EFFECTS OF REDUCED FREE FATTY ACID AVAILIBILITY ON HORMONE-SENSITIVE LIPASE ACTIVITY IN HUMAN SKELETAL MUSCLE DURING AEROBIC EXERCISE

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ABSTRACT

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This study used nicotinic acid (NA) to examine the effects of reduced free fatty acid availability on hormone sensitive lipase (HSL) activity in skeletal muscle during aerobic exercise. Eleven recreationally active males exercised at 55% VO_2max for 40 min following supplementation with NA or not (Control). Muscle biopsies were taken at rest and 5, 20, and 40 min of exercise. NA had no effect on HSL activity, despite increased plasma epinephrine concentrations and an apparent increase in IMTG oxidation. In both trials, HSL activity increased ~30% by 5 min, however did not increase further for the duration of the exercise. When respiratory exchange ratio (RER) was examined it appeared that some subjects were able to increase their IMTG oxidation in the NA trial, whereas others were not. Interestingly this ability to increase IMTG oxidation did not correlate with increased HSL activity. We concluded that HSL activation is only one step in the regulation of IMTG lipolysis and that, as in adipose tissue, other factors distal to this determine the actual rate of TG breakdown.

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Sincerely,
Marcus O'Neill

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ATP	adenosine triphosphate	**IMTG**	intramuscular triacylglycerol
ADP	adenosine diphosphate	**LCFA-CoA**	long chain fatty acyl – coenzyme A
AMP	adenosine monophosphate	**min**	minutes
AMPK	5'-AMP-activated protein kinase	**MG**	monoacylglycerol
bm	body mass	**MOME**	total neutral lipase concentration
Ca^{2+}	calcium	**MRS**	magnetic resonance spectroscopy
CaMKII	calmodulin-dependent kinase II	**NA**	nicotinic acid
CAMP	cyclic AMP	**PCr**	phosphocreatine
CHO	carbohydrate	**P_i**	inorganic phosphate
Cr	creatine	**PKA**	protein kinase A
dm	dry mass	**RER**	respiratory exchange ratio
EDL	extensor digitorum longus	**TG**	triacylglycerol
EMCL	extramyocellular lipids	**TO**	neutral lipase activity
ERK	extracellular signal-related kinase	**VCO_2**	volume of carbon dioxide output
FFA	free fatty acids	**V_E**	expired ventilation
G-6-*P*	glucose-6-phosphate	**VO_2**	volume of oxygen uptake
HSL	hormone-sensitive lipase	**VO_2max**	maximum oxygen uptake
HSLa	hormone-sensitive lipase activity		

CHAPTER 1 - INTRODUCTION

During aerobic exercise the major sources of fuel for skeletal muscle are carbohydrate (CHO) and fat. Mammals can obtain CHO and fat from both extra- and intramuscular sources. CHO is stored in muscle in the form of glycogen. Skeletal muscle can also uptake CHO in the form of glucose from the blood. Blood glucose levels are maintained by the release of glucose from the breakdown of liver glycogen or from the ingestion of CHO. The largest store of fat is found in adipose tissue, which is stored as triacylglycerol (TG). During exercise, hormonal and neural signals result in TG to be broken down into free fatty acids (FFA) and glycerol, which are released into the blood. The FFA are then transported to the muscle for uptake and oxidation. Also found in the blood are circulating TG, in the form of very-low density lipoproteins and chylomicrons, which can be taken up and oxidized by muscle. However, during exercise, circulating TG are only thought to minimally contribute to total fat oxidation. Lastly, a pool of TG exists inside the muscle (IMTG), which can be metabolized for energy. IMTG exists as lipid droplets that are generally located in close proximity to the mitochondria.

Several factors, including exercise intensity and duration, and training status of the individual determine the relative contribution of CHO and fat oxidation during exercise. In general, as one moves from a lower intensity to a higher intensity exercise the amount of CHO used for ATP provision increases. Conversely, fat tends to be the predominant energy source at low-moderate intensity exercise, with the maximal relative contribution occuring at ~ 65% VO_2max (Achten et al., 2002; Romijn et al., 1993). The contribution of fat to total energy provision decreases as exercise increases to higher

intensities. Exercise duration plays an important role in fuel selection. At the onset of exercise, CHO tends to be the major source of fuel. However, as the exercise duration increases fat becomes the dominant fuel source (Watt et al., 2002a). Training status is also a major determinant in fuel selection. Numerous studies have shown that endurance exercise training enhances the capacity of muscle to oxidize fat through an upregulation of fat metabolizing enzymes and improved cellular machinery (Holloszy and Coyle 1984; Saltin 1986).

Within the last two decades a tremendous amount of work has been done regarding the regulation of CHO metabolism. This has led to a great understanding of the metabolic processes involved in the utilization of CHO during exercise. There is far less information available regarding the role of fat during exercise. Fat oxidation appears to have multiple sites of regulation, including 1) adipose lipolysis and FFA delivery to the muscle, 2) transport of FFA across the muscle membrane, 3) IMTG lipolysis, and 4) transport of FFA across the mitochondrial membrane. Similar to the regulation of CHO oxidation, various extra- and intracellular signals are thought to be involved in the regulation of fat oxidation. These signals can include the sympathetic nervous system (neural), catecholamines and insulin (hormonal), Ca^{2+}, the energy state of the cell (ADP, AMP and P_i) and the redox potential of the cell (NAD, NADH). Clearly, the regulation of fat metabolism is complex and more study is required to fully elucidate the mechanisms of these potentially important regulators of fat metabolism during endurance exercise.

There have been numerous attempts to quantify the relative importance of the various sources of fat during exercise. Unfortunately, there have been conflicting reports

as to whether IMTG plays an important role in energy provision during exercise. As will be discussed later, Watt et al., (2002b) pointed out that this discrepancy is likely due to methodological limitations associated with the measurement of IMTG rather than a true reflection of its use as a substrate during exercise. Today, it is generally accepted that IMTG represents an important source of energy during endurance exercise, but a controversy still exists regarding the relative contribution of IMTG to total fat oxidation.

There is also little known of the regulation of IMTG lipolysis in human skeletal muscle. Recently, hormone-sensitive lipase (HSL) has been identified as the rate-limiting enzyme responsible for the breakdown of IMTG (Holm et al., 1987; Langfort et al., 1999). In an attempt to gain insight into the regulation of IMTG lipolysis, the purpose of this study was to examine the effects of reduced FFA availability on the activity of HSL, and to correlate HSL activity with whole body fat oxidation during aerobic exercise. The statement of the problem and a list of the hypotheses are given at the end of the literature review.

CHAPTER 2 - LITERATURE REVIEW

MUSCLE TRIACYLGLYCEROL CONTENT

Human skeletal muscle

Biochemical measurement of IMTG content obtained from muscle biopsy samples of the vastus lateralis, have reported that human males possess ~20-40 mmol/kg dm IMTG (Kiens et al., 1993; Kimber et al., 2003; Starling et al., 1997; Watt et al., 2002b; Wendling et al., 1996), which corresponds to ~ 70-100% of the energy available from muscle glycogen (Van der Vusse and Reneman, 1996). More recent work has used proton magnetic resonance spectroscopy (^{1}H-MRS) in an attempt to quantify IMTG content. Most studies have reported a lower IMTG content (~7-20 mmol/kg dm) than reported in the biopsy studies (Descombaz et al., 2000 & 2001; Howald et al., 2002; Hwang et al., 2001; Krssak et al., 2000). However, other studies report similar IMTG content (~22-45 mmol/kg dm) to the biopsy studies (Rico-Sanz et al., 1999 & 2000). This discrepancy is likely due to differences between laboratories in the calibration of the signal obtained from IMTG. Also, in contrast to biopsy studies, in which the vastus lateralis is almost always sampled, most ^{1}H-MRS studies have examined muscles of the lower leg, including tibialis anterior, soleus, gastrocnemius, and tibialis posterior. Regardless of the measurement used to measure IMTG, it nonetheless represents a significant store of energy and, as such, has the potential to be an important source of fuel during exercise.

When individual muscle fibres are examined using biochemical analysis it has been observed that type I muscle fibres possess the greatest concentrations of IMTG (Essen, 1977, Essen et al., 1977). Correspondingly, type IIa has an intermediate amount

of IMTG and type IIb has the least amount (Essen, 1977, Essen et al., 1977). Similarly, using ^{1}H-MRS Rico-Sanz et al. (1999 & 2000) reported that muscle possessing a higher proportion of oxidative fibres (soleus) had a higher resting IMTG content than muscle containing a higher proportion of glycolytic fibres (tibialis anterior). This pattern of IMTG distribution would seem to be advantageous, as oxidative fibres rely more on fat as a source of energy.

Non-human skeletal muscle

Dog muscle has comparable IMTG content to humans, but monkey and rat muscle contains a smaller amount of IMTG (for review see Gorski 1992). The differences in IMTG content between the fibre-types of rat muscle are similar to that observed in human muscle, however the differences are not as pronounced (Gorski et al., 1992).

Factors affecting resting IMTG content

Diet

The data regarding the effects of fasting on IMTG content in rodents is equivocal. Studies have reported increases, decreases and no change in IMTG levels, following 42-78 hr fasting periods (for review see Gorski, 1992). The only study to examine the effects of fasting on human IMTG content used ^{1}H-MRS to show that IMTG of the vastus lateralis increased following a 72 hr fast (Stannard et al., 2002).

Changes in dietary intake of CHO and fat can also play a role in determining the TG content in rat muscle. Rats fed lard-rich diets increased IMTG content in fast-twitch

muscle but not slow-twitch muscle when compared to rats fed a glucose-rich diet (Abumrad et al., 1978; Pratt, 1989). Unfortunately no "normal" control diets were used to compare against. Diet is also an important determinant of TG content in human skeletal muscle. It would appear that IMTG content in humans is directly related to dietary fat intake. Coyle et al., (2001) examined human IMTG content in response to one week of a control diet (32% fat) and two different reduced-fat diets (22% and 2% fat respectively). They reported that following ingestion of the 2% fat diet, resting IMTG decreased 21%, as compared with the 22% fat diet, however no differences were observed between the control and 22% fat diets. Additionally, Johnson et al., (2003) recently reported an increase in resting IMTG following 2 days of ingestion of a high-fat diet, compared to a high-CHO diet. The energy derived from CHO, fat and protein was 5.7, 56.3 and 37.0% vs. 63.0, 19.0 and 17.3% in the high fat and high-CHO diets respectively.

Following endurance exercise, diet is also an important factor in the replenishment of IMTG. Several studies have reported that IMTG replenishment is attenuated following the ingestion of a high-CHO diet (Coyle et al., 2001; Descombaz et al., 2000 & 2001; Larson-Meyer et al., 2002), whereas it is augmented following the ingestion of moderate- or high-fat diets (Descombaz et al., 2000 & 2001, Larson-Meyer et al., 2002).

Hormonal

Earlier work reported that adrenalectomy both increases (Maling et al., 1966) and has no effect on resting IMTG content (Gorski et al., 1987) in rats. More recently,

physiological concentrations of epinephrine have been shown to decrease resting IMTG content in rat skeletal muscle (Peters et al., 1999). This decrease was only observed in more oxidative muscle (soleus and flexor digitorum brevis) and not in more glycolytic muscle (epitrochlearis). Insulin is an antilipolytic hormone that has been shown to increase resting IMTG content in normal and diabetic rat skeletal muscle (Gorska et al., 1990; Hopp and Palmer, 1991). Leptin, a hormone secreted primarily by adipocytes, is also involved in the control of IMTG content. In normal individuals, leptin has numerous effects on skeletal muscle, which includes increasing TG degradation and oxidation, and decreasing TG esterification (Steinberg et al., 2002). The role of thyroid hormones in determination of IMTG content is uncertain. In two separate studies, subjects with both hypo- and hyperthyroidism showed increased TG content in the vastus lateralis (Kaciuba-Uscilko et al., 1980 & 1981).

Obesity and Diabetes

There are numerous reports that the insulin resistant skeletal muscle of obese and type 2 diabetic humans tends to have increased IMTG content. This has been observed when measuring IMTG using several different techniques, including ^{1}H-MRS (Szczepaniak et al., 1999), computed tomography (CT) imaging (Goodpaster et al., 1997; 2000), biochemical analysis (Pan et al., 1995) and volume density assessment using light microscopy (Goodpaster et al., 1999; Malenfant et al., 2001). It is unclear whether increased IMTG content is the cause or result of the insulin resistance in skeletal muscle.

Training status

In rats, training has been shown to decrease resting IMTG content compared to untrained (for review see Gorski, 1992). In humans, most studies have reported increased IMTG content in trained individuals. In a cross-sectional study, trained subjects had a 2.5 times greater lipid droplet volume density within their vastus lateralis, when compared to untrained subjects (Hoppeler et al., 1973). When subjects underwent a training protocol and individual fibre-types were examined it was reported that increased lipid droplet volume density was only apparent in fast-twitch red and white fibres and not in slow-twitch red fibres (Howald et al., 1985). Using ^{1}H-MRS, Descombaz et al. (2001) assessed the IMTG content of the tibialis anterior muscle in trained and untrained populations and reported that the trained individuals had a 71% greater resting IMTG content than the untrained. Most studies that have implemented a training protocol report increased resting IMTG content post-training (Hoppler et al., 1985; Kiens et al., 1993; Morgan et al., 1969; Phillips et al., 1996). However, two studies reported no change (Bergman et al., 1999; Hurley et al., 1986) in resting IMTG. It is unclear why this discrepancy exists, although it may be attributed to differences in the exercise training protocols and/or the technical difficulties in assessing the true TG content of muscle (Horowitz and Klein, 2000; Van der Vusse and Reneman, 1996).

