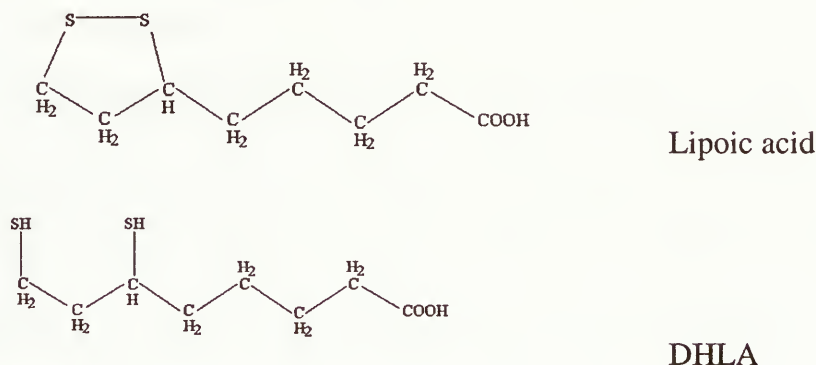
In our diet, lipoic acid is detected in the form of lipoyllysine [103], where the greatest concentration can be found in kidney and liver organ meats [80]. Lipoyllysine can also be found in garden peas, brussel sprouts, spinach, and rice bran [80].

In 1966, German physicians started to administer lipoic acid to patients with liver cirrhosis and heavy metal poisoning with the rationale that such patients had lower levels of endogenous lipoic acid [9]. It was assumed that supplementation would overcome the deficiency, thereby restoring the 2-oxo acid oxidation [9]. This notion was later supported by the discovery that lipoic acid, supplemented in the diet, accumulates in tissues with a substantial portion being converted to DHLA [81].

Currently, a diverse range of actions can be ascribed to lipoic acid. Exogenous lipoic acid has been found to possess therapeutic potential in neurodegenerative

16

disorders, radiation damage, heavy-metal poising, myocardial and cerebral ischemia-reperfusion injury [81, 80].

As noted previously, the naturally occurring enantiomer of lipoic acid is the R(+) configuration [4]. Various studies have examined whether there is a preference for the physiological R(+)-lipoic acid configuration, or if the non-physiological S(-) configurement of lipoic acid behaved in a similar manner. When studied in *Escherichia coli*, only the R (+) enantiomer could become covalently incorporated into the dihydrolipoamide acetyltransferase component of the PDH complex [78, 44]. These results offered an explanation for the in vivo results observed by Frolich et al. [29] in the treatment of vascular dementia and deficiency of brain PDH. They observed a stimulatory effect of PDH activity by the R(+) but not the S(-) form of lipoic acid [29]. Further, Streeper et al., [107] compared the individual effects of the two stereoisomers on skeletal muscle glucose metabolism in obese Zucker rats. It was demonstrated that following a single injection, the R(+) increased the rate of insulin-stimulated 2-deoxyglucose (2-DG) uptake, while further chronic administration was able to reduce plasma insulin and FFA concentrations. These effects were not demonstrated with the S(-) enantiomer [107].

While comparing the enantiomers of lipoic acid and their interactions with enzyme components of the purified PDH complex from porcine heart, it was noted that the R(+)-lipoic acid had a 24-fold greater maximum velocity than S(-)-lipoic acid when tested for its ability to act as substrates in the catalytic reaction of dihydrolipoamide dehydrogenase. Furthermore, it was suggested that if both enantiomers are present at the same time in equal concentrations (i.e. in a racemic mixture), the less active S(-) lipoic

acid would prevent the more active R(+) enantiomer from binding to the catalytic center, resulting in a reduction of enzymatic activity [67]. Dietary supplementation with the R(+) stereoisomer has also improved mitochondrial function in rat hepatocytes and increased ambulatory activity in aged rats [35]. However, despite these findings, lipoic acid is generally supplied and tested as a racemic mixture.

Animal studies examining the effect of lipoic Acid

Metabolic effects

Despite the numerous applications of lipoic acid, the most notable and widely examined therapeutic action of lipoic acid is to improve impaired glucose uptake. The most commonly used model to study this is the insulin-resistant obese Zucker rat. Jacob et al. [52] investigated the effect of lipoic acid *in vivo* (via intraperitoneal injection) on *in vitro* glucose uptake in isolated skeletal muscle from obese Zucker rats. Following a single treatment with lipoic acid (100 mg/kg body wt.), the rate of insulin-stimulated 2-DG uptake was increased by 34% [52]. Further, chronic administration over 10 days (30 mg/kg body wt./day) resulted in a 64% greater insulin-mediated increase in 2-DG uptake above basal levels when compared to the obese-control group [52].

Many studies have noted that the beneficial effects of lipoic acid are not limited to the obese, insulin-resistant rat model [42]. A study conducted by Henriksen et al. [42], demonstrated that following incubation of the epitrochlearis muscle of female obese Zucker rats, as well as that of their lean littermates with lipoic acid, 2-DG uptake was stimulated by 48% in the obese model, however there was a significantly greater stimulation of 2-DG in lean muscle as well (76%) [42]. Eason et al. (2002), also

18

demonstrated that short term incubation of L6 myotubes cultured from both normal, lean and severely insulin-resistant, obese-diabetic mice with lipoic acid increased glucose uptake, while insulin in addition to lipoic acid produced only slightly greater increases than either of the two agents alone [23]. Therefore, these results opened the controversy of whether lipoic acid was acting through an insulin-mediated mechanism or independently.

The finding that lipoic acid enhances glucose uptake in both insulin-resistant and lean muscle types is controversial. Using cultured L6 muscle cells and healthy lean, severely insulin resistant, and obese-diabetic rats skeletal muscle treated with lipoic acid, Eason et al., [23] demonstrated that lipoic acid could increase glucose uptake in all samples studied, including the healthy lean muscle [23]. However, in lean Zucker rats the combination of lipoic acid and exercise training did not result in a further improvement of insulin-stimulated glucose transport in skeletal muscle when compared to the effects of exercise training alone [101]. Such results dispute the notion that lipoic acid is able to enhance muscle insulin action in both healthy muscle and insulin resistant muscle, since lipoic acid alone was not able to improve insulin sensitivity in lean rat muscle [101].

From the divergent literature available, it becomes evident that the actions of lipoic acid are extremely widespread and effect many tissues other than just skeletal muscle. While examining the effects of lipoic acid on glucose metabolism in isolated perfused rat livers, Anderwald et al. [2] observed a dose-dependent reduction in rates of hepatic glucose production as well as lactate uptake. It was further illustrated that following lipoic acid perfusion, liver glycogen content was increased and there was a transient increase in bile flow [2].

19

Despite the promising finding regarding the positive effects of lipoic acid, little work has been conducted to determine the exact mechanism of action. Various studies have hypothesized that glucose transporters may be involved [57], while others suggest that the predominant effects of lipoic acid are exerted through an improvement in insulin signalling pathways, through an enhancement in the activation of phosphoinositol-3 kinase [42, 91]. Others suggest that alterations in glycogen storage and synthesis may be involved [52, 107]. Regardless of the theories suggested, work investigating the *in-vivo* effects of lipoic acid on human skeletal muscle has yet to be published.

Antioxidant effects

As an antioxidant, four properties of lipoic acid have been described: 1) a metal-chelating ability, 2) a reactive-oxygen species (ROS)-scavenging capacity, 3) a capacity to regenerate endogenous antioxidants and 4) a role in repair systems [9].

L6 muscle cells that have undergone oxidative stress lose reduced gluathione and have lower total concentrations compared to controls. Recently, Maddux et al. [69] observed that this decline was attenuated following treatment with lipoic acid, supporting the role for lipoic acid as a cellular antioxidant [69].

Hagen et al. (1999) found that hepatocytes from old rats had 59% lower oxygen consumption than their young counterparts. This reduction, was completely reversed following 2 weeks of lipoic acid supplementation. It was further demonstrated that the increase of oxidants with age (as measured by the fluorescence produced on oxidizing 2',7'-dichlorofluorescein) was significantly lower in the lipoic acid-treated compared to untreated aged rats [35].

In addition, Midaoui et al., [73], investigated whether dietary supplementation with lipoic acid could decrease mitochondrial superoxide production as well as formation of advanced glycation end-products in association with prevention of hypertension and insulin resistance in chronically glucose fed Sprague-Dawley rats. It was demonstrated that lipoic acid was able to prevent the increase in hyperglycemia-induced mitochondrial superoxide anion production and the rise in aortic advanced glycation end-product formation [73].

Clinical studies employing lipoic Acid

To determine the pharmacokinetics and the tolerability of lipoic acid, Evans et al. [25] studied 21 patients with type II diabetes and administered 900 mg of lipoic acid daily for 6 weeks and followed this with a further supplementation of 1200 mg for 9 weeks. It was demonstrated that the time to maximal plasma concentration was 1.25 hours and that there were no severe side effects or changes in liver and kidney function, or hematologic profiles [25]. However, lipoic acid induced slight hypoglycemia in some of these diabetic individuals probably due to an increase in muscle glucose disposal which was not quite balanced by liver output [51].

Studies have demonstrated that lipoic acid has a low bioavailability, implying that lipoic acid is slowly absorbed into the body and is gradually made available to skeletal muscle. A short elimination half-life after oral and intravenous administration indicates a high first-pass hepatic extraction for the drug. Food intake can significantly reduce bioavailability even further in healthy volunteers [118].

Following up on the positive results of lipoic acid on glucose homeostasis demonstrated in animal models, Jacob et al. [50] investigated the effects of a 10-day administration of lipoic acid in type II diabetics. A 30% increase in insulin-stimulated glucose disposal was observed via an isoglycemic glucose-clamp, making this one of the first clinical trials on the therapeutic value of lipoic acid [50].

One of the difficulties in comparing the results from various clinical studies is the lack of consistency in terms of study design. The duration of treatment, the administration method (oral versus intravenous), and finally the dosages vary greatly between studies. In a dose-response study, patients with type II diabetes were administered 600 mg of lipoic acid once, twice (1200 mg) or thrice daily (1800 mg) [51]. Following 4 weeks of treatment, no dose effect was demonstrated in the three treatment groups. Using an isoglycemic glucose-clamp, glucose uptake was significantly improved in all groups, and there was a slight trend towards lower levels of fasting plasma insulin concentration. No changes in fasting plasma glucose were noted [51].

To determine whether the beneficial results found previously were applicable to both lean and obese patients with type II diabetes, Konrad et al. [61] examined plasma pyruvate, lactate and glucose concentrations during an oral glucose tolerance test (OGTT) following 4 weeks of 1200 mg of lipoic acid daily. Lipoic acid supplementation resulted in an increase in insulin sensitivity during the OGTT in individuals with type II diabetes; and an increase in glucose clearance for both lean and obese diabetic individuals (with the effect being more prominent in the lean group). Reduced fasting plasma lactate and pyruvate concentrations were also demonstrated in both groups. Lipoic acid was also able to prevent the normally observed increase in pyruvate and lactate following the oral

glucose load [61]. The decrease in lactate and blood pyruvate following the glucose load can be ascribed to an increase in muscle oxidation possibly due to an increase in PDH activity. Instead of accumulating, muscle pyruvate would be converted to acetyl-CoA through the activity of PDH where it can then be shuttled to the TCA cycle and further oxidized to produce ATP. Decreased PDH activity would be expected to cause accumulation of muscle and possibly plasma lactate in response to an oral glucose load.

Similar findings were seen in two placebo-controlled studies evaluating the effects of an acute administration of lipoic acid (600 mg and 1000 mg) in patients with type II diabetes [42, 24]. After the treatment, glucose uptake (measured via 2-DG traffic) was markedly improved in the lipoic acid group, while there were no significant changes in the placebo group. It was noted that insulin sensitivity improved anywhere from 27% to 50% following the infusion of lipoic acid [42, 24].

Mechanism(s) of action

Surveying the clinical and experimental data, it is clear that lipoic acid has beneficial effects on insulin sensitivity and improves glucose uptake and disposal in both animal and human models. Despite this consensus, there are divergent theories regarding the mechanism(s) underlying the antidiabetic effects of lipoic acid.

Glucose uptake

Using primary human skeletal muscle cells, that were grown to differentiated myotubes, the effects of insulin and/or acute lipoic acid treatments on glucose transport were evaluated [5]. Following the exposure of the cells to the various treatments (basal, insulin, lipoic acid and lipoic acid + insulin), the cells were then incubated with 2-DG.

23

Lipoic acid or insulin exposure for 40 min resulted in increased glucose transport 2-3 fold over basal but was not additive when treatments were combined. Results from this and other studies support the suggestion that lipoic acid increases glucose uptake in an insulin-like manner [5, 52, 42, 60, 91, 74].

In patients with type II diabetes, it has been demonstrated that there is abnormally high hepatic glucose production causing hyperglycemia [19]. Thus, it is conceivable that lipoic acid could improve blood glucose levels by decreasing endogenous hepatic glucose production. In perfused livers of fasted rats, Anderwald et al. [2] found that infusions of lipoic acid decreased hepatic glucose production and increased bile flow and hepatic glycogen content, possibly through an insulin-like action [2].