INTRAMUSCULAR TRIACYLGLCEROL UTLIZATION DURING EXERCISE

Non-human skeletal muscle

The majority of animal studies using *in situ* or *in vitro* electrical stimulation have shown significant depletion in the IMTG stores (Barclay and Stainsby, 1972; Froberg,

1969; Gorski, 1992; Hopp and Palmer, 1990; Spriet et al., 1985 & 1986). When individual muscle fibre types were examined, it was generally reported that fast-twitch white muscle demonstrated minimal decreases in IMTG, whereas fast-twitch red and slow-twitch red muscle generally had a decline in IMTG content (Baldwin et al., 1973; Gorski and Kiryluk, 1980; Reitman et al., 1973; Spriet et al., 1985; Stankiewicz-Choroszucha and Gorski, 1978). This appears to coincide with the premise that red oxidative muscle is more dependent on fatty acid oxidation for energy production. However, the time course of IMTG utilization is unclear. Baldwin et al., (1973) reported a significant decline in IMTG content in the first 15 min of exercise and by 2 hr no further changes had been observed. A second study reported a steady decrease in IMTG over the entire 3 hr duration of exercise (Stankiewicz-Choroszucha and Gorski, 1978).

Human skeletal muscle

Human studies regarding IMTG utilization during exercise reported more inconsistent findings. There are three main techniques that have been used to quantify IMTG utilization during exercise; 1) muscle biopsies and subsequent biochemical analysis of IMTG, 2) estimation of IMTG use by subtracting the measured amount of FFA oxidation from whole body fat oxidation (estimated from respiratory exchange ratio (RER)) and, 3) ^{1}H-MRS.

Biopsy studies

In studies using muscle biopsies, net IMTG utilization is measured by the biochemical analysis of both pre- and post-exercise IMTG. Over the years, studies using

the biopsy technique to measure IMTG have reported differing results regarding the importance of IMTG as a source of energy during exercise. Earlier biopsy studies (prior to 1986) showed a significant decrease in IMTG content of ~20% following 60-100 min of moderate-intensity exercise (Carlson et al., 1971; Essen et al., 1977), and ~ 45% following greater than 2 hr of prolonged exercise (Bergstrom et al., 1973; Costill et al., 1973; Froberg and Mossfeldt, 1971). These studies implied an IMTG contribution of ~ 25% to total energy expenditure (Watt et al., 2002b). Intriguingly, more recent studies (1986-2001) generally reported minimal, statistically insignificant decreases in IMTG content following exercise (Guo et al., 2000; Kiens et al., 1993; Kiens and Richter, 1998; Roepstorff et al., 2002; Starling et al., 1997; Steffensen et al., 2002; Wendling et al., 1996). However, there were two studies that showed significant IMTG utilization (Cleroux et al., 1989; Phillips et al., 1996) and one that showed a paradoxical increase in IMTG with exercise (Bergman et al., 1999). The average contribution of IMTG to total energy expenditure from these recent studies was only ~5% (Watt et al., 2002b). In these studies, subjects generally exercised at a moderate intensity (60-70% VO_2max) for a duration of 90-120 min.

A major criticism of these biopsy studies is that they were unable to differentiate between IMTG and extramyocellular lipids (EMCL), resulting in EMCL being inadvertently sampled with the muscle tissue (Dobbins et al., 2001). Hurley et al. (1986) published the only study to dissect out individual muscle fibres and remove EMCL specifically for IMTG analysis. They showed that IMTG accounted for an approximately 6-fold greater total energy yield than previous reports that used similar exercise protocols where individual fibres were not dissected out (Cleroux et al., 1989; Guo et al., 2000;

Kiens & Richter, 1998; Phillips et al., 1996; Starling et al., 1997; Wendling et al., 1996). To circumvent the problem of EMCL contamination in muscle samples, the use of endurance-trained subjects has also been suggested (Wendling et al., 1996). Endurance training has been shown to markedly decrease the amount of TG found between muscle fibres (Szczepaniak et al., 1999). In untrained subjects the between-biopsy coefficient of variation (CV) of IMTG was 23.5% (n = 13) from three biopsies of the vastus lateralis of the same leg, sampled at the same time (Wendling et al., 1996). This would imply that a greater than 24% decline in IMTG content would be required to observe significant IMTG utilization during exercise (Watt et al., 2002b). In trained subjects the between-biopsy CV of IMTG was much less (12.3 ± 9.4% in 17 paired muscle samples)(Watt et al., 2002a), which is thought to be largely due to decreased EMCL. Trained subjects also exhibit more homogenous stores of IMTG, which could also contribute to the decreased variation between samples (Kelley et al., 2002). The result is that in trained individuals smaller decreases in IMTG are required for statistical significance (Watt et al., 2002a).

It should be noted that between biopsy variability of IMTG may in fact be real, and not due to EMCL contamination. Steffensen et al. (2002) reported that IMTG content of the vastus lateralis had a CV of 4% when five samples were obtained from one mixed freeze-dried sample, and a CV of 31% when a single wet biopsy was aliquoted into five samples prior to freeze-drying and analysis. This implied a large variability in IMTG content within one muscle sample independent of EMCL content.

Another possible source of variation between biopsy samples may be related to the differences in IMTG content among different fibre types. It has been shown that oxidative muscle fibres store a greater amount IMTG than glycolytic fibres (Essen 1977;

Essen et al., 1977). This itself can introduce a large variability in IMTG measurements because pre- and post-exercise biopsies are unlikely to contain the exact same proportion of muscle fibres. The skeletal muscle of trained individuals has a greater oxidative capacity and thus a smaller proportion of glycolytic fibres, which could also contribute to the decreased variability seen in IMTG (Watt et al., 2002b).

Isotopic tracer studies

Another approach for estimating the oxidation of IMTG during exercise is by subtracting the estimated plasma-derived FFA oxidation using isotopic tracers from whole body fat oxidation measured at the mouth via indirect calorimetry. There are several methods used to estimate plasma-derived FFA oxidation. Traditionally, studies introduced labelled palmitate (ie. ^{13}C) bound to albumin into the venous blood and measured the FFA rate of disappearance (R_d). This method assumed that 100% of the FFA taken up by the muscle is oxidized, and as such, represents the minimal contribution of IMTG to total fat oxidation. However, direct measures suggested that in fact not all the FFA taken up by the muscle is oxidized. To circumvent this problem, researchers developed a second method where they additionally measured the labelled CO_2 at the mouth. However, it is well known that CO_2 can be temporariliy fixed in isotopic exchange reactions in the tricarboxylic acid cycle before it is transferred to CO_2. Therefore, researchers also infused labelled acetate or bicarbonate and used the recovery of this label as a correction factor to improve the estimation of plasma FFA oxidation (Sidossis et al., 1995). A third method used to measure FFA turnover and oxidation is made by employing direct arteriovenous balance measures across the working muscle.

Whole body fat oxidation is calculated using the measured RER obtained via indirect calorimety. Non-plasma-derived fatty acid oxidation is calculated by subtracting the estimated FFA oxidation from the whole body fat oxidation. All non-plasma fatty acid oxidation is assumed to come from IMTG as the contribution of circulating TG to total fat oxidation during exercise under normal dietary conditions (~ 20% fat) has been estimated to be low (Havel et al., 1967; Helge et al., 2001; Terjung et al., 1983).

Unlike studies using the biopsy technique, most studies using the isotopic tracer methodology have reported significant IMTG utilization during exercise across a wide range of intensities (30-80% VO_2max) and durations (30-120min) in both trained (Coyle et al., 1998; Romijn et al., 1993 & 2000; Sidossis et al., 1997; Van Loon et al., 2001) and untrained subjects (Friedlander et al., 1999; Jansson et al., 1987; Martin et al., 1993; Phillips et al., 1996). These studies have reported an average contribution of 27% (trained) and 12% (untrained) of IMTG to total energy expenditure (Watt et al., 2002b).

The major criticism of the isotopic tracer methodology lies in the measurement of the RER. Numerous physiological and technical factors can lead to erroneous determinations of VO_2 and VCO_2. Small changes in VO_2 and VCO_2 result in large differences in RER. For example, an exercising subject may have a VO_2 of 2.5 l/min and a VCO_2 of 2.08 l/min, and thus an RER of 0.83, and an estimation of ~56% of the total energy production from fat (Watt et al., 2002b). A 70 ml underestimation of the VCO_2, such that the true value is 2.15 l/min, would result in an RER of 0.86, which corresponds to ~ 46% of the total energy production from fat (Watt et al., 2002b). Overestimations in VO_2 production would also give similar errors in the measurement of RER. It should also be noted that RER is a whole body measurement. During exercise it is often assumed

that skeletal muscle oxidation is equivalent to whole body oxidation. However, there is no way of determining the exact contribution of skeletal muscle to whole body fuel oxidation. Additionally, the measurement of RER can vary greatly between groups. Some groups report much lower RER than others in response to similar exercise intensities. This in itself can contribute to the discrepancy regarding the relative contribution of the various fat stores for ATP provision.

^{1}H-MRS studies

More recently, a third approach for measuring IMTG utilization during exercise has been developed. ^{1}H-MRS is a non-invasive technique whereby the resonances from methylene and methyl protons of TG are measured. These resonances appear as multiple peaks on the proton spectrum of skeletal muscle. It has been shown that IMTG and EMCL have distinct peaks on the proton spectrum (Szczepaniak et al., 1999), which advantageously allows differentiation between these fat depots.

Recent studies report that IMTG decreased ~ 25-50% in the vastus lateralis, tibialis anterior, and soleus muscle during prolonged (> 90 min), moderate-intensity exercise (~ 50-70% VO_2max) (Boesch et al., 1997 & 1999; Brechtel et al., 2001; Decombaz et al., 2001; Johnson et al., 2003; Krssak et al., 2000; Larson-Meyer et al., 2002; Rico-Sanz et al., 2000) in both trained and untrained men. IMTG does not appear to be utilized at higher intensities (85% VO_2max) (Brechtel et al., 2001).

There are however several limitations to ^{1}H-MRS methodology, including the technical difficulty associated with signal acquisition, the dependence of the signal on the muscle fibre orientation and the potential variability caused by large fat layers (Boesch et

al., 1999). Difficulties associated with the calibration of the signal of IMTG also make it difficult to quantify the absolute values of IMTG using ^{1}H-MRS.

Summary

In summary, most studies utilizing the isotope tracer and ^{1}H-MRS methodologies have shown that IMTG is an important source of fuel during prolonged, moderate intensity exercise. The direct measurement of IMTG utilization via the biopsy technique shows more ambiguous findings. Many early studies show significant decreases in IMTG during exercise, whereas more recent work has failed to show significant decreases in IMTG during exercise. However, it has been observed that when the variability of measuring TG between biopsies is reduced (ie. via training), biopsy studies also report a net utilization of IMTG. Today, it is generally agreed that the discrepancies in the literature regarding IMTG oxidation during exercise are the result of methodological problems and that IMTG is indeed an important metabolic substrate during endurance exercise (for review see Watt et al., 2002b).

Effect of endurance training on IMTG utilization during exercise

As previously mentioned, most studies report that trained muscle contains greater resting IMTG than untrained muscle (Descombaz et al., 2001; Hoppeler et al., 1973 & 1985; Kiens et al., 1993; Morgan et al., 1969; Phillips et al., 1996). This would appear to coincide with an increased ability to oxidize IMTG during exercise. However, studies examining IMTG utilization in trained and untrained individuals have yielded conflicting results. Some studies have shown an increased capacity of muscle to oxidize IMTG by

trained muscle during prolonged, moderate intensity exercise (Hurley et al., 1986; Martin et al., 1993; Phillips et al., 1996) whereas others have not (Bergmann et al., 1999; Descombaz et al., 2001; Kiens et al., 1993). The reason for this discrepancy is unclear, although it may be related to the technical difficulties associated with measuring IMTG (Horowitz and Klein, 2000). It is also unclear as to the mechanism by which training may increase IMTG oxidation, as plasma catecholamine concentrations are decreased (Winder et al., 1979) and β-adrenergic receptor density remains unchanged (Martin et al., 1989) in the trained state.

REGULATION OF TRIACYLGLYCEROL LIPOLYSIS

Regulation of TG lipolysis in adipose tissue

The breakdown of TG in adipose tissue is primarily catalyzed by three enzymes; TG lipase or hormone-sensitive lipase (HSL), diacylglycerol (DG) lipase and monoacylglycerol (MG) lipase. HSL is responsible for the cleavage of the first fatty acid from the glycerol backbone, whereas DG lipase and MG lipase release the final two fatty acids. HSL also has the ability to cleave the second fatty acid from the glycerol backbone. HSL is thought to be the rate-limiting enzyme for triacylglycerol lipolysis because the affinity of DG lipase and HSL for diacylglycerol, and MG lipase for monoacylglycerol far exceed the affinity of HSL for triaclyglycerol (Fredrikson et al., 1981; Langfort et al., 1999). Also, HSL has at least 5 known phosphorylation sites (Holm et al., 2000; Greenberg et al., 2001), which suggests that the activation of HSL is externally regulated.

The majority of the scientific literature regarding HSL has examined its regulation in adipose tissue. HSL is regulated by reversible phosphorylation. The sympathetic nervous system (ie. catecholamines) and plasma insulin are generally accepted as the major regulators of HSL activity in adipose tissue (Holm et al., 2000). β-adrenergic activation by catecholamines results in an increase in cyclic AMP (cAMP) and phosphorylation of HSL by protein kinase A (PKA) at one of three serine residues (Ser-563, Ser-659 and Ser-660) (Anthonsen et al., 1998; Holm et al., 1988). Insulin acts to decrease cAMP via a phosphodiesterase, which decreases HSL activity (Degerman et al., 1997). Additionally, several other intracellular signalling molecules have been shown to phosphorylate HSL. HSL activation is increased by extracellular signal-related kinase (ERK) 1/2, which phosphorylates HSL at Ser-600, (Greenberg et al., 2001). Also, 5'-AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), glycogen synthase kinase-IV are able to phosphorylate HSL at Ser-565 (Garton et al., 1989; Holm et al., 2000). When HSL is phosphorylated at Ser-565, PKA is unable to phosphorylate HSL at Ser-563, thus the activation of HSL is inhibited. It has also been reported that HSL is down-regulated allosterically by long chain fatty acyl (LCFA)-CoA (Jepson and Yeaman, 1992). At least two protein phosphatases (PP2A and PP1) also exist, which act to dephosphorylate HSL in vivo, thus decreasing HSL activity (Olsson and Belfrage, 1987; Wood et al., 1993). Clearly, HSL activity in adipose tissue is subject to complex regulation, presumably to handle various metabolic situations (Langin et al., 1996).

Regulation of TG lipolysis in skeletal muscle

TG lipases

Three different TG lipases have been identified in skeletal muscle, each with distinctly different pH optimums (Oscai et al., 1990; Van der Vusse and Reneman, 1996). The acidic lysosomal lipase has an in vitro optimal pH of approximately 5. Due to its low pH optimum, it is not thought to play a role in TG breakdown. Lipoprotein lipase, which for a decade was thought to be the lipase responsible for IMTG breakdown (Oscai et al., 1990) is also present, however several lines of evidence have recently contradicted this hypothesis (Van der Vusse and Reneman, 1996). The optimal pH of lipoprotein lipase is approximately 8.5, while the pH of the cytoplasmic milieu of skeletal muscle is 7 and during contraction can be even lower. It has also been shown that lipoprotein lipase is produced as a secretory enzyme and when inside the cell only resides within vesicles, where it would be unable to interact with the lipid droplet. A neutral, hormone-sensitive lipase (HSL) is also present in muscle and is now generally accepted as the lipase responsible for IMTG breakdown (Langfort et al., 1999). As its name implies, the neutral lipase has an optimal pH of 7, which is consistent with the conditions in the cytoplasm of skeletal muscle.

Approximately 15 years ago, Western and Northern blotting techniques were used to identify HSL mRNA and protein in skeletal muscle (Holm et al., 1987 & 1988). Unfortunately these studies were unable to confirm that the HSL originated from muscle and not adipocytes located between the muscle fibres, even though the amount of HSL measured was less than 10% of that found in adipose tissue. More recently, experiments have confirmed the presence of HSL in skeletal muscle (Langfort et al., 1999; Peters et

al., 1998). Oxidative muscle (soleus and diaphragm) was shown to have a ~ 4-fold greater resting HSL activity and total enzyme concentration than glycolytic muscle (EDL and epithroclearis) (Langfort et al., 1999). It appears that the differences in HSL activity and total concentration between muscle fibre types are directly correlated with known differences in TG content and oxidative capacity.

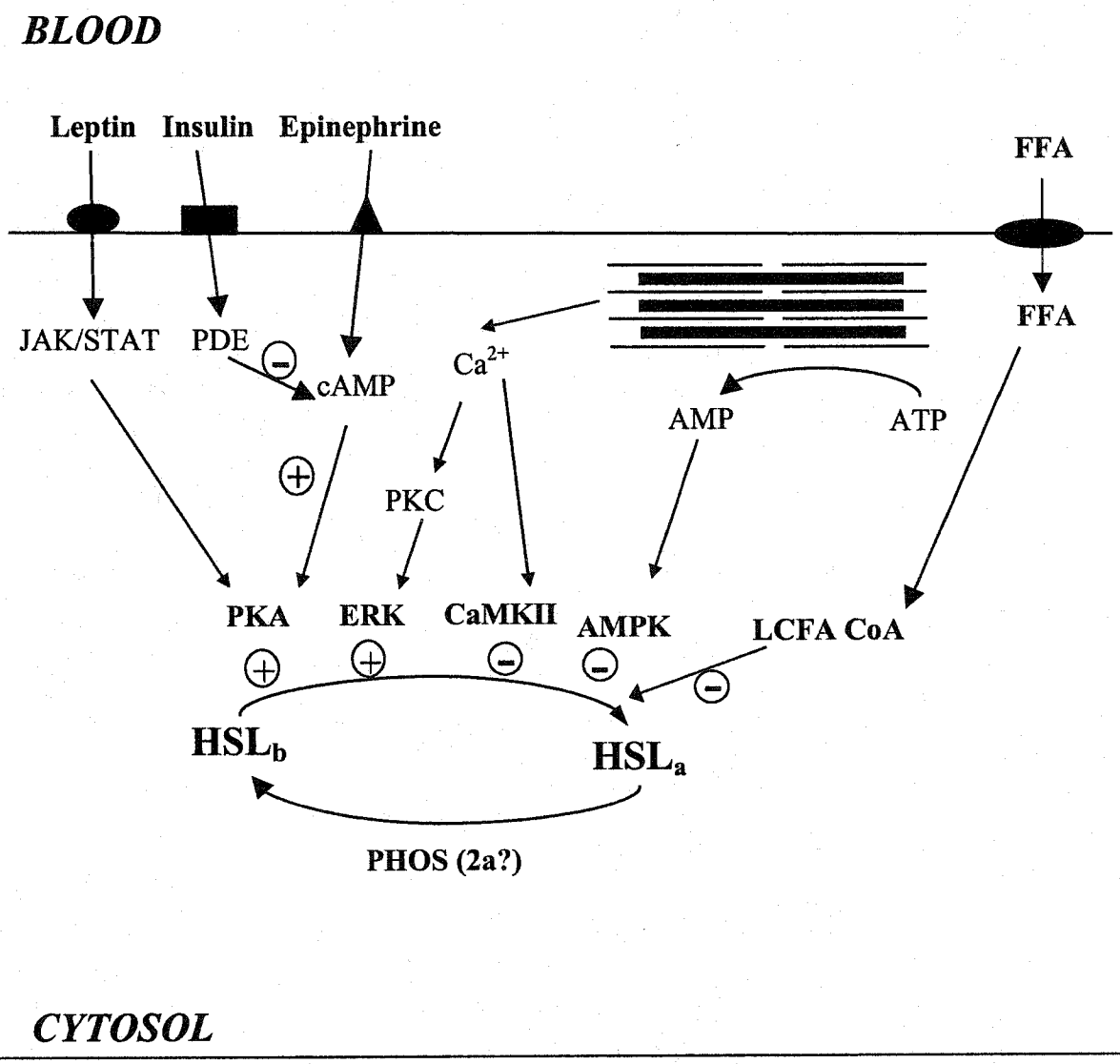
Within the past 5 years, several studies have examined the regulation of HSL in rat and human skeletal muscle. Many of the same signalling pathways present in adipose appear to also exist in muscle. A summary diagram of the proposed regulation of HSL in skeletal muscle is shown in Figure 1.

Rat skeletal muscle

Recently, Langfort et al. (1999 & 2000) examined HSL activity and total enzyme concentration in isolated rat soleus by using a previously developed method to quantify HSL (Osterlund et al., 1996). A diacylglycerol analogue (1(3)-mono[^{3}H]oleoyl-2-oleylglycerol; MOME) and a triaclyglycerol analogue (tri[^{3}H]olein; TO) were used as substrates for HSL. In the basal state HSL has a 10-fold greater affinity for DG as it does for TG. Upon activation of HSL (ie. phosphorylation), the activity of HSL towards TO increased, whereas MOME activity remained the same. Thus, MOME activity was considered to be a measure of total HSL enzyme concentration and TO activity a measure of the activated form of HSL.

Langfort et al. (1999 & 2000) reported that both epinephrine and electrical stimulation increased TO activity. When incubated with epinephrine, TO activity increased after 2 min and remained constant for the duration of the experiment (20 min).

Figure 1: Proposed acute regulation of HSL in skeletal muscle.



PDE, phosphodiesterase; PKA and PKC, protein kinases A and C; ERK, extracellular regulated protein kinase; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; LCFA-CoA, long-chain fatty acyl-CoA; PHOS, phosphatase.

Muscle contraction increased TO activity by 1 and 5 min, however, it returned to resting levels by 10 and 60 min. MOME activity did not change in response to either epinephrine or electrical stimulation. Muscle samples were incubated with HSL antiserum to confirm the neutral lipase that was being measured was in fact HSL (Langfort et al., 1999 & 2000). Although anti-HSL did not completely reduce all neutral lipase activity (TO or MOME activity), it did block the epinephrine and contraction-induced increases in TO activity, implying HSL is an important regulator of TG breakdown.

It should be noted that Langfort et al. (2000) electrically stimulated the muscle to perform repeated (1 Hz), maximal tetanic contractions (200-ms trains of 100 Hz, impulse duration 0.2 ms, 25 V), which are conditions similar to very high intensity exercise. It is curious why this stimulation protocol was chosen as TG breakdown and oxidation is not believed to be an important source of energy at such high intensities.

To determine if HSL in skeletal muscle, as in adipose, is regulated by reversible phosphorylation, Langfort et al. (1999 & 2000) conducted a series of experiments. Muscle was incubated with PKA-C (the catalytic subunit of PKA) and shown to increase HSL activity to the same degree as observed when incubated with epinephrine alone or with epinephrine + PKA-C (Langfort et al., 1999), which suggested that epinephrine was increasing HSL activity through phosphorylation by PKA. When incubated with a phosphatase, contraction-induced increases in HSL activity were completely reversed (Langfort et al., 2000). However, when incubated with okadaic acid, a powerful phosphatase inhibitor, HSL activity increased to levels above that of contraction alone (Langfort et al., 2000).

Interestingly, the time course of HSL activation by epinephrine and contraction is in parallel with that of glycogen phosphorylase (PHOS) (Langfort et al., 1999 & 2000). Activation of HSL and PHOS by epinephrine occurred by 2 min and remained constant for the duration of the experiment (20 min). Electrical stimulation increased HSL and PHOS activation by 1 min, and by 10 min of stimulation both HSL and PHOS activities had returned to resting levels. These observations suggested that no selectivity existed in the primary setting of mobilization of the major energy stores within the cell. This would seem to imply that other regulators must play a role in the ultimate determination of the relative contribution of CHO and fat toward energy provision in exercising muscle.

To study the effects of training on HSL activity and content, rats were swim trained for 18 weeks, following which soleus and EDL were dissected out and analyzed (Enevoldsen et al., 2001). Compared to a sedentary control group, the HSL activity in response to epinephrine stimulation was reduced in both soleus and EDL of trained rats. The total HSL content between sedentary and trained rats was not different. These results are consistent with a previous study that indicated epinephrine-induced lipolysis in muscle is reduced with training (Enevoldsen et al., 2000). It has been shown in some studies that training increases IMTG utilization during exercise (Hurley et al., 1986; Phillips et al., 1996). Since only epinephrine-induced training adaptations were examined by Enevoldsen et al. (2001), it is possible that the increased IMTG utilization seen in trained individuals may be mediated by an increase in the contraction-induced activation of HSL.

To gain more insight into the signals relating muscle contraction to HSL activation Watt et al. (2003c) examined the role of Ca^{2+} in isolated rat soleus, using

cyclopiazonic acid (CPA) to increase cytoplasmic Ca^{2+} to sub-contraction levels without causing muscle contractions or altering the energy charge of the cell. CaMKII, a downstream target of Ca^{2+} that is responsible for inhibiting HSL activation in adipose, was also examined. It was found that increases in Ca^{2+} resulted in decreased HSL activity, which appeared to be mediated by CaMKII. This is consistent with what is seen in adipose tissue (Xu et al., 2001; Zemel et al., 2000), but appears to contrast with observations previously made in skeletal muscle, where experiments have shown a stimulatory effect of muscle contraction on HSL activity (Langfort et al., 2000; Watt et al., 2003a, b, & d). The authors suggest that more powerful signals or higher Ca^{2+} concentrations during muscle contraction (perhaps via the protein kinase C (PKC)-ERK pathway) may override the CaMKII-mediated inhibition HSL activity caused by sub-contraction levels of Ca^{2+}.

Human skeletal muscle

To study the importance of epinephrine (adrenaline) for HSL activation in human skeletal muscle, Kjaer et al. (2000) recruited 6 adrenalectomized subjects and exercised them at ~70% VO_2max for 45 min, and then a further 15 min at ~85% VO_2max. During exercise subjects were infused with near-physiological levels of epinephrine (ADR+) or not (ADR-). The ADR+ group showed increased HSL activation at 45 and 60 min of exercise, whereas the ADR- group did not. These results led the authors to conclude that epinephrine is essential for the activation of HSL in the skeletal muscle of adrenalectomized patients. A control group of healthy subjects also completed the same exercise protocol without epinephrine infusion. This group also showed increased HSL

activity at 45 and 60 min. However, it could not be determined if this activation was the result of epinephrine and/or other factors.