Glucose transporters

Since cellular uptake in mammalian cells is mediated by a family of glucose transporters (GLUT) proteins, much of the literature to date has focused on the role of these transporters as a possible mechanism for the action(s) of lipoic acid. In skeletal muscle membranes, GLUT4 (and to a lesser extent GLUT1) are the predominant glucose transporters which mediate glucose uptake [41]. While both exercise and insulin causes a translocation of GLUT4 to the plasma membrane, GLUT1 is primarily insulin sensitive [41]. However, the GLUT4 transporters are located in two partitioned intracellular groups, whereby insulin activates one group, and exercise the other [41]. With stimulation, the transporters pass from their respective intracellular pool, and are recruited to the plasma membrane and T-tubules to allow for glucose uptake [41].

In the streptozotocin-treated diabetic rat, it has been demonstrated that muscle GLUT4 content is reduced depending on the duration of diabetes and on the muscle fiber type, in spite of the notion that insulin-stimulated GLUT4 translocation is unaltered [104]. However, following a 10 day treatment of lipoic acid, Khamaisi et al. [57] observed a significantly elevated GLUT4 content in the gastrocnemius muscle from both control and diabetic rats, although there was no associated change in GLUT4 mRNA levels. Based on their observations they hypothesized that lipoic acid interfered with GLUT4 degradation [57, 4].

Konrad et al. [60] expanded these findings through their research utilizing 3T3-L1 adipocytes in culture. It was demonstrated that treatment with lipoic acid promoted a greater activation of GLUT4 transporters as well as a greater translocation to the plasma membrane [60].

Yet despite these promising findings, the involvement of GLUT4 transporters in the positive effects of lipoic acid remains controversial. Using obese Zucker rats, Saengsirisuwan et al. [100] found that lipoic acid supplementation did not increase the expression GLUT4 protein levels or the activities of hexokinase (HK) or citrate synthase (CS), implying that an increase in GLUT4 was not the mechanism by which lipoic acid improved skeletal muscle glucose transport [100].

Separation of the cellular effects of insulin and lipoic acid

To evaluate the capacity of lipoic acid to modulate glycogen synthesis in comparison to insulin, rat soleus muscle was incubated for up to 60 min with 2.5 mmol/L lipoic acid in the presence or absence of insulin [22]. While insulin upregulated the

synthesis of glycogen, in the presence of lipoic acid, this effect was attenuated compared to insulin alone suggesting that lipoic acid regulates glucose metabolism in the muscle differently than insulin. To examine whether the counteracting effect of lipoic acid on insulin during glycogen synthesis was related to redox control, the soleus muscle was exposed to the oxidant t-butylhydroperoxide. t-Butylhydroperoxide decreased glycogen synthesis, and when combined with lipoic acid there was an additive effect, decreasing glycogen production by 80% [22]. These results suggest that the beneficial effects of lipoic acid may be related to its antioxidant capacities which may operate independently of insulin-dependent pathways.

The notion that lipoic acid works independently of insulin was further supported by the work of Khanna et al [58]. Using L6 myotubes, Khanna tested the hypothesis that cytokine-induced glucose uptake was redox sensitive. Following cell treatment with lipoic acid, the effect of tumour necrosis factor, lymphokine interferon, and lipopolysaccharides were examined on glucose uptake. Although a combination of all three cytokines caused a decrease in insulin sensitivity, the treatments did not affect the lipoic acid-stimulated increase in glucose uptake in the skeletal muscle cells [58].

Carbohydrate oxidation

While the improvements in glucose homeostasis demonstrated with lipoic acid supplementation is a commonality among the various studies, there is little agreement on the mechanism(s) responsible. However, due to the fundamental role of PDH in oxidative glucose disposal, the hypothesis that the regulation of PDH activity may be important in the interaction between lipoic acid and improved skeletal muscle glucose disposal is

possible. Increased disposal of glucose through oxidative means would create an increased transmembrane gradient for glucose, and enhance glucose uptake.

As discussed previously, starvation and diabetes induce a stable increase in PDK activity in skeletal muscle mitochondria, thereby allowing phosphorylation and inactivation of the complex [72, 126, 30]. A recent *in vitro* study by Korotchkina et al., [62] demonstrated that in a purified protein system, lipoic acid inhibited the autophosphoryation of PDK2, indicating that lipoic acid exerted its effects directly on PDKs. The inhibition of PDK activity was possibly due to the interaction between lipoic acid and E2. In the presence of lipoic acid, the binding site on PDK for the lipoyl moiety of the lipoyl domain of E2 would be occupied by free lipoic acid. With decreased availability of the lipoyl-binding domain, PDK would not be activated through E2 binding, resulting in inhibition [62]. A "stable" inhibition of PDK activity would allow increased PDHa activity, thereby inducing a greater flux through the PDH complex towards oxidative disposal.

It has been noted that lipoic acid levels are reduced in many type II diabetics, primarily due to an impairment in the biosynthesis of lipoic acid from linoleic acid as a result of a reduction of the conversion of linoleic acid to arachidonic acid [77, 12]. Therefore, it has been suggested that reduced liver lipoic acid levels may be responsible for the inhibition of the PDH complex. Lipoic acid supplementation would then allow patients to return to near normal levels, thus alleviating the inhibition of PDH activity [77, 122].

Some researchers have suggested that increased accumulation of intramitochondrial acetyl CoA in diabetes leads to a higher degree of acetylation of

27

enzyme-bound lipoic acid [124, 31]. This acetylation, stimulates the kinase activity by promoting binding to the E2 core, causing inhibition of the complex [31]. However, increasing concentrations of CoA can overcome this inhibition. There is evidence that DHLA can bind acetyl groups in a manner similar to CoA by de-acetylating the complex, thus releasing PDK from the E2 core, and enhancing PDHa activity [31]. Thus, lipoic acid supplementation and increased mitochondrial DHLA can reverse the acetylation and decrease kinase activity [31].

An increase in PDH activity has been illustrated in several animal studies in various tissues and models. Using cultured hepatocytes, Walgren et al. [123] were able to demonstrate a two-fold increase in PDH activation in cells treated with 50 µmol/L lipoic acid and a three fold increase in those treated with 100 µmol/L [123]. Similarly, in streptozotocin rats, Strodter et al. [108] found that lipoic acid not only increased ATP content significantly and improved glucose uptake by 66%, but also decreased muscle lactate and pyruvate in isolated heart muscle [108]. These improvements however were demonstrated in normal rats (without a deficiency in lipoic acid), therefore the hypothesis of a causal relationship between depressed lipoic acid levels in diabetic patients and PDH inactivation is questionable [108].

To further support the argument for the role of PDH regulation as the mechanism behind lipoic acid effects, researchers have examined the effects lipoic acid in conjunction with fatty acid oxidation in liver. In diabetic individuals, impaired fatty acid oxidation occurs in liver mitochondria [122] causing increased circulating plasma FFA and increasing liver FFA oxidation. Despite tremendous research, the mechanism(s) for this are unknown. However, this would result in augmented mitochondrial acetyl CoA

production and acetyl CoA accumulation in the liver mitochondria, further inhibiting PDH [122, 123]. Lipoic acid, in its DHLA form, can impair fatty acid oxidation by replacing CoA as discussed previously. Reduced rates of FFA oxidation have been observed when acetoacetyl DHLA replaced the naturally occurring acetoacetyl CoA, causing a reduction in liver ketone body production following lipoic acid supplementation [31, 123].

Walgren et al. [123] further tested this hypothesis using cultured hepatocytes, and demonstrated that cells exposed to physiological concentrations of FFA (0.1 mmol/L) treated with 25 μmol/L lipoic acid resulted in a 47% decrease in FFA oxidation suggesting that carbohydrate oxidation was increased. Greater concentrations of lipoic acid reduced FFA oxidation in a dose-dependent manner [123].

Given the available literature, it is apparent that the action of lipoic acid is difficult to isolate to one tissue or cycle, for as in carbohydrate and fat metabolism the two are so tightly interwoven, it is difficult to dissect out independent events. While research has focused on the improvements in glucose uptake in various muscle and whole body models, there has been a paucity of research pertaining to human skeletal muscle. Theories have been developed in regard to the role of PDH yet the exact relationship between lipoic acid and PDH in human skeletal muscle of a whole body model remains unknown. Therefore, a thorough investigation into the role of lipoic acid in carbohydrate metabolism is vital to developing a greater understanding of the functional significance of lipoic acid in both fat and carbohydrate utilization and oxidation in healthy individuals.

CHAPTER 3

STATEMENT OF THE PROBLEM

In the review of the literature, the actions of lipoic acid were discussed, with a particular emphasis being placed on the role of lipoic acid in the regulation of glucose uptake in human muscle. While the beneficial effects of lipoic acid on glucose uptake have been well documented, little is understood about the underlying mechanism(s), particularly in human skeletal muscle. To date, only a limited number of studies have examined this response with specific regard to PDH action and regulation. It is evident that PDH activity plays a fundamental role in glucose regulation in human muscle tissue, but the association between lipoic acid, glucose metabolism and PDH regulation remains elusive. Thus, to fully understand the effects of lipoic acid supplementation on skeletal muscle oxidative glucose disposal one must examine the PDH complex and its regulation.

Purpose

The purpose of this study was to determine if regulation of oxidative skeletal muscle carbohydrate disposal during an OGTT contributed to increased glucose clearance following 1000 mg/day of R(+)-lipoic acid for 28d. The primary purpose was to examine changes in total PDK activity in response to the supplementation in human skeletal muscle. A secondary purpose was to determine if these changes affected the activation of PDHa activity, both prior to and during an OGTT.

30

HYPOTHESES

1. As demonstrated in previous studies, subjects would have an improved blood glucose and plasma insulin response during a 3h OGTT following 28 days of supplementation with 1000 mg R(+)-lipoic acid (demonstrated by a smaller area under the glucose vs. time and insulin vs. time curves (AUC)).

2. Total skeletal muscle PDK activity would decrease with 28 days of supplementation with 1000 mg lipoic acid.

3. PDHa activity would increase in the basal state following 28 days of supplementation with 1000 mg lipoic acid.

4. PDHa activity would increase to a greater extent 75 min after the administration of the oral glucose load following 28 days of lipoic acid supplementation.

CHAPTER 4

METHODS

Subjects

Nine healthy, male university students volunteered for this study. All of the subjects were relatively inactive (<1-2 times wk^{-1}), and were informed of the study protocol and associated risks and benefits before giving written informed consent. The Research Ethics Boards of McMaster University and Brock University approved the study (REB 04-113 and REB 03-287 respectively).

Experimental protocol

Prior to the experiment, each subject monitored and recorded their diet for 3d by recording the type and quantity of food consumed. The subjects reported to the laboratory on two separate occasions before and after the 28d lipoic acid supplementation. For 3 d prior to their first visit, subjects consumed the standardized pre-diet which was based on their 3d habitual records. The subjects were restricted to activities of daily living, and were not permitted to exercise above their pre-study levels for the course of the study. Subjects also maintained their habitual diet including any consumption of caffeine and alcohol. Frequent contact was made by telephone to monitor compliance.

On the first visit, the subjects reported to the laboratory following an overnight fast (10-12 h). A catheter was inserted into an antecubital vein of one arm. A blood sample was obtained (-30 min) and patency was maintained with a sterile non-heparinized isotonic solution. Blood was sampled prior to the OGTT, and at 30, 60, 90, 120, 150, and 180 min. Both legs were prepared for two percutaneous needle biopsies

32

each from the vastus lateralis muscle under local anaesthetic, as previously described [8]. The first pair of muscle samples were taken (0 min). The first sample was dissected free of blood and connective tissue, and used for mitochondrial extraction for the determination of PDK activity. A second biopsy sample was taken immediately following the first, and frozen in liquid nitrogen (N_2) for determination of PDHa activity.

For the OGTT each subject was asked to drink 1 g/kg glucose to determine the individual's response to a carbohydrate load. 75 minutes after the ingestion of the oral glucose load, two further percutaneous needle biopsies were taken from the vastus lateralis muscle of the contralateral leg. The two samples were treated as those described above for PDK and PDHa activity.

The subjects began the 28 d supplementation with 1000 mg lipoic acid the morning following the first laboratory visit. Subjects consumed one 500 mg R(+)-lipoic acid capsule a minimum of a half hour prior to eating breakfast, and a second 500 mg capsule a minimum of a half hour prior to dinner. Following the 28 d of lipoic supplementation, the subjects fasted overnight (10-12 h) and returned to the laboratory (day 29). Subjects underwent a second OGTT, with blood and muscle sampling in an identical manner to the initial laboratory visit.

Figure 5. **Schematic of experimental protocol for both PRE and POST trial.**
Following the PRE trial, subjects began 1000 mg lipoic acid for 28 days followed by an overnight fast. Subjects then returned to the laboratory for the POST trial. LA, lipoic acid; OGTT, oral glucose tolerance test.
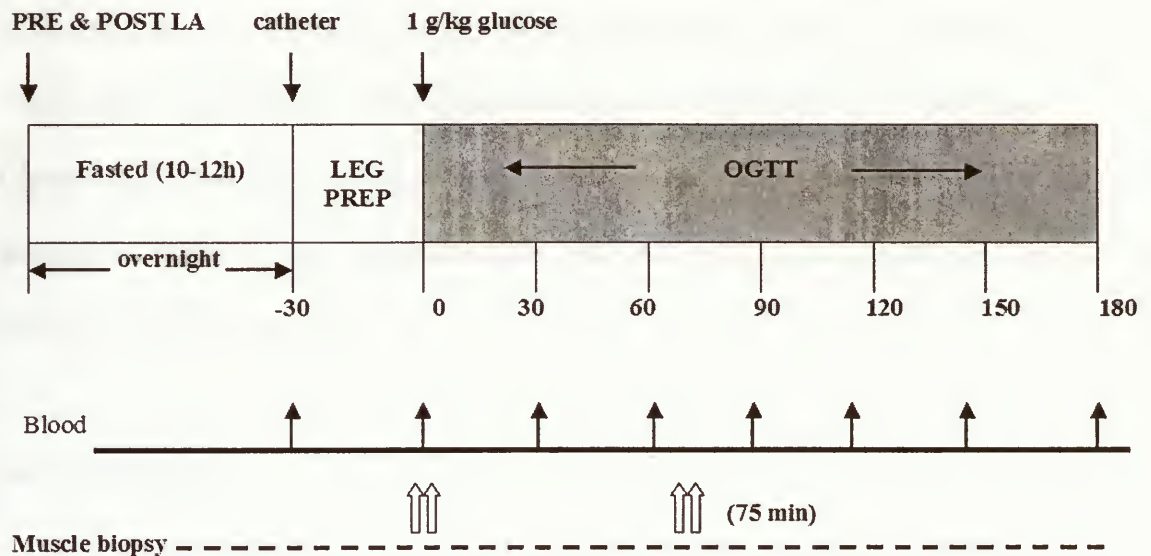
Blood analyses

Blood samples (4-5 ml) were drawn from the indwelling catheter. One portion of whole blood (200 µl) was added to 0.6 N perchloric acid (1 ml), vortexed and centrifuged at 10 000 rpm for 3 min. The supernatant was removed for analysis of glucose and lactate as previously described [7]. A second portion of whole blood was centrifuged and approximately 1 ml of plasma was removed for measurement of plasma FFA and insulin. Plasma FFA concentration, were measured using a Wako NEFA C test kit (Wako Chemicals, Richmond, VA). The remaining aliquots of plasma were analyzed for insulin using a commercially available insulin RIA kit (Coat-a-Count Insulin test kit Diagnostic Products, Los Angeles, CA).

Muscle analyses

The first sample (~75 – 100 mg) at each time point was used for fresh muscle extraction of mitochondria, and subsequently for the measurement of PDK activity and citrate synthase (CS) activities. The second muscle sample from each time point, was immediately frozen in liquid N_2 for analysis of PDHa activity and muscle metabolites.

Mitochondrial preparation for PDK activity

Intact mitochondria were extracted from fresh muscle homogenate using differential centrifugation, as previously described [48, 71]. Muscle was briefly minced and then homogenized using a glass-on-glass Potter homogenizer in 20 volumes of a buffer containing 100 mM KCL, 40 mM Tris HCl, 10 mM Tris base, 5 mM magnesium sulfate, 100 mM EDTA, and 1 mM ATP (pH 7.5). Following centrifugation at 700 g for

10 min, the supernatant was retained and was subject to a further centrifugation at 14,000 g (10 min), to pellet the mitochondria. The pellet was then washed, re-suspended, and pelleted twice for 10 min at 7,000 g in 10 volumes of 100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 1 mM magnesium sulfate, 0.1mM EDTA, and 0.25 ATP (pH 7.5). The first wash buffer included 1% (wt/vol) bovine serum albumin, and the second was protein free. The final mitochondrial pellet was re-suspended in a sucrose and manitol solution according to the weight of the original muscle sample obtained (e.g. 100 mg sample to 100:100 :1 sucrose and manitol). This was aliquoted for future measurement of PDK activity, extramitochondrial CS (CS_{em}) and total mitochondrial suspension (CS_{ts}). During the sample preparation, to drive the ATP concentration to zero in the intact mitochondria (by forcing the mitochondria to respire due to uncoupling) the PDK suspension was incubated in a buffer containing 10 μM carboyl cyanide m-chlorophenyl-hydrazone (a mitochondrial uncoupler), 20 mM Tris HCl, 120 mM potassium chloride, 2 mM EGTA, and 5 mM potassium phosphate, for 20 min (pH 7.4). To ensure complete conversion of PDH to the active form of PDHa, mitochondria were then further pelleted at 7,000g for 10 min and stored in liquid N_2 for future analysis of PDK activity.

PDK activity

Following the protocol outlined by Peters et al. [86], PDK activity was measured by initially suspending mitochondria in a phosphate buffer (pH 7.0) containing 30 mM KH_2PO_4, 5 mM EGTA, 5 mM DTT, 10% BSA, 25 mM oligomycin B, 1 mM tosyl-lypsyl-chloro-methyl-ketone (protease inhibitor), and 10% Triton X-100 (detergent). Mitochondria were then freeze-thawed twice following re-suspension to rupture all of the

mitochondria. Once the mitochondrial extract had been warmed to 30°C, two aliquots were removed and added to a buffer containing dichloroacetate and sodium fluoride (NaF), pH 7.8. The purpose of this buffer was to lock PDHa activity through PDK and PDP inhibition respectively. The two initial aliquots taken represent total PDH at zero time. To initiate the reaction, 3 mM magnesium-ATP was added to the remaining suspension. With the aid of a timer, samples were taken every 30 sec for 3 min. Upon removal, each sample was immediately placed in a dichloracetate-NaF buffer and stored on ice for future analysis of PDHa activity through the radioscopic measurement of acetyl-CoA production as previously described [17, 90]. PDK activity is reported as the apparent first-order rate constant on the inactivation of PDH (min^{-1}) or as the slope of ln [%(PDHa activity with ATP addition)/(total PDH without ATP addition)] vs. time [26, 121]. The slope was determined using linear regression analysis.

Mitochondrial and total homogenate CS activity

Using a spectrophotometer, CS activity was measured using the enzymatic method to link the release of free CoASH to the colourmetric agent dithiobis-2-nitrobenzoate, as described previously [106]. The CS activity in the mitochondrial suspensions and total muscle homogenate (CS_{hm}) were used to determine mitochondrial recovery and quality of the mitochondrial preparations [86]. CS_{ts} and CS_{em} were measured following the mitochondrial suspension. The suspension was freeze-thawed twice to break the mitochondria, allowing for a more accurate CS_{ts} measurement.

$$\% \text{ Fractional Recovery} = (CS_{ts} - CS_{em})/ CS_{hm}$$
$$\% \text{ Intact Mitochondria} = 100 \times (CS_{ts} - CS_{em})/ CS_{ts}$$

PDHa activity

Using the methods described by Constantin-Teodosiu et al., [17] and Putman et al., [90] to determine PDHa activity, approximately 10 mg of frozen wet muscle was chipped from the frozen biopsies under liquid N_2. To inhibit PDK and PDP, samples were initially homogenized in a pH 7.8 buffer containing dichloroacetate and NaF. A volume corresponding to the ratio $30\mu l$ buffer: 1 mg homogenate to a reagent mixture at 37°C. To drive the PDHa reaction as described by [90], the reagent mixture contained the required coenzymes (3 mM NAD, 1 mM CoA, and 1 mM TPP). To initiate the reaction, pyruvate was added to the reagent mixture and 200 μl aliquots were removed at timed intervals (1, 2, and 3 min) and mixed with 40 μl PCA to stop the reaction. Following a 5 min incubation, samples were neutralized with 1 M K_2CO_3. The neutralized extracts were then stored at -20°C for later analysis of acetyl-CoA using a radio-isotopic methods as previously described by [15, 17, 90]. To determine the reaction rates for PDHa, linear regression analysis of acetyl-CoA vs. time plots were performed.

According to the method of Bergmeyer and co-workers [7], TCr content was measured in PDHa muscle homogenate extracts following the neutralization with PCA. To control for the presence of blood and connective tissue in the muscle samples, the highest creatine content in each subject's set of biopsy samples was used as a correction factor for the PDHa activity.

Muscle Metabolites

The remainder of the frozen muscle was freeze-dried, powdered and dissected of all visible blood, connective tissue and fat. The muscle metabolites were measured using

enzymatic methods that link the concentration of the metabolite to a product that absorbs light or fluoresces at a given wavelength, or to a labelled product that can be measured radioisotopically. The metabolites were measured in fasting and 75-min biopsies on neutralized PCA extracts. Creatine (Cr), phosphocreatine (PCr), ATP, and lactate were determined by spectrophotometric analysis [7, 40], pyruvate was measured fluorometrically [82], and acetyl CoA and acetyl carnitine were determined by radioisotopic measurement [15]. All of the metabolites were corrected to the highest total creatine concentration from a set of biopsies to compensate for the presence of blood and connective tissue.

Calculations

A 180-min OGTT area under the curve (AUC) for blood glucose and plasma insulin concentrations was calculated respectively for each subject as follows; fasting glucose and insulin concentrations (-30 min) were used as baseline value and the total positive area between baseline and the curve was calculated between –30 and 180 min.

Statistical Analyses

PDK activity in muscle samples (PRE vs. POST), change in PDHa activity, and AUC's for blood-glucose and plasma-insulin concentrations were compared respectively using a one-tailed student's t-test. Basal concentrations of blood and plasma parameters were directly compared between PRE and POST lipoic acid using a paired student's t-test. Blood parameters and enzyme activities (PDHa), and muscle metabolite concentrations over time during OGTT were analyzed using a two-way repeated

measures ANOVA (time vs. lipoic acid) with a Fisher's post-hoc test for all pairwise multiple comparisons. All blood and muscle data are presented as means $\pm$ SE. Significance was accepted at $p < 0.05$.

CHAPTER 5

RESULTS

Subjects, when examined as a whole, displayed no significant changes PRE vs. POST in all muscle and blood parameters. However when examined individually, diverging trends emerged, where there were responsive subjects (n=4), and subjects where no alterations were observed during the OGTT following lipoic acid treatment (n=5). Because the purpose of the present study was to determine if improved glucose clearance was due to changes in PDK and PDHa activity, it was important to identify those subjects who had improved glucose clearance. Subject participants herein will be discussed as a whole initially and then will be referred to as Responders (RSP) and Non-Responders (N-RSP) when appropriate.

Criteria for responders vs. non-responders

Following the analysis of data, subjects who demonstrated a minimum of a 10% decrease in the AUC for plasma insulin concentration during the OGTT following lipoic acid supplementation were designated as responders (RSP) (average insulin improvement of RSP 33.7 ± 5.7%, range: 16.3-56.3%) while the remaining subjects were designated as non-responders (N-RSP).

Subject Characteristics

The participating subjects were young (20-27 yrs) and were all relatively inactive (Table 1). However there was a wide range of body mass index (BMI). Some subjects could be considered lean (BMI 22.1), while others could be considered obese (BMI 35.6).

Blood glucose and plasma insulin

No changes were demonstrated in fasting blood glucose and plasma insulin concentrations PRE vs. POST (following lipoic acid) in any subjects (5.3 ± 0.3 vs. 5.0 ± 0.3 mM and 5.21 ± 0.6 vs. 6.31 ± 0.9 µIU/ml respectively).

When the subjects were examined as a whole during the OGTT, no differences were observed between PRE and POST glucose (n=9). (Figure 6.) However, in the RSP subjects, the peak at 30 min was noticeably lower following treatment (PRE 8.6 ± 1.7 mM vs. POST 6.4 ± 0.5 mM), however this only approaching significance (p=0.10) (Figure 7). There was no change demonstrated in the N-RSP subjects when compared PRE vs. POST (Figure 8).

During the OGTT, the AUC for blood glucose concentration was lower POST vs. PRE in RSP subjects (n=4), although this was not significant (p=0.14). (Figure 9). No significant changes were demonstrated in N-RSP subjects (n=5).

The AUC for plasma insulin was lower POST lipoic acid supplementation, however this was limited to the four RSP subjects only and was only approaching significance (p=0.09)

Blood lactate and plasma free fatty acids

There were no significant changes in blood lactate in any subjects (Figure 10). No significant changes were demonstrated in plasma FFA. However, all subjects (n=9) had lower fasting plasma FFA following lipoic acid (0.41 ± 0.1 vs. 0.36 ± 0.04 mM), although this was not significant (p=0.19).

42

During the OGTT, RSP subjects exhibited a trend towards lower plasma FFA

POST vs. PRE, this was not significant (p=0.23) (Figure 11).

Figure 6. **Blood glucose and plasma insulin concentrations vs. time during OGTT for all subjects (n=9).** The blood glucose and plasma insulin remained unchained PRE vs. POST R(+)-lipoic acid supplementation for 28d (1000 mg).