Watt et al. (2003b) examined the early activation of HSL during exercise at varying power outputs. Subjects exercised on a cycle ergometer for 10 min at 30%, 60% and 90% VO_2max with muscle biopsies taken at rest, 1 and 10 min of exercise. At 1 min of exercise HSL activity increased at all intensities, however at 90% VO_2max it was significantly greater than at 30 or 60% VO_2max. The finding of an immediate increase in HSL activity at all intensities, and a greater elevation of HSL activity during 90% VO_2max is consistent with the premise that Ca^{2+} or some other contraction-related factor may be important in the regulation of HSL activity during early exercise. In adipose tissue, insulin is a powerful inhibitor of HSL activity. By 1 min of exercise at 90% VO_2max, insulin had decreased compared to rest, which could also contribute to the increased HSL activation during early exercise at 90% VO_2max. These observations contrast with the work of Kjaer et al. (2000), where adrenalectomized patients required epinephrine for the activation of HSL. In healthy humans it did not seem that epinephrine was essential for the early activation of HSL at 30% and 60% VO_2max as there were no changes in plasma epinephrine levels at these intensities. By 10 min of exercise, the HSL activity at 30 and 60% VO_2max remained constant, whereas at 90% VO_2max it decreased to values seen at 30 and 60% VO_2max. The authors suggested that by 10 min the energy state of the cell (ie free ADP and AMP), perhaps through AMPK, may have played a role in reducing HSL activity.

Watt et al. (2003b) noted that there was no apparent relationship between the initial activation of HSL and the subsequent IMTG lipolysis, suggesting there are likely

other factors that determine the actual rate of IMTG lipolysis. They suggested that, as in adipose tissue, factors distal to HSL activation might also be important in determining the actual rate of IMTG lipolysis. These factors may include the actual movement of HSL to the lipid droplet (Brasaemle et al., 2000; Clifford et al., 2000) and the phosphorylation of a phospho-protein coat (perilipins) surrounding the lipid droplet (Souza et al., 2002). It has been suggested that phosphorylation of perilipin A facilitates the access of HSL to the lipid droplet by altering the conformation of perilipin A and/or by causing perilipin A to translocate off the lipid droplet (Souza et al., 2002).

The role of exercise duration in the activation of HSL was also recently studied (Watt et al, 2003a). As previously mentioned, LCFA-CoA has been implicated as an allosteric inhibitor of HSL in adipose tissue (Jepson and Yeaman, 1992). Unfortunately, allosteric regulation cannot be quantified when using the current method for measuring HSL activity. However, by measuring the LCFA-CoA concentrations, Watt et al. (2003a) were able to investigate the potential role of LCFA-CoA on flux through HSL. Subjects cycled at 60% VO_2max for 120 min and had muscle samples taken at rest, 10, 60 and 120 min of exercise. At 10 min of exercise HSL activity increased above resting values and by 60 min had increased even further. However, at 120 min the HSL activity decreased back to resting levels. The increases between 10 and 60 min were attributed to increases in epinephrine and decreases in insulin. The decreases in HSL activity between 60 and 120 min were proposed to be related to the energy state of the cell (ie. AMP, ADP, P_i), which may have acted through AMPK to override the stimulatory effect of the plasma hormones. LCFA-CoA increased throughout the exercise, and as such may have decreased the flux through HSL towards the end of the exercise. This appeared to

coincide with previous data, which reported no further breakdown of IMTG past 120 min of endurance exercise (Watt et al., 2002a)

To further explore the dual activation of HSL by epinephrine and muscle contraction Watt et al. (2003d) recruited 7 recreationally active males and exercised them for 10 min at 60% VO_2max. Each subject participated in three trials that occurred in random order. The first was a control, where each subject rode for 10 min at 60% VO_2max with no epinephrine infusion (CON). The second trial consisted of the same exercise, however beginning at 3 min of exercise, epinephrine (adrenaline) was infused to high physiological concentrations (EXR + ADR). HSL increased by 3 min in both trials and increased even further by 10 min in EXR + ADR. Indeed, high physiological concentrations of epinephrine increased HSL activity above that caused by contraction alone. The third trial consisted of an epinephrine infusion to high physiological levels, beginning 20 min prior to the same exercise protocol and continuing for the duration of the exercise (ADR). In this trial, epinephrine infusion increased HSL activity at rest. Interestingly, the onset of exercise did not further increase HSL activation, suggesting that the order of the contraction and epinephrine stimuli are important in determining HSL activity.

The second purpose of this study was to examine the role of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in the determination of HSL activity. HSL has been recently shown in adipocytes to be a downstream target of ERK (Greenberg et al., 2001). It has been suggested that ERK 1/2 is involved in the early upregulation of HSL in skeletal muscle as both muscle contraction in rodents (Aronson et al., 1997a; Goodyear et al., 1996) and moderate intensity exercise (~ 70% VO_2max) in humans

(Aronson et al., 1997b; Widegren et al., 1998; Yu et al., 2001) have been shown to increase ERK activity. Watt et al. (2003d) reported increased ERK activity by 3 min of exercise in EXR + ADR and CON, which implied a potential role of ERK 1/2 in activating HSL during muscle contraction, possibly via PKC. An increase in ERK activity was also observed at rest in ADR. Previous work has indicated that epinephrine does not stimulate ERK in human skeletal muscle (Aronson et al., 1997b), however in this study epinephrine concentrations were not measured and were likely to be low. In ADR, epinephrine was infused to high physiological concentrations. This suggested that, when in sufficient concentrations, epinephrine was also able to regulate ERK activity via β-adrenegic stimulation. It should be noted that temporal relationships do not infer causality and more study is required to fully elucidate the role of ERK in the activation of HSL.

Summary

In summary, HSL in skeletal muscle appears to be regulated by many different factors. HSL activation occurs rapidly at the onset of muscle contraction, which is related to increased ERK 1/2 phosphorylation and/or possibly other contraction-related factors. Similar to adipose tissue, epinephrine and insulin are also able to regulate HSL activity in muscle. Stimulation of HSL by epinephrine appears to occur through the cAMP cascade and PKA. In addition, phosphorylation of HSL by AMPK and CaMKII may act to decrease IMTG lipolysis.

Evidently, the activation of HSL is essential for IMTG lipolysis during exercise, but it is not understood why HSL activity does not match well with the actual rate of

IMTG lipolysis. It has been suggested that HSL activation is a type of gross control, whereas factors distal to this are important in fine-tuning the rate of IMTG lipolysis. In adipose tissue, the translocation of HSL to the lipid droplet and the interaction of HSL with the lipid droplet are thought to be important in the determination of the rate of TG lipolysis. Clearly, there is a need for more data examining the relationship between HSL activity and TG lipolysis in skeletal muscle.

CHAPTER 3 – RATIONALE FOR STUDY AND STATEMENT OF THE PROBLEM

Nicotinc acid (NA) is often used to examine the effects of reduced free fatty acid availability on skeletal muscle. NA is a powerful antilipolytic agent that effectively inhibits the breakdown of TG stored in adipocytes by decreasing HSL activity, resulting in decreased plasma FFA concentrations. NA inhibits adipose tissue lipolysis in humans through recently identified HM74 receptors that are coupled to G_i proteins, which act to decrease cAMP levels and ultimately prevent HSL activation (Tunaru et al., 2003). It should be noted that although the possibility cannot be completely excluded, there are several lines of evidence suggesting that NA is not inhibiting HSL in skeletal muscle as well. The work by Tunaru et al. (2003) has only identified HM74 receptors in white adipose and spleen tissue and not in muscle. Additionally, if NA were indeed inhibiting skeletal muscle HSL, we would expect the RER to be dramatically increased following NA supplementation compared to Control. Previous work has indicated that this is not the case, as some individuals did not change their RER between NA and Control trials (Coyle et al., 1998; unpublished observations Stellingwerff et al., 2003). It should also be noted that NA supplementation has been shown to increase plasma epinephrine concentrations (Hawley et al., 2000; Howlett et al., 2001). It is unclear why this occurs, although Howlett et al. (2001) has suggested it is the result of increased sympathetic activity.

Previous work has reported that when supplemented with NA, subjects partially compensate for the reduced ability to derive energy from plasma FFA by increasing

whole body CHO oxidation (Bergstrom et al., 1969; Howlett et al., 2001; Murray et al., 1995; Stellingwerff et al., 2003). In these studies, whole body fat oxidation is only reduced ~20-45%. Recent work has reported that during moderate intensity exercise plasma FFA contributes ~50-100% to the total fat oxidation (Bergman et al., 1999; Friedlander et al., 1999; Van Loon et al., 2001). Assuming that the contribution of plasma FFA to total fat oxidation is minimal in the NA trial, it would seem that IMTG oxidation is also increased in the NA trials to provide FFA for oxidation. While several studies have examined the upregulation of CHO metabolism in skeletal muscle following NA ingestion, no studies to our knowledge have examined the effects of NA on fat metabolism.

The main purpose of this study was to examine the effects of NA supplementation on HSL activity in skeletal muscle, during moderate-intensity exercise in recreationally active males. The second purpose of this study was to examine whole body fat oxidation using indirect calorimetry during both NA and Control trials, in an attempt to correlate HSL activity with change in RER. IMTG oxidation was estimated from the magnitude of the increase in RER following NA ingestion. Large compensation for reduced availability of plasma FFA by IMTG would result in only small increases in RER, whereas minimal compensation would result in large increases in RER.

We hypothesized that:

1. HSL activity in skeletal muscle would be greater during exercise in the NA trial (vs. Control), due to increased epinephrine concentrations and an increased reliance on IMTG for energy provision.

2. RER would be slightly increased in the NA trial, implying increased oxidation of IMTG. These increases in estimated IMTG oxidation would be directly correlated to HSL activity.

CHAPTER 4 - METHODS

Subjects

Eleven active male subjects volunteered to participate in this study. All subjects engaged in at least four, 30 min aerobic workouts per week, which included activities such as running or cycling. Their mean (± SE) age, weight and maximal oxygen uptake (VO_2max) were 22.5 ± 1.4 years, 72.1 ± 3.0 kg, 54.1 ± 2.2 ml/kg/min respectively. All subjects were informed of the experiment protocol, and the possible associated risks of the study were explained to subjects both orally and in writing before written informed consent was obtained. The ethics committee's of the University of Guelph and McMaster University approved the experimental protocol.

Pre-experimental Protocol

All subjects reported to the University of Guelph testing laboratory on two separate occasions prior to the experimental trials. The first visit consisted of a VO_2max test, whereby each subject completed an incremental cycling test (Quinton Excalibur, Quinton Instrument, Seattle, WA) until volitional exhaustion was reached. The second visit was a familiarization trial, where subjects cycled for 20 min to confirm the exercise power output of ~55%. The mean power output was 144 ± 8 W.

Dietary analysis for each subject was completed prior to the experimental trials. Based on 1 day of dietary recall a "pre-experimental diet" was devised (~ 50% carbohydrate, 30% fat and 20% protein), where each subject would consume the same pre-determined diet, 1 day prior to each experimental trial. Subjects were also instructed

to abstain from intense exercise and caffeine and alcohol ingestion 1 day prior to each experimental trial.

Experimental Protocol

All experimental trials were conducted exactly 1 week apart with the subjects reporting to the McMaster University laboratory following an overnight fast (~ 10 hrs). Each subject completed 4 experimental trials. In the first 2 experimental trials subjects either supplemented with NA during the 60 min before the exercise or did not (Control) in a randomized fashion. Subjects then exercised on the cycle ergometer for 40 min at 55% VO_2max. No blood samples or muscles biopsies were taken during these trials. The purpose of these rides was two-fold; 1) to assure that subjects tolerated the NA before any invasive measures were made, 2) to collect expired gases at 10, 20, 30 and 40 min (Quinton Q-plex 1, Quinton Instruments, Seattle, WA) for the measurement of ventilation and expired fractions of O_2 and CO_2, for the determination of whole body fat and carbohydrate oxidation rates.

Upon arrival for the subsequent 2 experimental trials, subjects had an indwelling catheter inserted into an antecubital vein for blood sampling and a saline drip was attached to maintain a patent line. One leg was prepared for muscle biopsies by making four incisions in the skin and underlying connective tissue over the vastus lateralis muscle under a local anaesthetic (2% Lidocaine, no epinephrine). During the 60 min before the exercise, subjects either supplemented with NA or did not (Control) in a randomized fashion. Before exercise commenced, resting blood samples (-60, 0 min) and a resting muscle biopsy (0 min) were taken. As with the previous rides, subjects then

cycled for 40 min at 55% VO_2max. Additional blood samples were taken at 5, 10, 20, 30 and 40 min of exercise. Muscle biopsies were taken at 5, 20 and 40 min of exercise. When taking biopsies, ~30 s elapsed between the cessation of exercise, obtaining the muscle biopsy, and recommencing cycling. All muscle samples were immediately frozen in liquid N_2 for future analysis.

NA Administration

Each subject ingested a total of 20 mg/kg body mass (bm) NA (ICN Canada Ltd, Montreal, Canada), in three doses. The first dose of 10 mg/kg bm was given 60 min prior to exercise and the following two doses of 5 mg/kg bm were administered 30 min prior to and immediately prior to exercise. NA is a powerful anti-lipolytic agent that inhibits the activation of HSL in adipose tissue, thus significantly reducing plasma FFA and glycerol concentrations. This dosing protocol, as outlined by Hawley et al. (2000) and Stellingwerff et al. (2003) was intended to minimize adverse side effects, which can include gastrointestinal distress and headaches. All subjects experienced redness/flushing of their skin over most of their body, beginning ~10-15 min after their first dose. The flushing was often associated with a tingling or itchy sensation. These side effects usually began to subside by the commencement of exercise and in most cases were minimal by the end of the trial. Although we do not believe that these symptoms affected whole body or skeletal muscle responses to exercise, we cannot completely rule this out.

Analysis

Venous blood samples were placed in a heparinzed tube and partitioned into three fractions. An aliquot of 200 µl of whole blood was added to 1 ml of 0.6% (wt/vol) $HClO_4$ and centrifuged. The deproteinized supernatant was stored at -80°C and later analyzed for glucose, lactate and glycerol (Bergmeyer, 1974). A second aliquot of 1.5 ml of blood was added to 30 µl of EGTA/reduced glutathione and thoroughly mixed and centrifuged. The supernatant was analyzed for plasma epinephrine by radioimmunoassay (Adrenaline RIA, Labor Diagnostika Nord, Germany). The remaining blood (~ 1.5 ml) was immediately centrifuged and the plasma removed for analysis of plasma free fatty acids (Wako NEFA C test kit; Wako Chemicals, Richmond VA).

Muscle samples were removed from liquid N_2 and freeze-dried, dissected free of blood and connective tissue, powdered and stored at –80°C until analysis. A 6-8 mg aliquot of powdered muscle was used for the determination of hormone-sensitive lipase activity (HSLa). The method has been described by Langfort et al., (1999) and modified by Watt et al (2003). Briefly, the powdered muscle was homogenized on ice in 20 volumes of homogenizing buffer using a rotating teflon pestle on glass. After centrifugation the supernatant was removed and stored on ice for immediate analysis of HSL activity. A substrate consisting of 5 mM triolein, 14 x 10^6 dpm [9, 10-^{3}H] triolein, 0.6 mg phospholipid (phosphatidylcholine / phosphatidylinositol 3:1 w/w), 0.1 M potassium phosphate and 20% BSA was emulsified by sonication (Fredrikson et al., 1981; Osterlund et al., 1996). The muscle homogenate supernatant (14 µl) was incubated at 37°C with enzyme dilution buffer (86 µl) and 100 µl of triolein substrate. The reaction

was stopped after 20 min by the addition of 3.25 ml of a methanol/chloroform/heptane (10:9:7 v:v:v) solution and 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid were added to facilitate the separation of the organic and aqueous phases. The mixture was mixed on a vortex, centrifuged at 1100 *g* for 20 min and 1 ml of the upper phase containing the released fatty acids was removed for determination of radioactivity on a beta spectrometer (Beckman LS 5000TA). All measurements were made in triplicate and the mean of these values reported.

A second aliquot of 10-12 mg was extracted with 0.5 M $HClO_4$ (1 mM EDTA) and neutralized with 2.2 M $KHCO_3$. The supernatant was subsequently analyzed for phosphocreatine (PCr), creatine, lactate, glucose-6 phosphate (G-6-*P*) and ATP by enzymatic spectrophotometric analysis (Bergmeyer, 1974; Harris et al., 1974) and pyruvate by fluorometric analysis (Passoneau and Lowry, 1993). A third aliquot of muscle (~3 mg) from the rest and 40 min biopsies was used to determine muscle glycogen content (Harris et al., 1974).

Calculations

Free ADP (ADP_f) and AMP (AMP_f) were calculated assuming equilibrium constants for the creatine kinase and adenylate kinase reactions. Specifically ADP_f was calculated using the measured contents of PCr, creatine and ATP, the estimated H^+ concentration (Sahlin et al., 1976) and an equilibrium constant for creatine kinase of 1.66×10^9. AMP_f was calculated using the measured contents of ATP, the estimation of ADP_f and the equilibrium constant for adenylate kinase of 1.05. Free inorganic phosphate (P_{if}) was calculated by adding the resting P_{if} content of 10.8 mmol/kg dry mass (Dudley et al.,

1987) to the difference in PCr content minus the accumulation of G-6-*P* between rest and 5, 20 and 40 min of exercise.

Carbohydrate (CHO) and fat oxidation rates (g/min) were calculated according to the following equations (Peronnet and Massicotte, 1991):

CHO oxidation $= 4.585\ VCO_2$ (l/min) $- 3.226\ VO_2$ (l/min)

Fat oxidation $= 1.695\ VO_2$ (l/min) $- 1.701\ VCO_2$ (l/min)

To convert oxidation rates to kJ/min, values were multiplied by 16.19 for carbohydrate and by 40.80 for fat.

Statistics

Data are presented as means ± SEM. A two way repeated-measures ANOVA (treatment × time) was used to determine significant differences between treatments. When a significant *F*-ratio was obtained, post hoc analysis was completed using a Student-Newman-Keuls test. A single-tailed paired t-test was used to assess net glycogen utilization between trials. Statistical significance was accepted at $P < 0.05$.

CHAPTER 5 - RESULTS

Respiratory Measurements

At 30 and 40 min of exercise VO_2 was significantly greater than at 10 min for both Control and NA trials (Table 1). Throughout the NA trial, VO_2 was significantly greater than Control (treatment effect). The mean percent VO_2max for the Control and NA trial were 55 ± 1% and 57 ± 1%, respectively. At 40 min of exercise, ventilation (V_E) was significantly greater than at 10 min for both Control and NA trials (Table 1). V_E was significantly greater in the NA trial compared to Control (treatment effect). RER decreased significantly at 20, 30 and 40 min when compared to 10 min of exercise for both trials (Figure 2). RER in the NA trial was significantly greater than Control throughout exercise. CHO and fat oxidation rates during both trials were significantly different at 20, 30 and 40 min when compared to 10 min of exercise (Figure 3). In the NA trial, the CHO oxidation rates were elevated and the fat oxidation rates were decreased throughout the exercise compared to the Control trial.

Blood Measurements

Blood glucose remained constant throughout the Control trial (Table 2). Glucose was increased significantly at rest following NA ingestion and remained elevated for the initial 20 min of exercise. Thereafter, blood glucose returned to resting levels. Blood lactate was unaffected by NA supplementation at rest (Table 2). During exercise lactate levels increased significantly above resting values in both trials; however, at 5, 10 and 20

Table 1. Respiratory measurements during 40 minutes of exercise at 55% VO_2max, with or without NA supplementation

Measure	Trial	Time, min			
		10	20	30	40
VO_2 (l/min)	Control	2.10 ± 0.09	2.14 ± 0.09	2.13 ± 0.10 *	2.19 ± 0.09 *
	NA ‡	2.16 ± 0.09	2.17 ± 0.09	2.21 ± 0.10 *	2.24 ± 0.09 *
V_E (l/min)	Control	51.1 ± 1.4	52.9 ± 1.6	51.2 ± 2.0	53.5 ± 2.0 *
	NA ‡	55.9 ± 2.1	55.7 ± 1.8	56.4 ± 1.5	57.5 ± 1.4 *

Values are means ± SE; $n = 11$. NA, nicotinic acid; Control, no supplementation; VO_2, oxygen uptake; V_E, expired ventilation. *Significantly different from 10 min of same condition ($P < 0.05$); ‡ NA trial significantly different from Control ($P < 0.05$).

Table 2. Blood measurements at rest and during 40 minutes of exercise at 55% VO_2max, with or without NA supplementation

Time, min	Measure					
	Lactate (mM)		Glucose (mM)		Glycerol (μM)	
	Control	NA	Control	NA	Control	NA
-60	0.9 ± 0.1	0.9 ± 0.1	4.1 ± 0.2	4.3 ± 0.2	75 ± 16	60 ± 11
0	0.9 ± 0.1	1.2 ± 0.1	4.3 ± 0.3	5.1 ± 0.3 *†	57 ± 9	20 ± 2 *†
5	2.1 ± 0.2 *	3.8 ± 0.3 *†	4.3 ± 0.2	5.0 ± 0.3 *†	56 ± 7	23 ± 3 *†
10	3.1 ± 0.3 *	4.4 ± 0.5 *†	4.5 ± 0.2	4.8 ± 0.3 *	79 ± 6	33 ± 4 *†
20	3.2 ± 0.5 *	3.9 ± 0.6 *†	4.3 ± 0.2	4.7 ± 0.3 *†	92 ± 5	48 ± 6 †
30	2.8 ± 0.4 *	3.2 ± 0.6 *	4.3 ± 0.2	4.4 ± 0.2	119 ± 9 *	62 ± 8 †
40	2.7 ± 0.5 *	2.9 ± 0.6 *	4.4 ± 0.2	4.4 ± 0.2	143 ± 12 *	61 ± 8 †

Values are means ± SE; $n = 11$. *Significantly different from rest (-60 min) of same condition ($P < 0.05$); † significantly different from corresponding time point for Control ($P < 0.05$); ‡ NA trial significantly different from Control ($P < 0.05$).

Figure 2. Respiratory exchange ratio (RER) during 40 min of cycling at 55% VO_2max with or without NA supplementation.

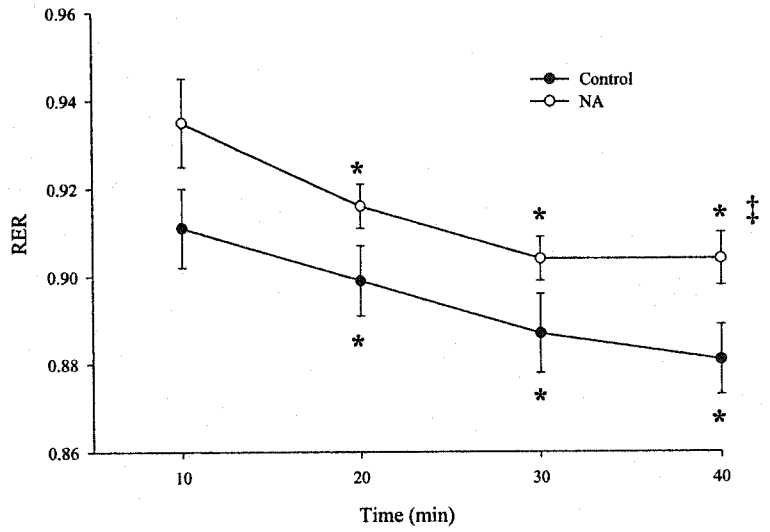
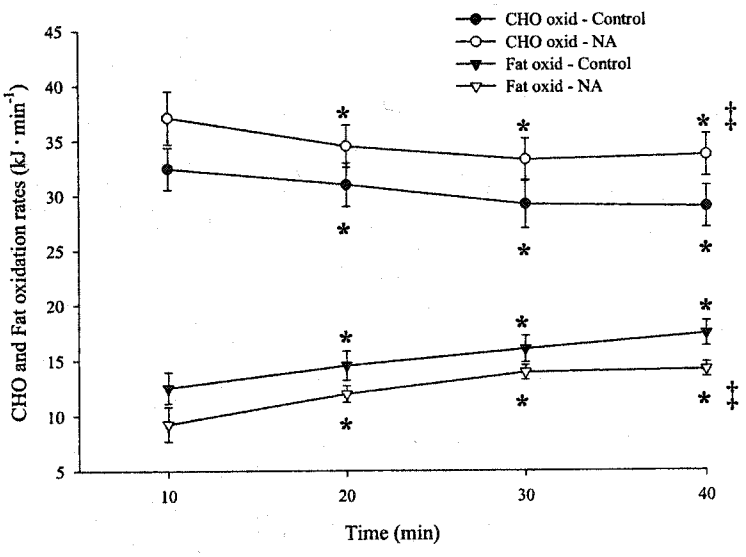
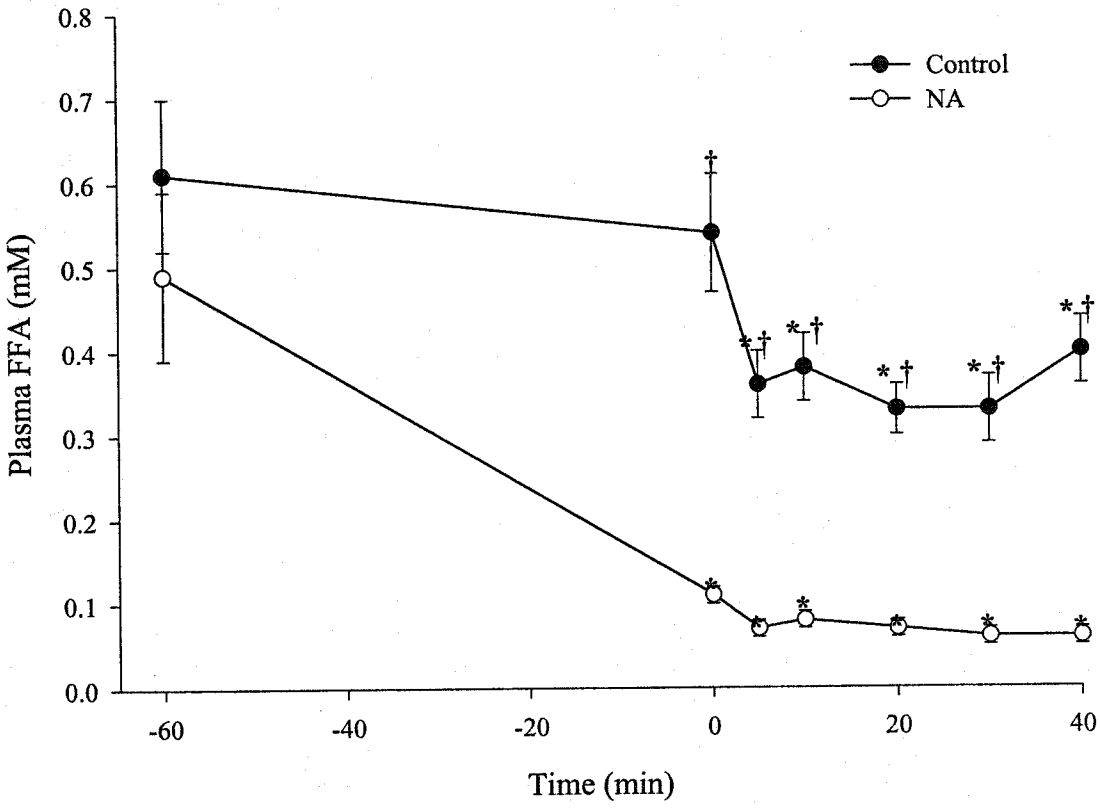


Figure 3. Calculated carbohydrate (CHO) and fat oxidation rates during 40 min of cycling at 55% VO_2max, with or without NA supplementation.