Figure 7. **Blood glucose and plasma insulin concentrations vs. time during OGTT for RSP (n=4).** The blood glucose and plasma insulin remained appeared lower POST R(+)-lipoic acid supplementation for 28d (1000 mg) vs. PRE, although this was not significant.
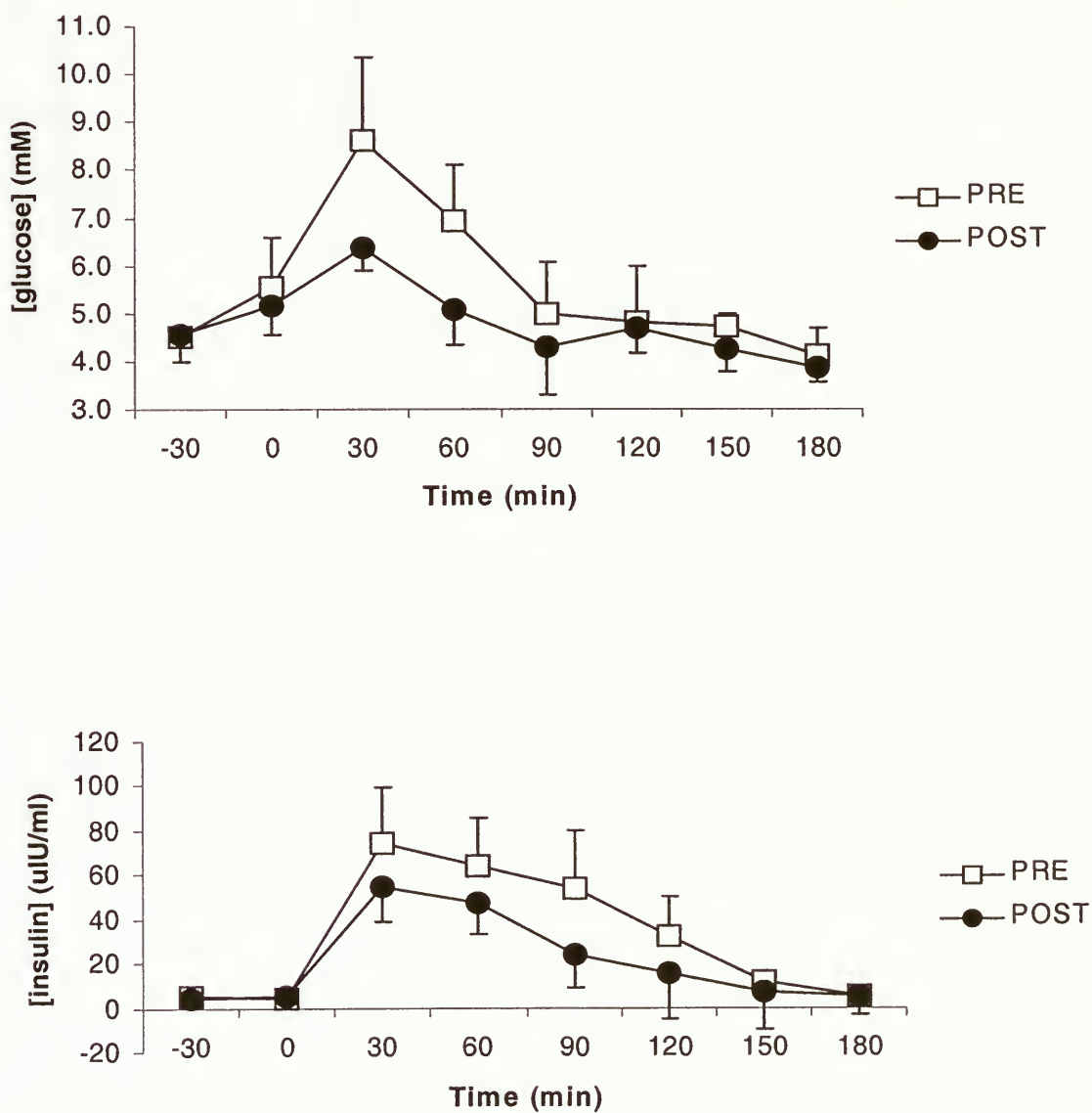
Figure 8. **Blood glucose and plasma insulin concentrations vs. time during oral glucose tolerance test for N-RSP (n=5).** The blood glucose and plasma insulin remained appeared unchanged PRE vs. POST R(+)-lipoic acid supplementation for 28d (1000 mg).
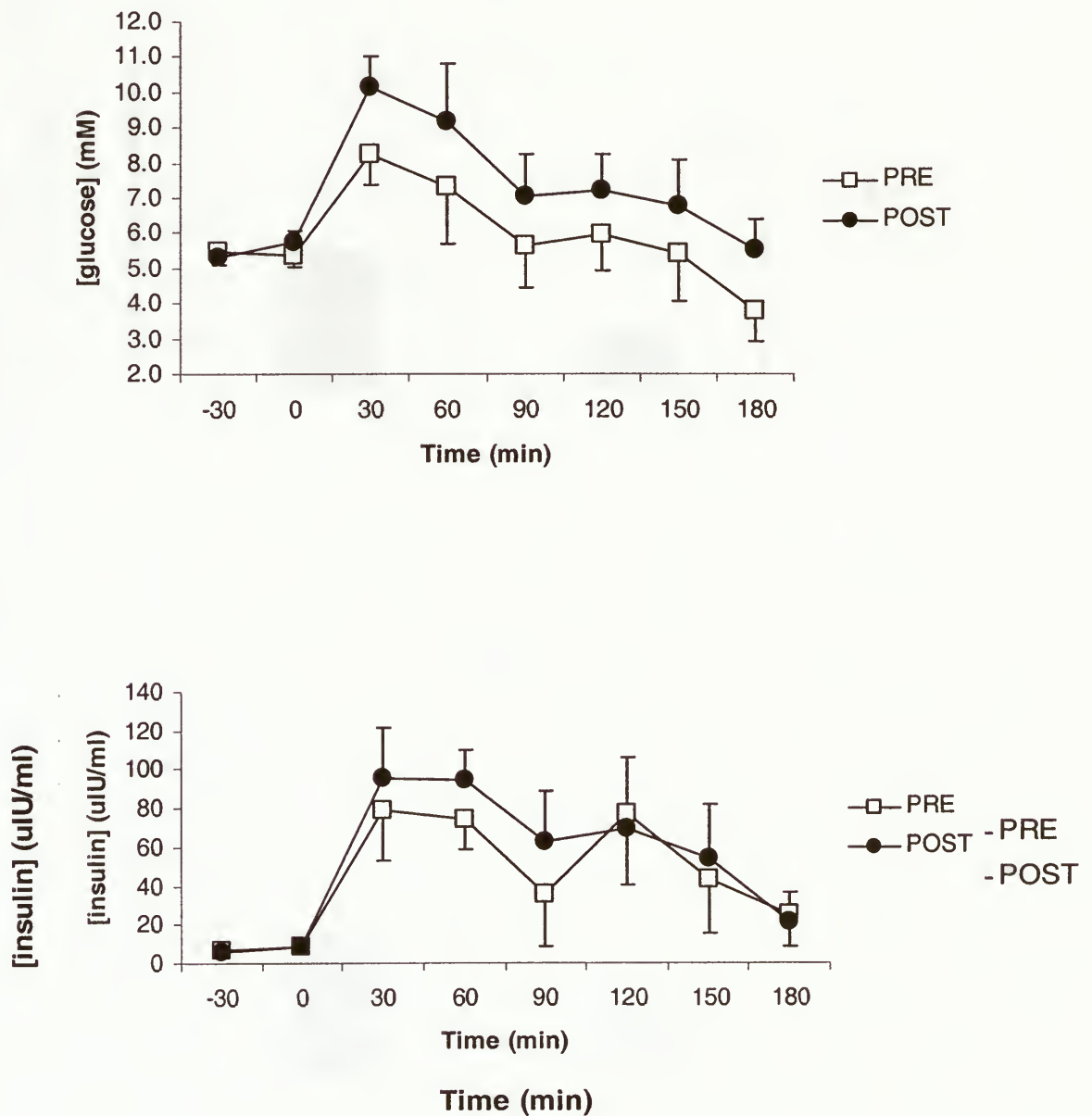
Figure 9. **Blood glucose and plasma insulin concentrations area under the curve (AUC) during the oral glucose tolerance test (OGTT).** During the OGTT, the AUC for blood glucose and plasma insulin concentrations were lower following 28d R(+)-lipoic acid supplementation (1000 mg) in RES subjects (n=4), although this was not significant.
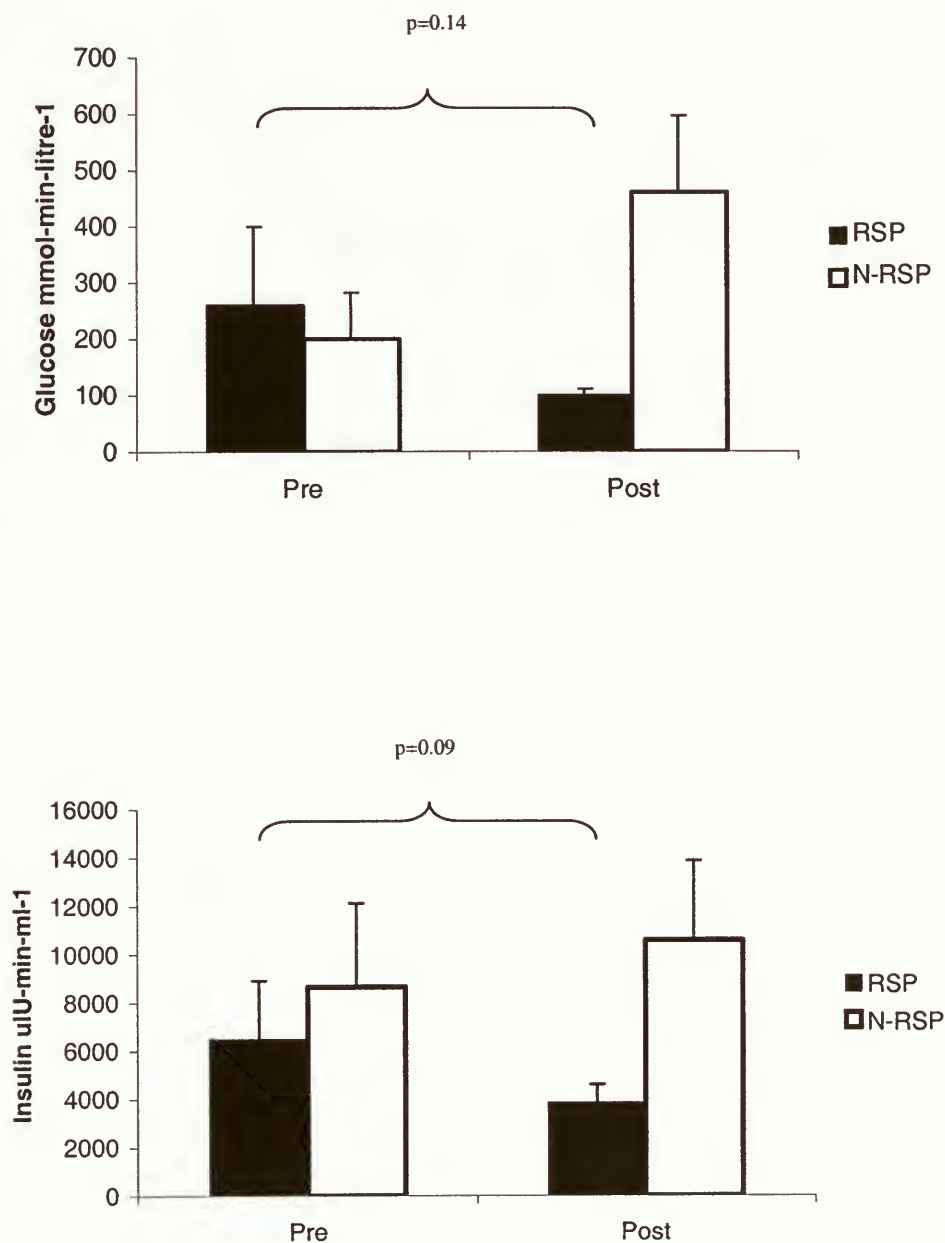
Figure 10. **Plasma FFA concentration vs. time during the oral glucose tolerance test (OGTT).** Plasma FFA concentrations (top panel) remained unchanged in the entire subject population (n=9) (p=0.19). Plasma FFA concentrations appeared to be lower prior to the OGTT following 28d lipoic acid supplementation (1000mg) in RSP subjects, although this was not significant.

47
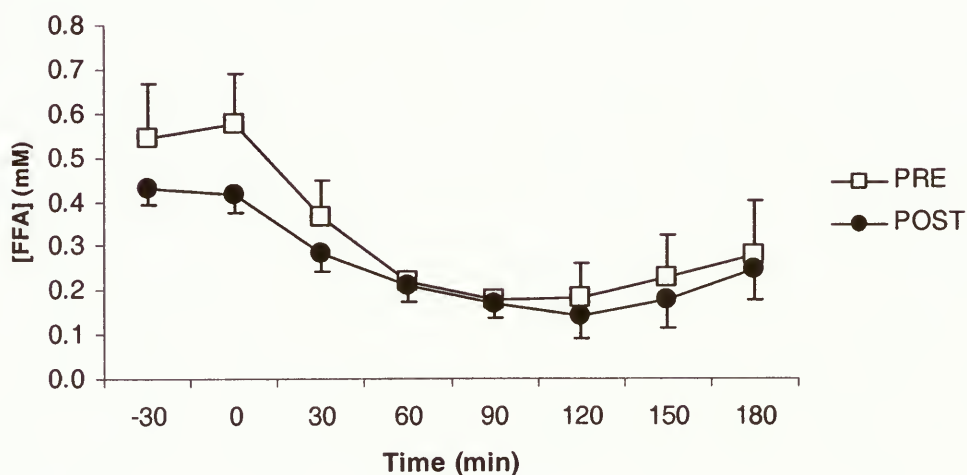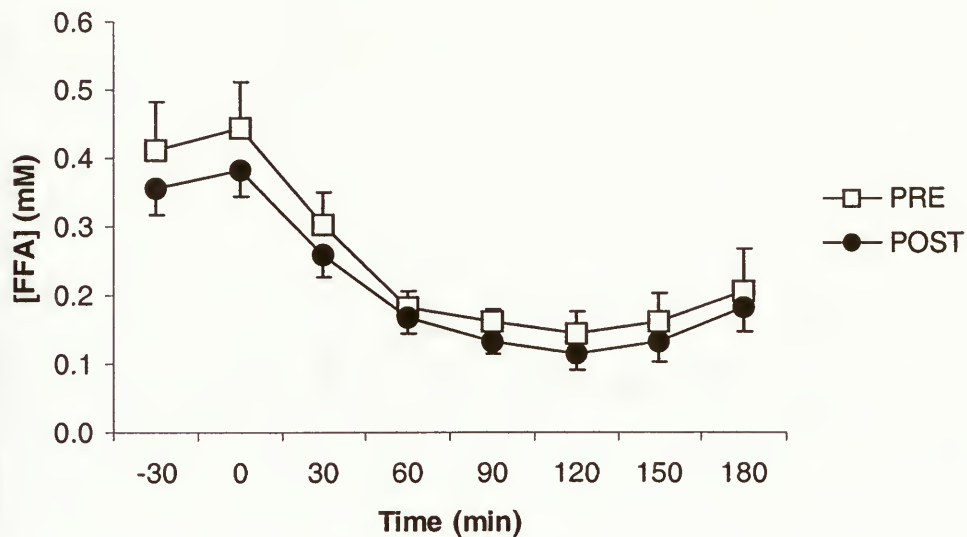
Figure 11. **Plasma lactate concentration during oral glucose tolerance test.** No changes were noted in any subjects following 28d R(+)-lipoic acid treatment (1000 mg).
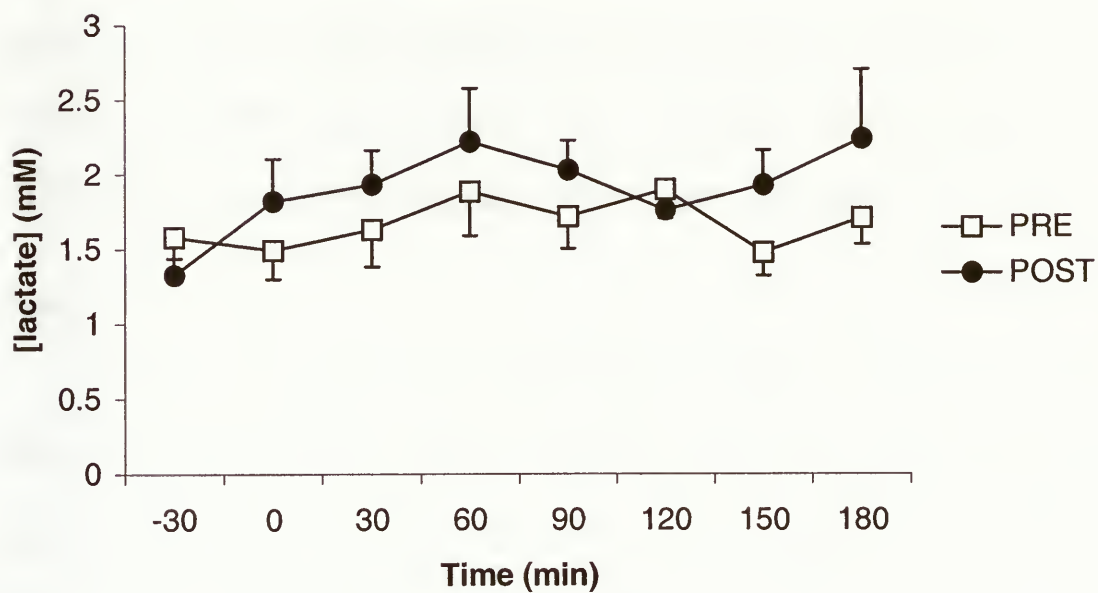
PDK and PDHa activity

PDK

PDK activity was measured at time 0min and 75 min during the OGTT. There was no significant difference between the two time points within the same trial. Therefore, PDK activity for PRE and POST is representative of an average of the time throughout the OGTT. (0.134 ± 0.0011 min^{-1}; 0.120 ± 0.0127 min^{-1}) Resting PDK activity was not altered PRE vs. POST lipoic acid supplementation in the full sample population (n=9). However, PDK activity was lower POST vs. PRE lipoic acid supplementation (0.137 ± 0.023 min^{-1} vs. 0.09 ± 0.024 min^{-1}.) in RSP subjects (p=0.0009) (Figure 12).

PDHa

As a whole, there were no significant changes in PDHa activity (n=9). Basal PDHa activity appeared lower following supplementation in RSP (0.82 ± 0.32 mmol·min^{-1}·kg^{-1} vs. 0.42 ± 0.13 mmol·min^{-1}·kg^{-1}), however this was not significant (p=0.19). During OGTT at 75 min, PDHa appeared to increase in RSP subjects following lipoic acid supplementation (0.81 ± 0.13 mmol·min^{-1}·kg^{-1} vs. 1.21 ± 0.34 mmol·min^{-1}·kg^{-1}), again however, significance was not achieved (p=0.19). Similar changes were noted in the N-RSP however this was not significant. (Figure 13).

When the change in PDHa activity was compared within each trial (0 and 75 min) using a one tailed t-test for all subjects (n=9), the delta was greater POST (0.49 ± 0.17 mmol·min^{-1}·kg^{-1}) vs. PRE lipoic acid supplementation (0.04 ± 0.15 mmol·min^{-1}·kg^{-1}) (p=0.059). The delta for the change in PDHa activity during the OGTT (0 and 75 min) in the RSP subjects (n=4) was PRE lipoic acid supplementation (0.01 ± 0.29 mmol·min^{-1}·kg^{-1})

vs. POST (0.79 ± 0.26 mmol·min^{-1}·kg^{-1}) vs (p=0.09). Whereas the delta for the change in PDHa activity during the OGTT (between 0 and 75 min) in the N-RSP subjects (n=5) was PRE lipoic acid supplementation (0.10 ± 0.20 mmol·min^{-1}·kg) vs. POST (0.25 ± 0.19 mmol·min^{-1}·kg^{-1}) (p=0.14). (Figure 14).

Muscle metabolites.

No significant changes were noted in any of the muscle metabolites. Following lipoic acid supplementation there appeared to be a slight decrease in fasting muscle pyruvate in all subjects (n=9) (PRE 0.43 ± 0.11 vs. POST 0.29 ± 0.09 mmol·kg^{-1} dw), although this was not significant (p=0.16) The muscle metabolite concentrations may be found in Table 2.

Citrate Synthase (CS)

Total muscle CS was unchanged in subjects PRE vs. POST lipoic acid supplementation in all subjects (n=9) (11.91 ± 1.14 vs. 12.06 ± 1.29 μmol·min^{-1}·g^{-1}) .The percent fractional mitochondrial recovery averaged $22 \pm 2.6\%$ of the total mitochondria, and the quality of extraction (percentage of intact mitochondria) was $87 \pm 22\%$.