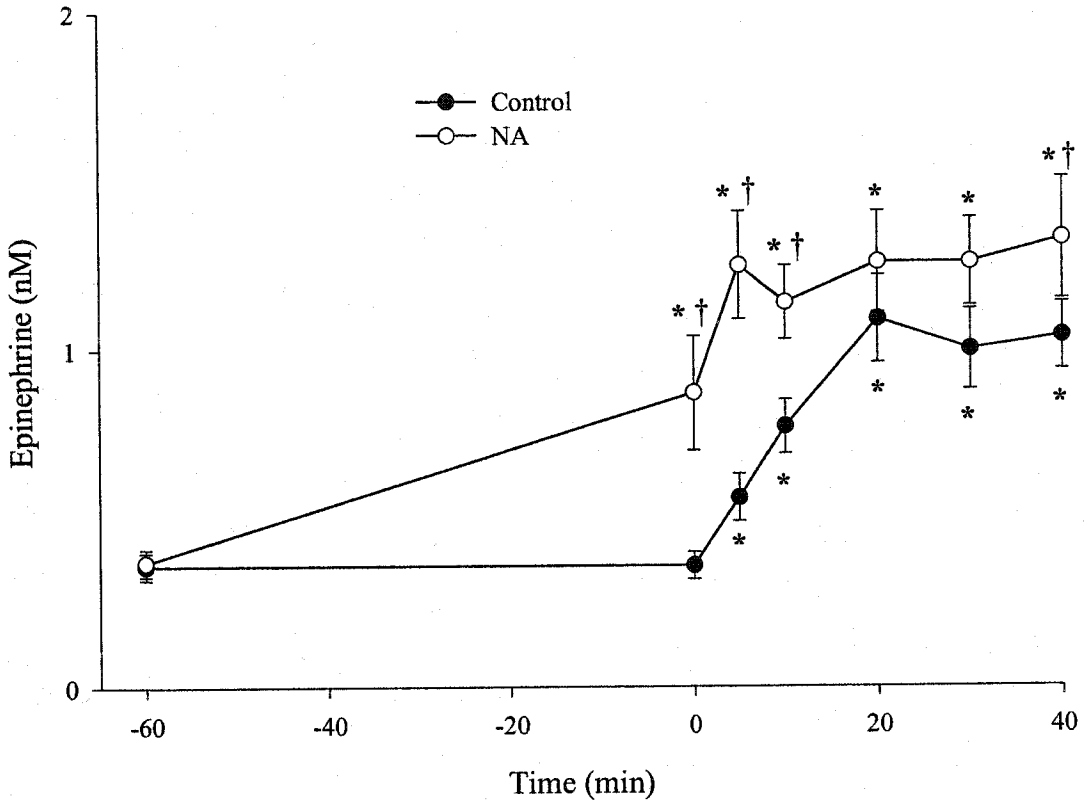
Values are means ± SE, n = 11. * Significantly different from 10 min of same condition ($P<0.05$). ‡ NA trial significantly different that Control ($P<0.05$).

Figure 4. Plasma free fatty acids (FFA) during 40 min of cycling at 55% VO_2max, with or without NA supplementation.



Values are means ± SE, n = 11. * Significantly different from pre-ingestion (-60 min) of same condition ($P<0.05$). † Significantly different than corresponding time point for NA ($P<0.05$).

Figure 5. Plasma epinephrine during 40 min of cycling at 55% VO_2max, with or without NA supplementation.



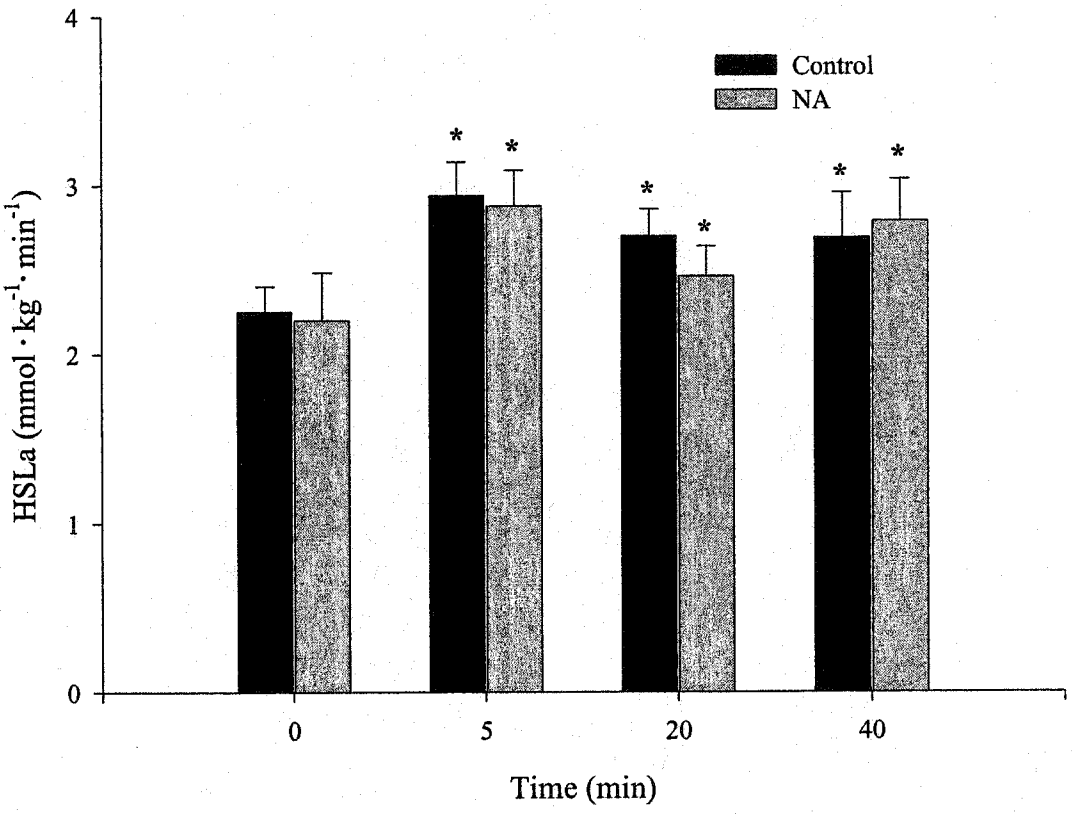
Values are means ± SE, n = 11. * Significantly different from pre-ingestion (-60 min) of same condition ($P<0.05$). † Significantly different than corresponding time point for NA ($P<0.05$).

min of the NA trial, lactate was significantly greater than the Control trial. Resting plasma FFA concentrations were not different between trials (Figure 4). Following NA supplementation, FFA decreased significantly for the duration of the exercise. At all time points following NA ingestion FFA were significantly less than that observed in the Control trial. Prior to NA ingestion, there was no difference in blood glycerol concentration between NA and Control trials (Table 2). In the control trial, glycerol was significantly greater than rest by 30 min of exercise. Following NA supplementation, glycerol decreased significantly, but returned to resting levels by 20 min of exercise. At all time points following NA ingestion blood glycerol was significantly lower than in the Control trial. Plasma epinephrine concentrations were not different prior to NA supplementation (Figure 5). In the both the NA and Control trials, plasma epinephrine was elevated from resting levels during exercise, however the NA trial was significantly higher than Control at 0, 5, 10 and 40 min.

HSLa

There were no differences in HSLa between NA and Control trials (Figure 6). NA ingestion had no effect on resting HSLa. Resting HSLa for both trials averaged 2.22 ± 0.15 mmol/kg dm/min and significantly increased by a mean of 30.8 % by 5 min. No further increases were observed at 20 or 40 min.

Figure 6. Hormone-sensitive lipase activity (HSLa) during 40 min of cycling at 55% VO_2 max, with or without NA supplementation.



Values are means ± SEM, n = 11. * Significantly different from 0 min of same condition ($P<0.05$).

Muscle Metabolites

Phosphocreatine decreased significantly during exercise in both NA and Control trials (Table 3). No differences in ATP occurred during either NA or Control trials (Table 3). ADP_f, AMP_f and P_{if} increased significantly at 5 min and remained elevated for the duration of the exercise in both trials (Table 3). Muscle glycogen contents were not different between trials at rest or following 40 min of exercise (Table 4). However, net glycogen breakdown was significantly greater during exercise in the NA compared to the Control trial (197 ± 26 vs. 154 ± 26). G-6-*P*, pyruvate and lactate increased significantly during exercise when compared to rest in both NA and Control trials and there were no differences between trials (Table 4).

Correlation of shift in RER and degree of HSL activation

When examining the group as a whole, the difference in RER between NA and Control trials (ie. shift in RER) was 0.020 ± 0.008 (Figure 1). However, when individual differences in RER between trials were examined it was evident that a continuum of responses was present (Figure 6A). The shift in RER ranged from –0.009 to 0.054 among the subjects. Clearly, some subjects were able to increase their IMTG oxidation such the that total fat oxidation during the NA trial was no different than in the Control trial (subjects 1-3), whereas other subjects compensated for their reduced ability to oxidize plasma FFA by upregulating their CHO oxidation to varying degrees (subjects 4-11). We expected the individuals who upregulated their IMTG oxidation during NA supplementation to also increase their HSL activity in the NA trial, however no

Table 3. High energy phosphate measurements and calculations at rest and during 40 minutes of exercise at 55% VO_2max, with or without NA supplementation.

Measure	Trial	Time, min			
		0	5	20	40
PCr	Control	78.3 ± 2.6	44.9 ± 5.6 *	52.9 ± 5.7 *	53.4 ± 4.0 *
	NA	75.8 ± 3.3	51.4 ± 5.8 *	49.0 ± 4.3 *	52.1 ± 4.9 *
ATP	Control	24.9 ± 0.7	24.5 ± 1.0	25.0 ± 1.2	24.4 ± 0.8
	NA	24.1 ± 0.9	24.5 ± 0.9	24.3 ± 0.8	24.6 ± 1.1
ADP_f	Control	65 ± 5	228 ± 66 *	172 ± 38 *	153 ± 18 *
	NA	70 ± 7	191 ± 66 *	173 ± 22 *	163 ± 24 *
AMP_f	Control	0.16 ± 0.03	1.63 ± 0.73 *	1.08 ± 0.42 *	0.91 ± 0.21 *
	NA	0.18 ± 0.02	0.72 ± 0.17 *	1.23 ± 0.29 *	0.98 ± 0.23 *
P_{if}	Control	10.8	42.8 ± 4.1 *	35.4 ± 4.1 *	35.3 ± 2.2 *
	NA	10.8	34.0 ± 3.5 *	36.6 ± 3.2 *	34.0 ± 2.9 *

Values are means ± SE; n = 11. PCr, phosphocreatine; ADP_f, AMP_f, and P_{if}, free ADP, AMP, and P_i respectively. All values are expressed as mmol/kg dry mass except for ADP_f and AMP_f, which are expressed as μmol/kg dry mass. Resting P_{if} of 10.8 assumed from Dudley et al., (1987). *Significantly different from 0 min of same condition ($P <$ 0.05).

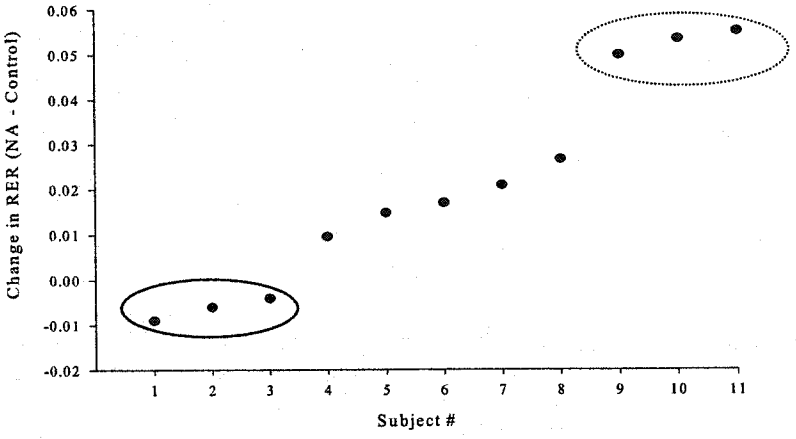
Table 4. Muscle metabolite measurements at rest and during 40 minutes of exercise at 55% VO_2max, with or without NA supplementation.