Figure 12. **PDK activity.** PDK activity was measured in intact mitochondria at rest (0-min) and during OGTT (75-min). The top panel refers to the subject pool as a whole. The bottom panel separates the subject population into Responders (RSP) and Non-responders (N-RSP).
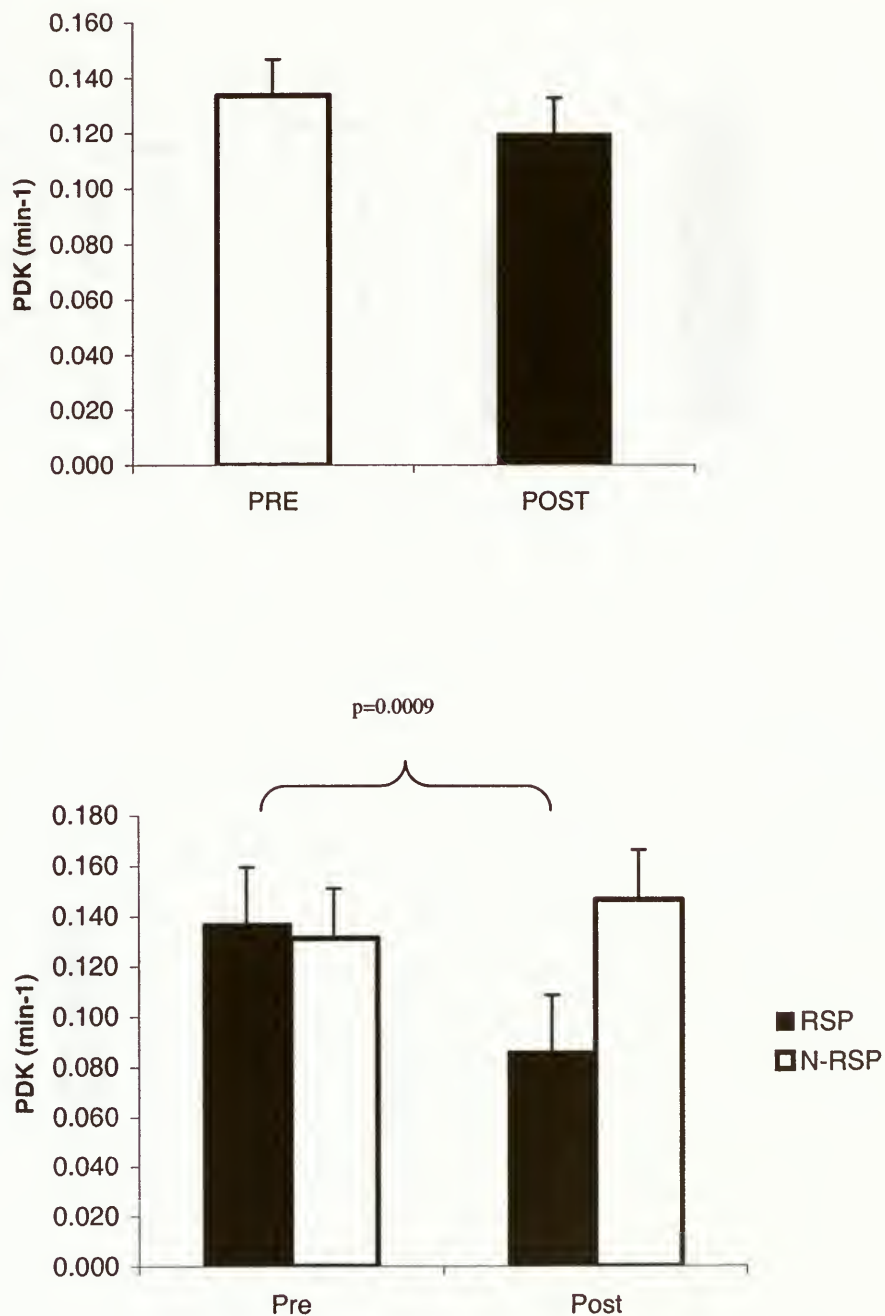
Figure 13. **PDHa activity.** PDHa activity was measured in intact mitochondria at rest (0-min) and during the oral glucose tolerance test (75-min). The top panel refers PDHa activity in subjects as a whole. The bottom panel separates the subject pool into Responders (RSP) and Non-responders (N-RSP).
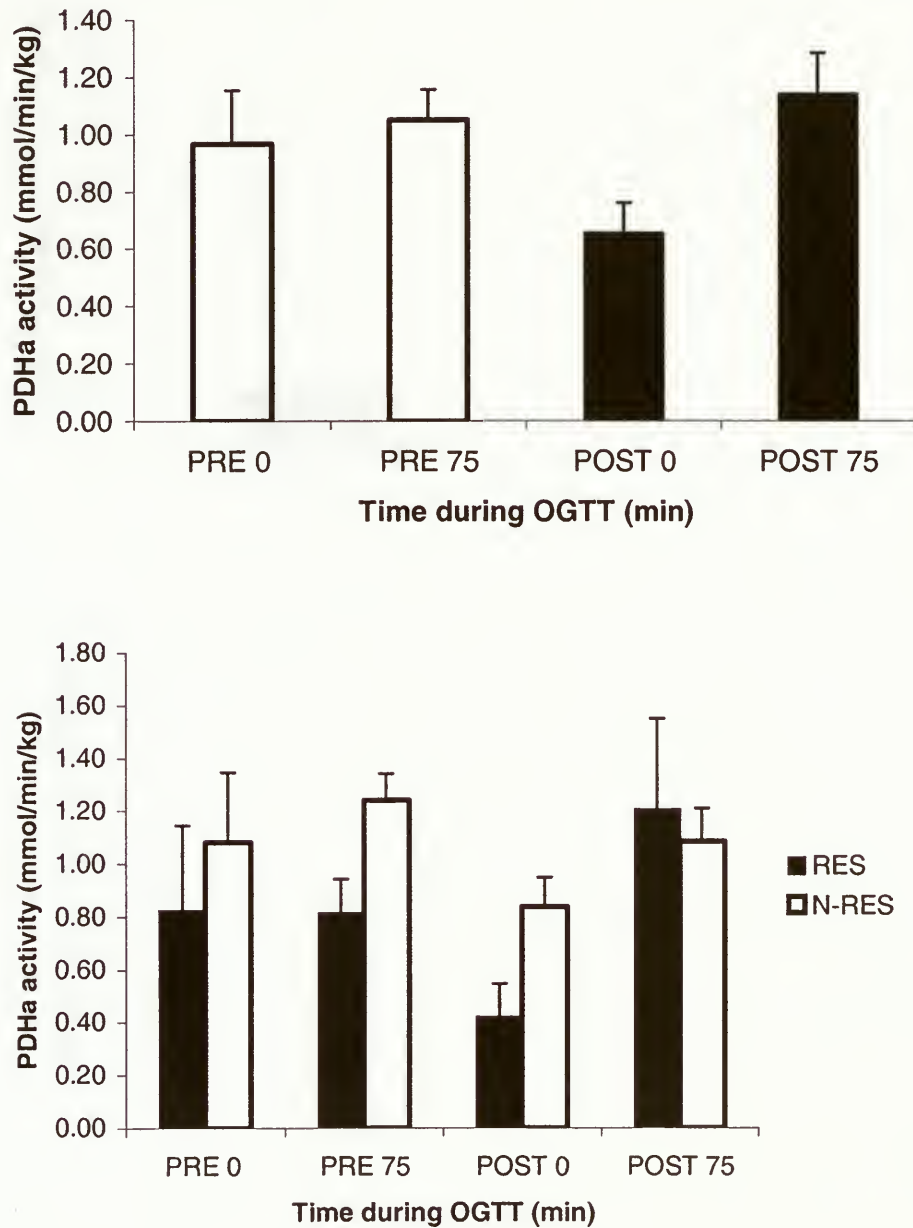
Figure 14. **Change in PDHa activity.** PDHa activity was measured in intact mitochondria at rest (0-min) and during the oral glucose tolerance test (75-min). The top panel refers to the change PDHa activity between time points (0 and 75 min) during the OGTT in all 9 subjects. The bottom panel refers to the change in PDHa activity between time points (0 and 75 min) during the OGTT in responding subjects (RSP).
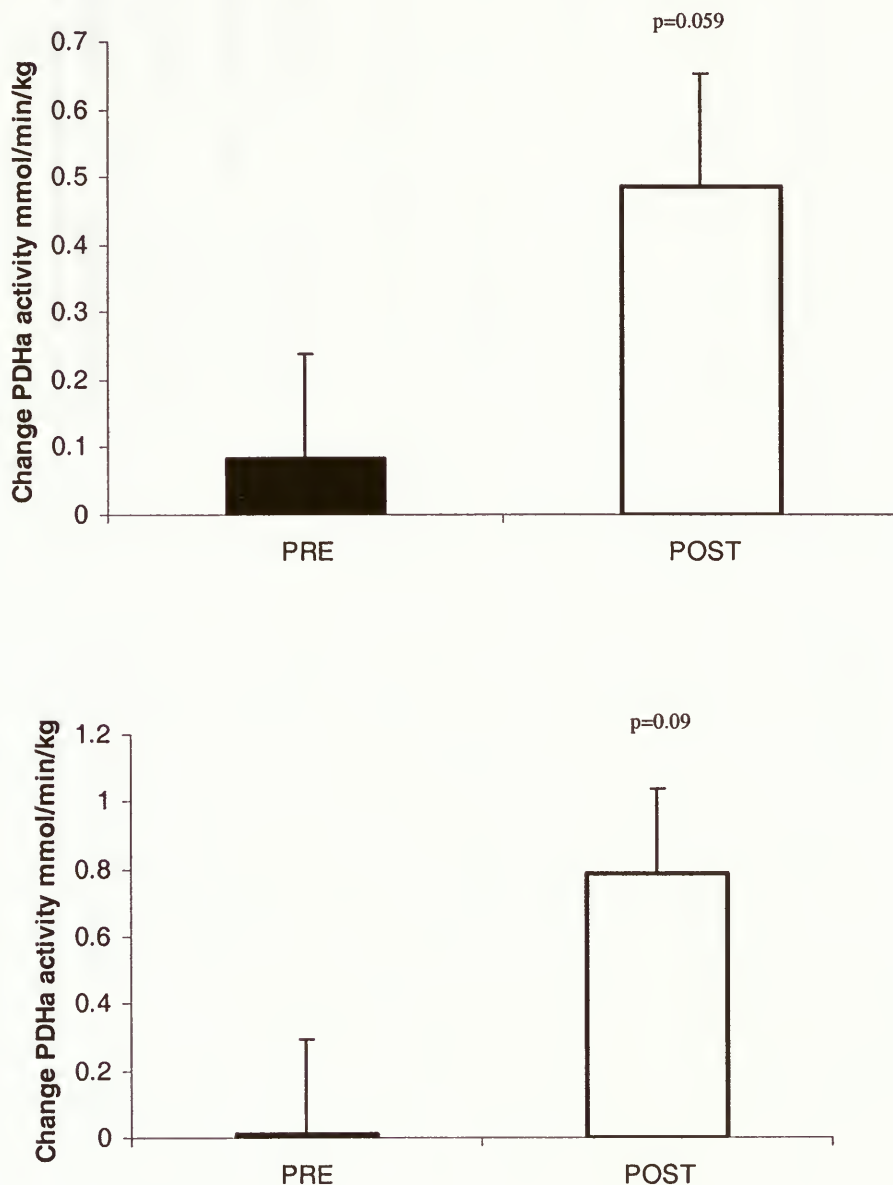
Table 1. **Subject characteristics.** Subjects basic measurements are recorded in years, kg, m respectively. The mean and SE for the subjects were calculated using RSP, N-RSP and the subject population as a total.

Subject	Age	Weight (kg)	Height (m)	BMI	Activity Level	Type of Activity
1	21	77.0	1.83	23.0	approx. 1x/wk	squash, leisure bike riding
2	21	79.0	1.80	24.4	approx. 2x/wk	walking, recreational beach volleyball
3	23	84.0	1.83	25.1	approx. 1x/wk	recreational basketball
4	23	73.9	1.70	25.6	< 1/wk	none during summer months
RSP Mean	22.0 ± 0.7	78.5 ± 2.4	1.79 ± 0.04	24.5 ± 0.65		
5	27	120.0	1.97	30.9	< 1/wk	none
6	22	80.3	1.86	23.2	approx. 1x/wk	recreational basketball
7	20	80.7	1.91	22.1	< 1/wk	none
8	20	81.0	1.81	24.7	approx. 1x/wk	recreational volleyball, walking
9	22	109.0	1.75	35.6	< 1/wk	none
N-RSP Mean	22.2 ± 1.4	94.2 ± 9.5	1.86 ± 0.04	27.3 ± 2.87		
Total Mean	22.1 ± 0.67	78.7 ± 10.3	1.66 ± 0.19	23.62 ± 3.03		

Table 2. **Muscle metabolites.** Metabolites are creatine corrected, reported as mean and $\pm$ SE mmol·kg^{-1} dry wt. except for Ac-CoA which is reported as μmol/kg dry wt.

	PRE 0	PRE 75	POST 0	POST 75
ATP	24.2 ±1.4	23.9 ± 1.7	21.1 ± 1.2	23.8 ± 1.6
PCr	52.9 ± 5.9	64.2 ± 4.1	54.1 ± 6.9	60.8 ± 5.2
Cr	29.7 ± 3.4	33.6 ± 3.9	37.9 ± 1.7	33.6 ± 2.6
Ac-CoA*	8.5 ± 1.5	5.3 ± 1.3	5.2 ± 0.7	5.6 ± 0.5
Ac-carn	2.0 ± 0.7	0.5 ± 0.1	1.1 ± 0.3	0.6 ± 0.1
Lactate	4.4 ± 0.8	4.9 ± 0.8	4.0 ± 0.5	4.1 ± 0.3
Pyruvate	0.43 ± 0.1	0.38 ± 0.1	0.29 ± 0.1	0.37 ± 0.1

CHAPTER 6

DISCUSSION

The primary purpose of this study was to examine the response of human skeletal muscle PDK and PDH activity to 28d supplementation with 1000 mg of (R+)-lipoic acid to determine if it could contribute to enhanced muscle glucose clearance. The anti-diabetic effects of lipoic acid supplementation in regards to blood glucose and plasma insulin had been demonstrated previously in humans [49, 51, 50, 61]. However, in the present study an important finding was that lipoic acid supplementation was limited in its ability to increase whole-body insulin sensitivity in sedentary human subjects, and a heterogeneity of responses were observed. Following supplementation, no changes were identified in non-responding subjects. However, in responding subjects, the POST AUC for both blood glucose and plasma insulin concentrations decreased, although this was not statistically significant. Further, this coincided with a trend towards decreased PDK activity POST lipoic acid. This was accompanied by PDHa activity that was lower prior to the OGTT following lipoic acid treatment but increased above PRE conditions during the OGTT, suggesting that increased PDHa activity contributed to increased glucose disposal and was activated more readily following an oral glucose load, but only in a portion of the subjects.

Responding versus non-responding subjects

Previous studies examining the effects of lipoic acid have consistently reported homogenous results amongst subjects. In the present study, it is difficult to explain why there were inconsistent results among the subject pool. The subjects recruited were

chosen to represent a homogeneous group, in that they were male, between 18 and 35 years of age and relatively inactive (< 3 times·wk^{-1}). While relative differences existed between individual subjects, when examined as responders versus non-responders, these variations were negligible. No statistical differences were demonstrated when subject groups (responders vs. non-responders) were examined for differences in PRE lipoic acid CS activity (11.73 ± 2.30 vs. 12.32 ± 1.97 μmol·min·g w.w.) and basal total PDK activity (0.137± 0.02 vs. 0.131 ± 0.02 min^{-1}). Given these findings, it must be assumed that the reason for the divergent responses is not due to skeletal muscle oxidative capacity.

However, while all subjects consumed 1000 mg R-(+)-lipoic acid per day for 28d, due to individual differences in body mass amongst the subject population, the dose of lipoic acid in milligrams (mg) per kilogram (kg) varied between subjects. The range of dose per/kg fluctuated from 8.33 mg/kg to 13.52 mg/kg where the average was 12.08 ± 0.6 mg/kg. The average dose for the RSP was 12.77 ± 0.39 mg/kg while, the average dose for the N-RSP was 10.94 ± 1.01 mg/kg, however, this difference was not significant.

Variations in the level of consumption of lipoic acid through dietary sources was also examined, although no significant differences were noted between RSP and N-RSP.

Insulin sensitivity

The AUC for blood glucose and plasma insulin concentrations during the OGTT appeared to decrease for the responding subjects, however this was not demonstrated in the non-responding subjects.

The majority of an oral glucose load (approx 71%) is taken up by skeletal muscle, as illustrated using a direct measurement of leg glucose uptake using femoral venous catheterization [55]. The increase in insulin sensitivity seen amongst the responders

would suggest that there was a greater insulin-stimulated skeletal muscle glucose disposal following treatment. Therefore, subsequent to lipoic acid supplementation in responding subjects, keeping the oral glucose load consistent, the responding subjects required less insulin and cleared the blood glucose more readily. Therefore, consistent with previous reports that lipoic acid appeared to enhance glucose disposal, at least in the responding subjects. This could be due to increased glucose uptake, increased glycogen storage and/or increased glucose disposal through oxidation [49, 107, 31]. In this study we focused on changes in glucose oxidation through PDH in these subjects.

In the present study, the primary focus of lipoic acid was on its effects on skeletal muscle. However, it is also important to consider the involvement of the liver in determining the blood glucose level during the OGTT. Through previous research it has been demonstrated that, in healthy subjects after an overnight fast, hepatic glucose accounted for 25% of the disposal of an oral glucose load [55] and hepatic glucose output was suppressed by 50% during an OGTT [28]. Thus, in addition to an increase in muscle glucose uptake, factors that may contribute to a decreased AUC for blood glucose concentration following lipoic acid, are an increase in hepatic glucose uptake or a decrease in hepatic glucose output. Anderwald et al. [2] used isolated livers of fasted rats treated with lipoic acid and demonstrated a decrease in hepatic glucose production and lactate uptake, an increase in liver glycogen content, in addition to a transient increase in bile flow [2]. Therefore, although hepatic glucose output and uptake were not measured in the present study, they cannot be fully discounted as contributing factors to the improved glucose clearance. Subsequent research must be considered to fully understand hepatic contribution to the effects of lipoic acid on blood glucose homeostasis in humans.

PDK activity

Following lipoic acid supplementation, no changes in PDK activity were illustrated in the complete subject pool (n=9), however, a trend towards an overall decrease in fasting PDK activity as well during the OGTT was observed (p=0.0009); n=4). The blunting of PDK activity may be one mechanism involved in the increased disposal of an oral glucose load following lipoic acid supplementation. However, due to the fact that these results were limited to responding subjects only, it is difficult to make this conclusion. This finding is supported by recent research using an in vitro purified protein system. [62]. When synthesized PDH components were reconstituted into a complex, the observed decrease in PDK activity was suggested to be due to competitive binding of PDK to the E2 core. Previous research has demonstrated that E2 binding increases PDK activity thus inactivating the PDH complex [66, 127]. During the binding of PDK to the lipoyl domain of E2, a conformational changed occurs in PDK resulting in its activation, however in the presence of lipoic acid, free lipoic acid is able to occupy the binding site on PDK of lipoyl moiety of the lipoyl domain of E2. Therefore, E2, in the absence of the lipoyl domain would not allow for PDK activation and could cause inhibition. The inhibitory effect of lipoic acid on PDK would result in less phosphorylation of E1 and thereby allow for greater PDH activity [62]. Therefore, the decrease in PDK activity following lipoic acid in responding subjects could offer *in vivo* support to this *in vitro* observation.

While it was not quantified in the present study, alterations in PDK activity may be the result of changes in protein levels as the time course for the study took place over

28 days. Research conducted in Pima Indians has illustrated that the expression of PDK isoforms was correlated with the degree of severity of a subject's insulin resistance [70]. It was shown that insulin had a direct effect in the expression of PDK2 and 4, suggesting that in insulin-resistant individuals, there may be insufficient insulin-mediated downregulation PDK. Therefore, the trend towards an increase in insulin sensitivity demonstrated following lipoic acid supplementation in the responding subjects of the present study could account for decreased PDK expression and/or activity.

This agrees with previous research, which demonstrated that, a stable increase in PDK in rat skeletal muscle in starvation and diabetes is correlated with a decrease in circulating insulin concentrations (or in insulin sensitivity) [79].

PDHa activity

The mechanism(s) responsible for previously observed reductions in blood glucose and plasma insulin in prior studies were not fully understood. Possible explanations have suggested altered regulation of PDH activation.

An important trend in this study was an apparent decrease in POST PDHa activity prior to the oral glucose load below PRE conditions. This was demonstrated in all subjects. This was is contrast to our hypothesis, as it was expected that fasting PDHa activity would be higher following supplementation.

PDH, as mentioned previously, is subject to regulation by PDK activity, whereby the inhibition of PDK allows for activation and thus a greater flux through PDHa. Therefore, in the present study, one would then anticipate the apparent decrease in PDHa activity to be associated by an increase in total PDK activity. However, this was not

observed. Therefore in the basal POST lipoic acid condition, acute regulators must dictate PDHa activity.

PDH activity is acutely regulated covalently through the PDP/PDK-dependent phosphorylation state, which will be examined separately for possible contributions to this acute regulation [112]. PDP mediated dephosphorylation and activation of PDH is modulated by insulin and Ca^{2+} concentrations. PDP1 is sensitive to changes in muscle Ca^{2+} concentration, we would expect that this remained unchanged through the study and therefore would not contribute to differences in PDHa activity in the basal state. However recent evidence from animal work suggests that insulin stimulates PDP2 in rat heart and kidney, which activates the complex [46]. It should be noted that while the PDP1 concentration is more prominent in skeletal muscle, there is evidence to support the notion that insulin is able to activate the complex in a similar manner [27]. Plasma insulin concentrations were similar in the basal condition following the 12h fast. If lipoic acid had increased muscle insulin sensitivity, meaning that the effect of a given insulin concentration was greater, it would be anticipated that this would increase PDP2 activity and therefore stimulate PDHa activity. However, this is contrary to what was observed since basal PDHa activity was lower POST lipoic acid than before supplementation.

While PDK2 activity is sensitive to the ratio of acetyl CoA/CoA, this remained relatively stable throughout the study. Muscle pyruvate however, appeared to decrease prior to the OGTT following lipoic acid ($p=0.16$; $n=9$). PDK2 is sensitive to pyruvate inhibition, therefore it would be expected that a decrease in pyruvate would allow for a greater activation of PDHa, however this was not demonstrated.

In the present study, PDHa activity appeared to increase to a greater extent during the OGTT following lipoic acid supplementation in the four responding subjects. In addition, the difference between the two time points (0 and 75 min) was greater POST lipoic acid (0.79 ± 0.26 mmol·min^{-1}·kg) versus PRE (0.01 ± 0.29 mmol·min^{-1}·kg). The trend towards an increase in PDHa activity was in accordance with our hypothesis, as it was anticipated that PDHa activity would increase in response to the demands of oxidizing the oral glucose load. This would contribute to the increase glucose clearance in these subjects and the decreased AUC for plasma insulin and blood glucose.

Further, the increase in PDHa activity is in parallel with the decrease in total PDK activity noted previously. With the inhibitory action of PDK suppressed, PDHa activation is able to prevail.

In terms of the influence of PDP activity on PDHa activity during the OGTT, although muscle Ca^{2+} concentrations would remain stable throughout the trial, the influence of the increased insulin would have profound effects on PDP activity. The increase in insulin sensitivity following lipoic acid would activate PDP activity and thus stimulate PDHa activity, as seen in the present study.

To fully understand the increase in PDHa activity seen POST lipoic acid following the oral glucose load, one must examine the potential influence of PDK activity in response to allosteric regulators. Mentioned previously, PDK2 is sensitive to the ratio of acetyl CoA/CoA, this remained relatively stable throughout the study, therefore its influence can be minimized. Unfortunately, while PDK4 activity is sensitive to changes in the ratio of NADH/NAD^{+}, it is not possible to measure alterations in mitochondrial changes in redox with current technology. The importance of any fluctuations in

NADH/NAD$^+$ following supplementation would be critical to understanding of the mechanism(s) behind lipoic acid. Both lipoic acid and its reduced counterpart, DHLA have been effective against oxidative stress [76]. It has been suggested that lipoic acid is transported across the mitochondrial membrane for reduction and subsequently imported into the mitochondrial compartment as DHLA, at the expense of mitochondrial-reducing equivalents such as NADH and NADPH [38, 103]. Therefore, as the reduction of lipoic acid has been found to be NADH-dependent, it would be anticipated that NADH/NAD$^+$ would be reduced following lipoic acid treatment. A decrease in mitochondrial NADH/NAD+ would increase PDHa activity through a decrease in PDK4 activity. It would be enlightening to measure the ratio of NADH to NAD$^+$, as it is fundamental in both the regulation of PDK4 as well as determining the uptake and reduction of lipoic acid, and hence its ability to activate PDH.

Plasma FFA concentration following lipoic acid treatment

In the present study, plasma FFA concentration were not altered significantly following lipoic acid supplementation. In the responding subjects, it would be anticipated that there would be a decrease in FFA concentrations following lipoic acid supplementation, as an improvement in insulin sensitivity was noted in these individuals following supplementation. It has been demonstrated that during a euglycemic hyperinsulinemic clamp with infused intralipid during the hyperinsulinemia, elevated plasma FFA and induced a state of insulin insensitivity [10, 56]. Similarly Groop et al. [33] infused human subjects with ^{14}C palmitate to trace plasma FFA metabolism with indirect calorimetry in order to quantify total body lipid oxidation. In diabetic and obese

subjects, the ability of both physiological and pharmacological levels of insulin to suppress FFA levels and to inhibit total FFA turnover, and non-oxidative disposal (reesterification) was impaired. The elevated rates of FFA/lipid oxidation showed a strong inverse correlation with the decreased rates of basal and insulin-stimulated glucose oxidation [33]. Further, Sargard et al. (2005) noted that a decrease in plasma FFA is associated with an increase in insulin sensitivity, whereas raising plasma FFA levels decrease insulin sensitivity in a dose-dependent fashion [102]. Sargard et al., [102] continued to assert that a decrease in plasma FFA is likely related to improvements in insulin sensitivity [102].

Further, Boden et al. [10] illustrated that an increase in FFA is associated with an increase in intramyocellular triglycerides and a subsequent decrease in insulin sensitivity [10]. As recently reviewed by Hulver and Dohm [47] recent work suggests that fatty acyl-CoA, ceramides and diacylglycerols accumulates in diabetic and insulin resistant models and directly alter various aspects of the insulin-signalling pathway [47]. However, the reason for an accumulation of intramyocellular fatty acyl-CoA and diacylglycerol are currently unknown as it appears that reduced fatty acid oxidation is not responsible, as fatty acyl-CoA continues to accumulate in skeletal muscle with a normal fatty acid oxidative capacity [47]. Although the present study did not measure intramuscular fats, this accumulation of lipid correlated with higher plasma FFA. Therefore, the apparent decrease in plasma FFA in the responding subjects could suggest a link to lipid-induced changes in muscle sensitivity.

Future Studies

Future research should be aimed at examining measures of plasma and muscle concentrations of lipoic acid in study populations, both pre- and post-supplementation. Previous studies have demonstrated that following supplementation, lipoic acid is able to be taken up into the muscle and is detectable via high performance liquid chromatography [119, 117, 54, 36]. Unfortunately, such methods have not been used in a manner to explain the potential for a heterogeneity of response. The notion that lipoic acid has the ability to be taken up and incorporated into human systems has not been debated nor was it the focus of the present study. However, measurements of lipoic acid concentrations would have allowed for a greater insight into potential differences between the responders and non-responders.

Differences in muscle oxidative metabolism have been noted previously between the genders [98]. The present study focused exclusively on PDK and PDHa activity in male subjects only. It is not clear whether there are gender differences with respect to changes in PDK and PDHa activity in response to lipoic acid supplementation. The PDH complex plays a pivotal role in regulating oxidative carbohydrate disposal, and its regulation by PDK through lipoic acid perturbations could vary between genders.

Further, the fact that the subjects who comprised this study were of varying ethnicity may have contributes to the heterogeneity of the findings. A greater incidence of type II diabetes and impaired glucose metabolism are more prevalent among ethnic populations such as the Pima Indians of Arizona discussed previously as well as migrant Asian Indians (individuals from Pakistan, India and Bangladash) [70, 1]. While it is not know whether the greater incidence of type II diabetes arises from a westernization of

lifestyle with dietary changes and lack of exercise, it has been noted that through various epidemiological studies, that these factors alone are not sufficient enough to explain this trend and a genetic factor may play a role [1]. Researching ethnic differences in PDK activity and isoform expression between population following lipoic acid supplementation may contribute to a better understanding of type II diabetes and its effects.

Previous studies have examined the effect of lipoic acid supplementation in humans over various time frames, ranging from one week to six, all of them demonstrating evidence of increased insulin sensitivity [49, 51, 61]. The current study took place over 28 days of lipoic acid supplementation and within that time frame we observed divergent results. Future studies could examine the time course of the effect of lipoic acid on insulin sensitivity and blood glucose homeostasis. It would be interesting to examine the responding subjects after 14 days of treatment to see if the benefits of lipoic acid could be identified in as quickly as 14 days. Conversely, future research could focus on the non-responding subjects to see whether they are immune to the effects of lipoic acid or if more time of supplementation (i.e. six weeks) would be necessary to see any alterations.

Finally, it would be valuable to determine how transient the changes demonstrated in the responders were. Recovery studies could examine the responders immediately POST lipoic acid supplementation and then again several days later to identify how lasting the benefits of lipoic acid supplementation were, and to illustrate the time course of reversal.

Summary and Conclusions

This study examined the PDH response to 28 days of 1000 mg R(+)-lipoic acid supplementation in human skeletal muscle. In contrast to previous literature, improvements in glucose clearance and plasma insulin concentrations in response to an OGTT were not homogenous following lipoic acid supplementation. Although, no statistical changes were noted amongst the entire subject population, once subjects were delineated as responders or non-responders according to their improvements in the insulin AUC during the oral glucose load following treatment, several trends began to emerge. In agreement with our hypothesis, improved glucose clearance following lipoic acid treatment appeared to be associated with alterations in PDH and PDK activity. Contrary to our hypothesis, there was a trend towards decreased PDHa activity following an overnight fast after lipoic acid supplementation in all responding subjects. However, total PDK activity was lower in these subjects after treatment, and therefore basal regulation appears to be mediated through increased insulin sensitivity and decreased muscle pyruvate concentration. However, during the OGTT, increased blood glucose clearance in responding subjects was associated with increased activation of PDHa activity and decreaed PDK activity. These results suggest that improved glucose clearance during an OGTT following lipoic acid supplementation is assisted by increased muscle glucose oxidation through increased PDHa activation and decreased PDK activity in certain individuals.