Measure	Trial	Time, min			
		0	5	20	40
Glycogen	Control	455 ± 30	ND	ND	301 ± 24 *
	NA	496 ± 34	ND	ND	299 ± 31 *
G-6-*P*	Control	0.60 ± 0.06	1.94 ± 0.22 *	1.41 ± 0.12 *	0.99 ± 0.21 *
	NA	0.59 ± 0.09	1.74 ± 0.32 *	1.60 ± 0.21 *	1.04 ± 0.24 *
Pyruvate	Control	0.22 ± 0.01	0.39 ± 0.03 *	0.42 ± 0.03 *	0.39 ± 0.04 *
	NA	0.26 ± 0.03	0.38 ± 0.03 *	0.36 ± 0.04 *	0.37 ± 0.04 *
Lactate	Control	4.0 ± 1.8	25.9 ± 4.8 *	17.2 ± 4.9 *	11.5 ± 3.0 *
	NA	4.4 ± 1.4	25.4 ± 5.6 *	18.0 ± 3.4 *	14.0 ± 2.9 *

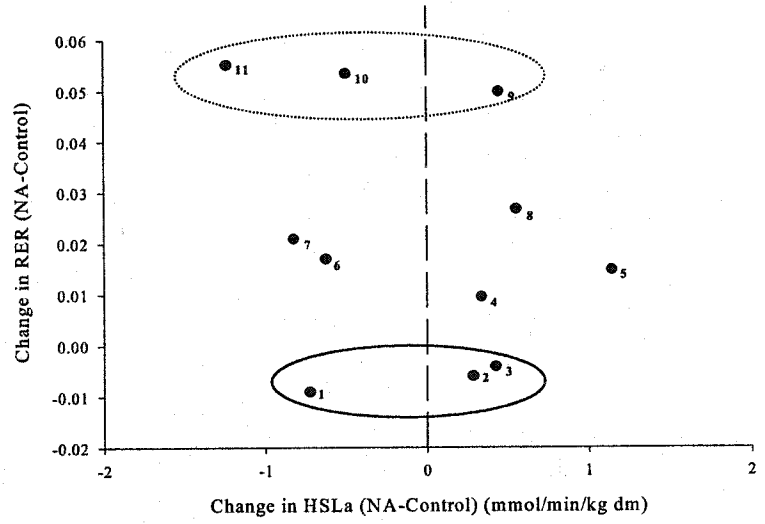
Values are means ± SE; $n = 11$. G-6-*P*, glucose 6-phosphate; ND, no determination. All values are expressed as mmol/kg dry mass. *Significantly different from 0 min of same condition ($P < 0.05$);

Figure 7. Individual RER shift in response to NA supplementation (A) and correlation of RER shift with difference in HSLa between trials (B)

A)



B)



Numerical subscripts represent subject numbers. Data is grouped on both graphs depicting individuals who did not shift the RER (solid line) and those who greatly shifted their RER (dashed line) between trials.

relationship between the shift in RER and the differences in HSLa between NA and Control trials was observed ($r^2 = 0.07$) (Figure 6B).

CHAPTER 6 - DISCUSSION

Nicotinic acid supplementation effectively inhibited adipose tissue lipolysis as shown by the decreased plasma FFA and glycerol concentrations. NA also increased plasma epinephrine concentrations. As expected during exercise, RER increased in the NA trial compared to the Control trial, such that 26% of the total energy used originated from fat, as opposed to 33% in the Control trial. This implied that there was a greater reliance on CHO and IMTG for energy provision in the NA trial. We hypothesized that HSL activity would be elevated during the NA trial due to increased plasma epinephrine and/or factors inside the muscle related to the need for greater IMTG oxidation. However, HSL activation was not elevated despite increased plasma epinephrine concentrations and an apparent increase in IMTG oxidation following NA supplementation. An interesting finding was the large inter-subject variation of the response of RER to NA supplementation. Some subjects had a large increase in RER in the NA trial, suggesting an inability to upregulate IMTG oxidation. These subjects responded to NA by upregulating their CHO oxidation. Other subjects did not shift their RER between trials, suggesting that IMTG lipolysis and oxidation increased in the NA trial. However, even in these subjects, HSL activity was not increased during the NA trial. This data strongly suggests that while HSL activity increases with exercise, it does not predict IMTG lipolysis and oxidation, implying that factors distal to HSL activation are also involved in the regulation of IMTG lipolysis.

Effects of NA on HSL activity

Nicotinic acid administration had no effect on HSL activity, despite the increased plasma epinephrine concentrations observed in the NA trial. In the present study, during the NA trial, plasma epinephrine increased by a mean of 44%, which is similar to that seen in previous studies using similar dosing protocols (Hawley et al., 2000; Howlett et al., 2001). Howlett et al. (2001) suggested the increased plasma epinephrine concentrations observed following NA supplementation could be due to increased sympathetic activity, although this has yet to be confirmed. The precise role of epinephrine in the activation of HSL in skeletal muscle is also unclear. Studies that have artificially elevated plasma epinephrine concentrations to levels seen at the high end of the physiological spectrum and beyond have observed an increase in HSL activity both at rest and during exercise (Kjaer et al., 2000; Langfort et al., 1999; Watt et al., 2003d). As in adipose tissue, the stimulation of HSL appears to be mediated by β-adrenergic activation, resulting in an increase in cAMP and phosphorylation of HSL by PKA (Langfort et al., 1999). Other studies have shown that normal epinephrine concentrations do not necessarily predict HSL activity (Watt et al., 2003a & 2003b). Watt et al., (2003a) reported a constant increase in plasma epinephrine concentrations throughout 120 min of moderate intensity exercise (60% VO_2max). HSL activity increased for the first 60 min of exercise and then decreased back to resting levels by 120 min of exercise, despite the elevated plasma epinephrine concentrations. Additionally, Watt et al. (2003b) reported no difference in HSL activity following 10 min of exercise between higher intensity (90% VO_2max) and lower intensity (30% and 60% VO_2max), despite increased epinephrine concentrations at the higher intensity. It would appear that epinephrine

possesses the ability to regulate the activation of HSL at high physiological and supra-physiological concentrations. However, under normal situations other signals, such as contraction related events, are able to override epinephrine's stimulatory effect on HSL. In the present study we observed an activation of HSL early in exercise, which is likely due to contraction-related factors, although a permissive effect of epinephrine cannot be discounted. However, as the exercise progressed the increased plasma epinephrine concentrations in the NA trial did not further stimulate HSL activity.

As mentioned previously NA inhibits adipose tissue lipolysis in humans through recently identified HM74, which act to ultimately prevent HSL activation (Tunaru et al., 2003). This study provides additional evidence that inhibition of HSL does not also occur in skeletal muscle following NA supplementation. If NA were inhibiting skeletal muscle HSL, we would expect the HSL activity during early exercise to be decreased in the NA trial compared to the Control trial, which it was not.

Effects of NA on IMTG oxidation

Using RER measurements, the calculated average energy derived from fat during the Control and NA trials was 33% and 26% of the total, respectively. Recent work using isotopic tracers has estimated that during moderate intensity exercise (55-65% VO_2max) of 30-60 min, FFA contribute ~50-100% of the total energy derived from fat (Bergman et al., 1999; Friedlander et al., 1999; Van Loon et al., 2001). Thus in our Control trial we expected that a large proportion (~50-100%) of the total fat oxidation came from plasma FFA. In the NA trial, we assumed that plasma FFA would make a minimal contribution to the total fat oxidation, which meant that a large proportion of the total fat being

oxidized was originating from nonplasma fatty acid sources. Most investigators assume that the majority of the nonplasma fatty acid oxidation is derived from IMTG because the oxidation of FFA from circulating very low-density lipoproteins and TG has been shown to be low during exercise under normal dietary conditions (~20% fat) (Havel et al., 1967; Helge et al., 2001; Terjung et al., 1983). If no upregulation of IMTG oxidation had occurred during the NA trial one would have expected the total fat oxidation to decrease by ~50-100% (ie. by the same amount that was contributed by plasma FFA in the Control trial), which it did not. To further illustrate this point, we reported an average RER of 0.896 and 0.916 during the Control and NA trials, respectively. If for example, total fat oxidation had decreased by 50% in the NA trial (vs. Control), we would have observed an average RER of 0.945. Additionally, if total fat oxidation had decreased by 75% in the NA trial (vs. Control), we would have seen an average RER of 0.971. Our results imply that in order to account for such a small shift in RER between trials, there must have been some upregulation of IMTG oxidation following NA supplementation.

An interesting finding of this study was the large variability among individual's RER responses to NA administration. As illustrated in Figure 6A, there was a continuum of responses to NA supplementation. In subjects 9-11 the total energy derived from fat in the Control and NA trials was 42% and 25% respectively. On the other end of the continuum, the total energy derived from fat in the Control and NA trials for subjects 1-3 was 26% and 28% respectively. Thus, in the NA trial, subjects 9-11 decreased their total energy derived from fat by an average of ~17%, whereas subjects 1-3 actually increased their total energy from fat by ~2%, compared to Control. Having assumed that plasma FFA makes a minimal contribution to total fat oxidation following NA supplementation,

one can conclude that subjects 1-3 must have greatly increased their relative IMTG oxidation, and that subjects 9-11 likely only increased their relative IMTG oxidation minimally. All of the subjects were aerobically fit individuals engaging in at least four, 30 min exercise bouts per week and there was no correlation between maximal oxygen uptake and the degree of the shift in RER between trials (results not shown). It is unclear why some individuals possess the ability to upregulate their IMTG oxidation where others cannot. It should be noted that subjects who seemed to possess this ability (ie. subject 1-3) had much less total fat oxidation in their Control trial (26% vs. 42%).

Regulation of HSL activity and IMTG lipolysis

HSL activity increased by ~30% by 5 min of exercise in both Control and NA trials. This increase in HSL activity is consistent with previous reports of HSL activity during early exercise in human skeletal muscle (Watt et al., 2003a, 2003b & 2003d) and likely the result of phosphorylation by Ca^{2+}/calmodulin-dependent kinase II (Langfort et al., 2000; Watt et al., 2003b) and/or other contraction-related factors, such as ERK 1/2 (Watt et al., 2003d). As the exercise progressed HSL activity did not increase to levels beyond that observed at 5 min. It has been suggested that extracellular factors, such as epinephrine and insulin, determine HSL activity in the first 60 min of prolonged exercise (Kjaer et al., 2000; Watt et al., 2003a). Epinephrine increases HSL activity through β-adrenergic receptor stimulation, which increases cAMP and phosphorylates HSL by PKA (Langfort et al., 1999). Between 5 and 40 min of the present study, epinephrine increased only minimally and although insulin was not measured, a previous study from our lab

using an identical experimental protocol measured small decreases in insulin over the same time period (Stellingwerff et al., 2003).

When the RER shift between NA and Control trials was correlated with the difference in HSL activity between trials, no relationship was present (figure 6B). If HSL activation were the sole determinant of IMTG lipolysis, we would have expected subjects who increased their IMTG oxidation to concomitantly increase their HSL activity during the NA trial. Clearly this was not the case, and IMTG oxidation increased in some subjects despite their HSL activity not increasing above levels observed at 5 min. It has been suggested that the activation of HSL is critical for IMTG breakdown, however it is only one factor in the regulation of IMTG lipolysis (Watt et al., 2003b). Recently, data obtained from adipose tissue suggested that the translocation of HSL to the lipid droplet (Brasaemle et al., 2000; Clifford et al., 2000) and the phosphorylation of perilipins, a family of proteins that coat the lipid surface, (Souza et al., 2002) may be essential for TG lipolysis. Data obtained from the present study supports the hypothesis that multiple factors also regulate the rate of IMTG lipolysis.

Effects of NA on CHO metabolism

Previous work from our laboratory using an identical experimental protocol reported a statistically insignificant increase in glycogen breakdown in the NA vs. Control trial (Stellingwerff et al., 2003). However, they theorized that this was due to subject variability and their sample size (n = 8), and that if more subjects had been used a statistically significant relationship would be apparent. Using a larger sample size (n = 11), we observed increased glycogen breakdown in the NA trial, which is consistent with

earlier work (Bergstrom et al., 1969). Increased glycogen utilization coincides with our RER measurements, which implied elevated whole body CHO oxidation with NA.

Summary

In summary, despite a shift towards increased CHO metabolism, fat oxidation only decreased from 33% to 26% of the total energy consumed in the NA trial. Even in three individuals who did not increase their RER with NA, where it appeared that IMTG lipolysis contributed to and maintained fat oxidation, HSL activity did not increase. Contrary to our hypothesis, NA supplementation had no effect on the exercise-induced activation of HSL despite elevated plasma epinephrine concentrations. In both trials HSL activity increased early in exercise and remained constant for the duration of the exercise. The present data strongly suggests that HSL activation is only one factor regulating IMTG lipolysis and that, as in adipose, other factors such as HSL translocation to the lipid droplet and phosphorylation of perilipins may also be important in determining the amount of IMTG that is oxidized during exercise.