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APPENDIX

BLOOD RESULTS over time, during OGTT (-30 to 180-min)

1. Blood glucose, mmol/l

PRE		-30	0	30	60	90	120	150	180
	1	4.82		9.33	5.77	4.22	3.47	4.80	5.30
	2	4.15	4.08	5.15	4.85	3.46	4.06	4.60	2.89
	3	4.83	5.55	7.83	7.73	4.65	3.98	5.27	3.94
	4	4.18	6.99	12.21	9.39	7.66	7.81	4.28	4.30
	5	6.05	5.47	9.07	5.47	3.02	5.47	5.33	5.47
	6	4.16	4.51	8.54	6.52	4.53	3.96	3.53	3.68
	7	4.93	4.92	6.59	4.67	4.70	4.40	5.15	4.48
	8	6.46	6.44	7.92	9.95	7.43	7.33	5.00	
	9	5.67	5.65	9.01	10.15	8.41	8.69	8.02	5.26
	MEAN	5.03	5.45	8.41	7.17	5.34	5.46	5.11	4.42
	SE	0.30	0.34	0.69	0.78	0.69	0.69	0.43	0.34

POST		-30	0	30	60	90	120	150	180
	1	4.57	6.65	6.39	6.03	4.67	4.44	3.92	4.46
	2	5.30	4.50	6.61	5.31	6.56	5.81	5.45	3.74
	3	3.21	4.36	5.25	3.24	2.43	3.78	3.66	3.32
	4	5.08	5.22	7.16	5.72	3.57	4.68	3.99	3.89
	5	4.81	5.83	10.45	9.46	6.78	7.51	9.86	7.63
	6	4.60	4.75	8.81	6.52	5.56	6.32	4.88	4.01
	7	5.09	5.64	8.24	5.37	4.10	4.64	4.41	4.23
	8	6.50	6.00	12.71	13.09	9.55	7.42	5.28	4.57
	9	5.52	6.58	10.41	11.53	9.28	10.21	9.46	7.19
	MEAN	4.96	5.50	8.45	7.36	5.83	6.09	5.66	4.78
	SE	0.31	0.30	0.85	1.15	0.87	0.72	0.83	0.54

2. Plasma Insulin, uIU/ml

PRE		-30	0	30	60	90	120	150	180
	1	3.42	3.32	62.33	30.58	40.72	10.22	3.80	4.13
	2	10.80	6.51	135.32	112.42	120.96	76.18	11.82	8.59
	3	3.70	4.20	69.97	44.42	24.82	18.50	19.03	4.34
	4	4.19	4.62	27.70	69.62	28.36	25.26	13.15	4.38
	5	8.30	6.68	125.27	39.56	41.62	24.64	20.65	10.57
	6	3.86	4.56	25.99	27.45	24.59	30.99	5.44	5.66
	7	6.65	8.46	78.44	53.87	33.50	23.15	13.61	11.82
	8	7.76	9.11	65.11	89.60	41.31	86.28	24.04	
	9	8.07	15.29	103.21	162.84		219.37	152.08	71.30
	MEAN	6.31	6.97	77.04	70.04	44.49	57.18	29.29	15.10
	SE	0.93	1.30	13.61	15.82	11.99	23.43	16.45	8.66

POST		-30	0	30	60	90	120	150	180
	1	3.42	3.32	41.80	29.61	14.20	10.17	3.93	3.93
	2	7.21	8.39	64.04	86.93	33.15	26.12	10.85	8.16
	3	4.53	6.68	70.92	30.80	25.45	19.00	7.24	4.79
	4	3.15	2.83	41.80	42.90	23.91	7.79	4.60	3.05
	5	7.07	8.66	185.25	112.44	26.50	18.69	50.24	7.79
	6	3.61	5.35	57.70	50.52	37.52	18.59	21.05	6.26
	7	5.88	8.74	59.69		19.33	15.23	23.29	8.94
	8	4.22	6.32	73.81	91.05	86.05	120.65	24.03	6.62
	9	7.82	15.12	101.23	123.39	143.94	173.04	150.98	77.57
	MEAN	5.21	7.27	77.36	70.96	45.56	45.48	32.91	14.12
	SE	0.64	1.29	15.65	14.04	15.02	20.92	16.46	8.44

3. FFA, mmol/l

PRE		-30	0	30	60	90	120	150	180
	1	0.70	0.76	0.40	0.23	0.23	0.38	0.46	0.59
	2	0.50	0.53	0.34	0.23	0.19	0.13	0.13	0.18
	3	0.72	0.69	0.54	0.24	0.15	0.13	0.10	0.19
	4	0.25	0.32	0.19	0.17	0.14	0.09	0.22	0.15
	5	0.24	0.25	0.21	0.12	0.09	0.11	0.13	
	6	0.40	0.44	0.44	0.30	0.21	0.16	0.14	0.21
	7	0.21	0.31	0.16	0.12	0.10	0.07	0.08	0.11
	8	0.21	0.25	0.18	0.11	0.12	0.13	0.11	0.13
	9	0.48	0.45	0.26	0.12	0.21	0.09	0.09	0.09
MEAN		0.41	0.44	0.30	0.18	0.16	0.14	0.16	0.21
SE		0.07	0.07	0.05	0.02	0.02	0.03	0.04	0.06

POST		-30	0	30	60	90	120	150	180
	1	0.41	0.52	0.22	0.20	0.22	0.27	0.34	0.43
	2	0.50	0.41	0.34	0.31	0.21	0.12	0.15	0.18
	3	0.36	0.38	0.36	0.17	0.11	0.08	0.11	0.21
	4	0.45	0.36	0.22	0.16	0.14	0.10	0.11	0.17
	5	0.21	0.25	0.18	0.11	0.12	0.13	0.11	0.13
	6	0.27	0.37	0.24	0.17	0.14	0.13	0.14	0.17
	7	0.20	0.22	0.14	0.09	0.06	0.07	0.07	0.07
	8	0.40	0.54	0.40	0.15	0.07	0.06	0.06	0.15
	9	0.41	0.40	0.22	0.14	0.12	0.07	0.08	0.13
MEAN		0.36	0.38	0.26	0.17	0.13	0.11	0.13	0.18
SE		0.04	0.04	0.03	0.02	0.02	0.02	0.03	0.04

4. Lactate, mmol/l

PRE		-30	0	30	60	90	120	150	180
	1	1.02	0.51	1.87	1.75	1.48	1.87	1.79	1.13
	2	0.86	1.21	1.09	1.13	1.60	1.37	1.09	1.48
	3	0.99	1.30	0.69	0.12	1.03	1.94	0.84	2.40
	4	1.18	2.06	1.75	2.70	2.02	2.13	1.87	1.68
	5	0.87	1.42	1.20	2.10	0.68	1.24	0.87	1.35
	6	1.83	0.98	1.24	1.80	1.98	2.06	1.57	1.54
	7	1.59	2.10	3.16	2.29	2.73	1.35	1.51	2.41
	8	2.92	1.86	1.74	2.80	2.02	2.14	1.74	
	9	2.99	2.04	1.96	2.28	1.92	2.99	2.00	1.68
MEAN		1.58	1.50	1.63	1.89	1.72	1.90	1.48	1.71
SE		0.30	0.20	0.25	0.30	0.21	0.19	0.15	0.18

POST		-30	0	30	60	90	120	150	180
	1	0.94	1.79	1.09	1.13	1.60	1.37	1.09	1.48
	2	0.86	1.25	1.44	2.10	2.42	1.13	2.65	1.83
	3	1.41	1.34	1.53	1.30	1.49	1.53	1.41	1.45
	4	1.87	2.13	1.94	2.13	1.98	1.87	1.83	1.56
	5	1.28	1.54	1.95	1.72	1.65	0.87	1.35	1.57
	6	1.24	1.05	1.69	2.17	1.83	2.21	3.07	2.13
	7	1.67	1.82	3.32	2.33	1.82	2.02	2.22	2.88
	8	1.19	1.70	2.53	4.61	3.32	2.53	2.22	1.74
	9	1.49	3.78	1.92	2.48	2.16	2.36	1.56	5.53
MEAN		1.33	1.82	1.93	2.22	2.03	1.76	1.93	2.24
SE		0.11	0.28	0.23	0.36	0.20	0.20	0.23	0.46

MUSCLE RESULTS

1. PDK activity, min $^{-1}$

	PRE 0	PRE 75	POST 0	POST 75
1	0.101	0.084	0.046	0.045
2	0.185	0.185	0.145	0.143
3	0.158	0.136	0.082	0.083
4	0.116	0.130	0.080	0.063
5	0.088	0.137	0.146	0.154
6	0.116	0.074	0.079	0.066
7	0.147	0.060	0.165	0.113
8	0.187	0.188	0.137	0.139
9	0.113	0.133	0.244	0.162
MEAN	0.135	0.125	0.125	0.108
SE	0.013	0.016	0.021	0.016

2. PDHa activity, mmol acetyl CoA $kg^{-1}min^{-1}$ (corrected to total muscle Cr)

	PRE 0	PRE 75	POST 0	POST 75
1	1.415	0.853	0.733	2.035
2	1.173	0.902	0.244	1.237
3	0.233	0.493	0.299	0.805
4	0.477	1.010	0.411	0.749
5	2.005	1.410	1.044	0.665
6	0.686	0.938	0.645	1.164
7	0.950	1.050	0.740	1.208
8	0.997	1.365	0.701	1.138
9	0.780	1.131	1.108	1.330
MEAN	0.968	1.017	0.658	1.148
SE	0.186	0.106	0.106	0.145

Muscle Metabolites

1. ATP, mmol/kg dm

	PRE 0	PRE 75	POST 0	POST 75
1	24.52	25.74	18.23	17.27
2	20.20	21.75	18.56	30.80
3	23.96	21.49	27.45	27.62
4	23.42	24.75	18.24	24.01
5		24.03	19.21	19.67
6	32.78	23.93	25.59	22.81
7	22.46	34.19	23.53	28.30
8	21.50	16.19	19.17	23.77
9	24.38	22.61	19.86	19.86
MEAN	24.15	23.85	21.09	23.79
SE	1.43	1.68	1.24	1.58

2. PCr, mmol/kg dm

	PRE 0	PRE 75	POST 0	POST 75
1	59.25	59.25	55.67	55.83
2	59.25	73.58	75.53	44.76
3	32.88	51.11	33.53	48.35
4	72.70	82.63	74.68	89.26
5		63.81	59.74	63.16
6	27.35	78.46	57.95	78.79
7	65.44	53.72	16.93	48.83
8	48.67	52.25	43.63	62.02
9	57.95	63.00	69.34	56.32
MEAN	52.94	64.20	54.11	60.81
SE	5.94	4.11	6.94	5.21

3. TCr, mmol/kg dm

	PRE 0	PRE 75	POST 0	POST 75
1	91.08	92.74	94.16	91.73
2	84.05	108.73	110.87	74.55
3	54.72	87.93	70.72	75.18
4	102.12	118.90	122.98	125.71
5		94.71	96.75	108.68
6	49.37	131.01	100.14	115.05
7	116.69	65.74	47.64	71.04
8	79.20	85.56	78.23	100.51
9	83.67	95.01	106.91	87.59
MEAN	82.61	97.81	92.04	94.45
SE	8.47	6.83	8.14	6.86

4. Ac-CoA

	PRE 0	PRE 75	POST 0	POST 75
1	12.86	4.61	6.30	5.19
2	3.66	4.94	4.72	6.62
3	11.79	15.10	9.38	8.67
4	8.46	3.75	5.37	3.85
5		4.07	2.03	5.07
6	5.66	3.56	4.27	4.13
7	3.05	5.67	5.84	5.53
8	12.06	2.83	4.89	5.36
9	10.55	3.04	4.23	6.22
MEAN	8.51	5.29	5.23	5.63
SE	1.48	1.34	0.70	0.51

5. Ac-Carn

	PRE 0	PRE 75	POST 0	POST 75
1	4.65	0.45	1.04	0.33
2	0.6	0.21	0.42	0.68
3	5.37	0.33	3.07	1.3
4	1.12	1.12	1.36	0.51
5		0.24	0.63	0.39
6	0.84	0.56	0.86	0.43
7	0.7	0.47	0.55	0.29
8	1.43	0.43	1.59	0.41
9	1.56	0.39	0.34	1.05
MEAN	2.10	0.48	1.19	0.54
SE	0.76	0.10	0.30	0.12

6. Lactate, mmol/kg dm

	PRE 0	PRE 75	POST 0	POST 75
1	3.47	5.76	3.86	3.14
2	2.31	2.12	2.89	4.06
3	2.80	4.02	2.37	6.41
4	5.84	5.04	5.69	4.75
5		3.69	3.55	3.05
6	5.07	3.05	4.21	3.26
7	2.72	10.05	6.28	4.47
8	5.11	4.71	4.32	4.67
9	8.47	4.14	4.18	4.12
MEAN	4.48	4.73	4.15	4.21
SE	0.53	0.85	0.47	0.40

7. Pyruvate, mmol/kg dm

	PRE 0	PRE 75	POST 0	POST 75
1	0.11	0.22	0.10	0.12
2	0.17	0.15	0.08	0.00
3	0.55	0.38	0.32	0.89
4	0.07	0.09	0.08	0.10
5		0.21	0.35	
6	0.62	0.22	0.17	0.28
7	0.38	0.73	0.77	0.56
8	0.94	0.89	0.66	0.56
9	0.61	0.55	0.10	0.45
MEAN	0.43	0.38	0.29	0.37
SE	0.11	0.10	0.09	0.11