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APPENDIX - INDIVIDUAL SUBJECT DATA

SUBJECT CHARACTERISTICS

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
Age (years)	22	27	23	33	18	18	26	19	19	20	23	**22.5**	**1.40**
Weight (lbs)	175	167	150	175	164	113	159	150	134	168	193	**158.9**	**6.56**
Weight (kg)	79.4	75.7	68.0	79.4	74.4	51.3	72.1	68.0	60.8	76.2	87.5	**72.1**	**2.98**
VO2 peak (l/min)	4.000	3.724	3.945	4.470	3.763	2.359	4.606	3.587	4.224	3.652	4.340	**3.879**	**0.18**
VO2 peak (ml/kg/min)	50.3	49.1	57.8	58.5	50.5	45.9	64.1	52.6	69.4	47.8	49.5	**54.1**	**2.24**
Trial workload (watts)	140	140	150	150	140	75	178	140	170	140	160	**143.9**	**7.98**

GAS MEASUREMENTS

VO2 (L/min)

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	2.042	2.146	2.009	2.338	2.021	1.319	2.289	2.163	2.292	1.988	2.507	**2.10**	**0.09**
20	2.143	2.092	2.022	2.360	2.093	1.380	2.471	2.030	2.396	2.033	2.501	**2.14**	**0.09**
30	2.120	2.142	2.032	2.343	2.044	1.408	2.412	1.911	2.461	2.041	2.541	**2.13**	**0.10**
40	2.182	2.194	2.060	2.377	2.259	1.479	2.448	1.958	2.446	2.123	2.583	**2.19**	**0.09**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	2.064	2.148	2.135	2.375	2.204	1.391	2.274	2.056	2.448	2.159	2.510	**2.16**	**0.09**
20	2.082	2.086	2.204	2.386	2.172	1.443	2.406	1.991	2.445	2.096	2.602	**2.17**	**0.09**
30	2.095	2.133	2.304	2.389	2.237	1.377	2.417	2.132	2.497	2.163	2.577	**2.21**	**0.10**
40	2.201	2.108	2.267	2.466	2.262	1.422	2.447	2.244	2.508	2.161	2.581	**2.24**	**0.09**

%VO2 max

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	51.1	57.6	50.9	52.3	53.7	55.9	49.7	60.3	54.3	54.4	57.8	**54.4**	**1.00**
20	53.6	56.2	51.3	52.8	55.6	58.5	53.6	56.6	56.7	55.7	57.6	**55.3**	**0.67**
30	53.0	57.5	51.5	52.4	54.3	59.7	52.4	53.3	58.3	55.9	58.5	**55.2**	**0.88**
40	54.5	58.9	52.2	53.2	60.0	62.7	53.1	54.6	57.9	58.1	59.5	**56.8**	**1.03**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	51.6	57.7	54.1	53.1	58.6	59.0	49.4	57.3	58.0	59.1	57.8	**56.0**	**1.01**
20	52.0	56.0	55.9	53.4	57.7	61.2	52.2	55.5	57.9	57.4	60.0	**56.3**	**0.89**
30	52.4	57.3	58.4	53.4	59.4	58.4	52.5	59.4	59.1	59.2	59.4	**57.2**	**0.88**
40	55.0	56.6	57.5	55.2	60.1	60.3	53.1	62.5	59.4	59.2	59.5	**58.0**	**0.85**

VCO2 (L/min)

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	1.806	2.044	1.826	2.086	1.896	1.214	2.094	1.831	2.094	1.915	2.319	**1.92**	**0.08**
20	1.873	1.958	1.854	2.066	1.919	1.206	2.242	1.724	2.211	1.873	2.205	**1.92**	**0.09**
30	1.827	1.946	1.848	2.018	1.838	1.203	2.184	1.585	2.263	1.820	2.259	**1.89**	**0.09**
40	1.862	1.978	1.856	2.061	2.020	1.263	2.194	1.642	2.223	1.858	2.242	**1.93**	**0.09**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	1.941	2.075	1.999	2.121	2.083	1.324	2.114	1.776	2.328	1.983	2.474	**2.02**	**0.09**
20	1.935	1.911	2.052	2.146	2.026	1.300	2.176	1.773	2.249	1.912	2.452	**1.99**	**0.09**
30	1.917	1.905	2.105	2.164	2.074	1.202	2.138	1.923	2.288	1.951	2.369	**2.00**	**0.09**
40	1.987	1.912	2.074	2.233	2.061	1.226	2.156	2.083	2.298	1.945	2.364	**2.03**	**0.09**

VE (L/min)

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	44.34	56.73	48.59	52.41	53.80	45.16	55.27	45.23	53.36	49.54	57.18	**51.06**	**1.43**
20	48.93	60.15	51.98	53.30	53.80	46.90	59.44	43.14	58.48	49.55	56.46	**52.92**	**1.65**
30	44.73	57.51	50.92	52.59	49.33	48.34	56.67	38.63	61.70	45.62	57.14	**51.20**	**2.04**
40	48.67	61.22	50.76	53.79	57.26	51.46	56.98	41.20	63.78	47.66	55.88	**53.51**	**1.95**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	50.23	64.57	50.00	57.57	57.68	51.64	57.02	42.89	64.50	53.95	64.42	**55.86**	**2.10**
20	50.45	56.84	54.73	58.30	57.72	53.08	58.60	43.89	62.38	51.84	64.86	**55.70**	**1.76**
30	51.53	53.35	54.48	59.30	60.43	49.85	57.99	50.44	63.48	56.28	62.87	**56.36**	**1.46**
40	51.52	57.05	55.93	61.57	60.42	49.22	56.74	57.42	66.33	55.26	60.68	**57.47**	**1.43**

RER

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	0.885	0.952	0.909	0.894	0.938	0.921	0.915	0.847	0.914	0.926	0.925	**0.911**	**0.009**
20	0.874	0.936	0.917	0.876	0.920	0.874	0.907	0.851	0.923	0.924	0.881	**0.899**	**0.008**
30	0.862	0.908	0.910	0.862	0.904	0.855	0.906	0.828	0.920	0.913	0.889	**0.887**	**0.009**
40	0.853	0.901	0.901	0.867	0.894	0.854	0.896	0.839	0.909	0.907	0.868	**0.881**	**0.008**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	0.941	0.965	0.937	0.897	0.945	0.952	0.929	0.864	0.951	0.918	0.985	**0.935**	**0.010**
20	0.930	0.916	0.931	0.899	0.934	0.901	0.905	0.891	0.919	0.912	0.942	**0.916**	**0.005**
30	0.915	0.893	0.914	0.906	0.926	0.872	0.884	0.896	0.916	0.902	0.919	**0.904**	**0.005**
40	0.903	0.907	0.914	0.905	0.911	0.862	0.882	0.929	0.916	0.900	0.916	**0.904**	**0.006**

CHO Oxidation (kJ/min)

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	27.429	39.629	30.662	32.779	35.193	21.252	35.846	22.994	35.716	34.142	41.174	**32.438**	**1.915**
20	27.148	36.089	32.030	30.140	33.099	17.468	37.369	21.975	39.014	33.208	33.015	**30.959**	**1.966**
30	24.942	32.575	31.045	27.458	29.681	15.788	36.129	17.807	39.458	30.952	35.000	**29.167**	**2.207**
40	24.239	32.202	30.190	28.817	31.977	16.533	35.028	19.623	37.258	30.913	31.508	**28.935**	**1.909**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	36.290	41.796	36.905	33.427	39.494	25.621	38.128	24.446	44.948	34.426	52.503	**37.089**	**2.414**
20	34.926	32.891	37.196	34.677	36.942	21.168	35.889	27.615	39.276	32.463	46.115	**34.469**	**1.919**
30	32.898	30.018	35.946	35.849	37.148	17.264	32.415	31.370	39.418	31.850	41.226	**33.218**	**1.914**
40	32.592	31.826	35.498	36.907	34.842	16.698	32.278	37.472	39.563	31.508	40.700	**33.626**	**1.938**

Fat Oxidation (kJ/min)

Control

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	15.865	6.565	12.159	16.867	8.169	6.935	13.019	22.461	13.194	10.126	12.461	**12.529**	**1.418**
20	18.174	8.788	11.153	19.780	11.605	11.718	15.288	20.712	12.225	10.122	19.972	**14.503**	**1.331**
30	19.766	13.088	12.284	21.952	13.796	13.848	15.245	22.198	13.139	11.588	18.921	**15.984**	**1.193**
40	21.686	14.488	13.653	21.378	16.020	14.601	17.005	21.451	14.891	12.754	23.047	**17.361**	**1.139**

NA

TIME	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
10	8.021	4.587	8.881	17.012	7.868	4.323	10.571	18.929	7.743	11.698	1.939	**9.234**	**1.558**
20	9.658	11.648	10.019	16.082	9.601	9.536	15.350	14.642	12.969	12.257	9.773	**11.958**	**0.752**
30	11.829	15.290	13.219	15.042	10.735	11.854	18.831	14.003	13.894	14.184	13.839	**13.883**	**0.651**
40	14.258	13.098	12.899	15.624	13.396	13.296	19.555	10.569	13.995	14.462	14.400	**14.141**	**0.663**

BLOOD MEASUREMENTS

Blood Glucose (mmol/L))

Control

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	5.53	5.19	4.18	4.25	4.45	3.56	3.80	3.61	3.55	3.80	3.60	**4.14**	**0.21**
0min	6.00	5.52	4.93	4.52	4.23	3.67	3.70	3.61	3.68	3.70	3.61	**4.29**	**0.26**
5min	5.95	5.11	5.16	4.61	4.11	3.62	3.80	4.04	3.62	3.79	4.04	**4.35**	**0.23**
10min	5.97	5.16	5.52	4.49	4.20	4.00	3.78	4.03	4.00	3.78	4.03	**4.45**	**0.23**
20min	5.75	5.41	5.35	4.42	4.00	3.73	3.64	3.86	3.72	3.63	3.84	**4.30**	**0.24**
30min	5.58	4.81	5.02	4.47	4.07	3.67	3.79	4.09	3.67	3.80	4.09	**4.28**	**0.19**
40min	5.63	5.66	5.20	4.66	4.05	3.70	3.57	4.19	3.71	3.57	4.18	**4.37**	**0.24**

NA

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	5.55	4.84	4.62	4.33	5.06	3.74	4.01	3.67	3.74	4.00	3.66	**4.29**	**0.19**
0min	7.06	5.76	4.78	5.74	5.46	3.95	5.01	4.47	3.95	5.00	4.47	**5.06**	**0.28**
5min	6.37	5.70	5.22	5.84	5.37	3.89	5.72	3.61	3.90	5.72	3.60	**4.99**	**0.31**
10min	6.28	5.30	5.55	5.84	5.48	3.86	4.87	3.31	3.86	4.86	3.31	**4.77**	**0.31**
20min	6.21	5.53	5.53	5.66	5.06	3.96	4.39	3.65	3.96	4.38	3.64	**4.72**	**0.27**
30min	5.81	5.09	4.85	5.37	4.53	3.53	4.10	3.61	3.52	4.09	3.60	**4.37**	**0.24**
40min	5.65	4.98	4.92	4.88	4.13	3.82	4.11	4.04	3.81	4.11	4.04	**4.41**	**0.18**

Blood Lactate (mmol/L)

Control

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	0.77	1.13	1.37	1.02	1.17	0.86	0.59	0.53	0.80	0.61	0.69	**0.87**	**0.08**
0min	1.16	0.98	1.24	1.07	1.15	0.99	0.58	0.58	0.74	0.57	0.62	**0.88**	**0.08**
5min	3.31	1.93	2.03	1.48	2.59	2.40	2.53	1.39	1.91	1.09	2.44	**2.10**	**0.19**
10min	3.53	4.44	2.63	1.67	3.60	4.46	2.63	2.07	2.65	2.57	4.17	**3.13**	**0.29**
20min	2.43	6.86	2.49	1.44	3.01	4.36	2.58	1.63	3.17	2.29	4.48	**3.16**	**0.47**
30min	2.19	6.04	2.44	1.49	3.29	3.87	2.22	1.51	2.82	1.82	3.60	**2.84**	**0.40**
40min	2.12	7.35	2.07	1.44	2.90	3.27	1.78	1.48	2.52	1.72	3.35	**2.73**	**0.50**

NA

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	0.15	1.62	1.21	1.31	1.48	1.02	0.81	0.60	0.61	0.73	0.84	**0.94**	**0.13**
0min	0.38	1.49	1.78	1.66	1.60	1.03	1.00	1.02	1.11	1.04	1.18	**1.21**	**0.12**
5min	3.86	6.48	4.36	3.80	4.17	3.20	4.16	3.71	2.47	2.40	3.75	**3.85**	**0.33**
10min	3.62	8.51	4.45	3.75	5.24	4.53	4.21	2.92	2.36	2.65	6.03	**4.39**	**0.53**
20min	2.42	8.69	3.79	3.34	3.88	3.27	3.78	2.40	2.27	2.76	6.05	**3.88**	**0.58**
30min	1.97	7.79	2.68	2.59	3.11	2.93	2.86	1.77	1.89	2.39	5.75	**3.25**	**0.56**
40min	1.52	7.92	2.54	2.33	2.41	2.12	2.23	1.80	1.78	2.05	5.40	**2.92**	**0.59**

Blood Glycerol (mmol/L)

Control

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	188.45	153.93	28.44	45.75	54.43	92.56	39.98	65.50	56.13	19.90	82.22	**75.21**	**15.84**
0min	112.68	44.49	23.97	44.40	43.13	98.86	37.48	68.12	56.67	26.22	72.08	**57.10**	**8.60**
5min	91.39	54.57	29.60	45.60	55.51	98.00	44.70	49.84	65.98	21.21	56.90	**55.75**	**6.94**
10min	92.70	81.55	54.80	83.89	83.43	102.32	53.80	83.13	101.92	44.93	87.29	**79.07**	**5.86**
20min	102.45	93.50	91.76	107.87	102.05	90.66	61.16	115.86	93.72	63.56	92.17	**92.25**	**5.04**
30min	117.58	103.89	125.52	173.88	150.75	105.83	72.97	154.99	113.85	82.79	110.25	**119.30**	**9.19**
40min	127.67	140.22	145.82	220.60	187.19	121.90	77.23	179.83	129.96	103.69	141.12	**143.20**	**12.09**

NA

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	145.39	80.40	30.32	24.26	66.10	37.42	18.36	71.88	71.44	81.81	37.66	**60.46**	**11.03**
0min	26.35	28.06	27.69	18.61	21.55	21.26	7.59	20.95	16.58	19.67	17.90	**20.56**	**1.75**
5min	27.99	35.90	22.20	37.24	26.04	21.75	20.78	14.18	12.30	16.54	16.32	**22.84**	**2.50**
10min	43.26	55.57	31.96	53.52	34.06	30.03	32.99	19.09	20.18	18.34	27.28	**33.30**	**3.87**
20min	68.11	75.95	40.75	81.58	37.61	35.33	44.91	32.71	39.41	31.33	37.11	**47.71**	**5.52**
30min	93.73	90.01	38.63	114.20	80.19	56.18	47.41	33.88	51.53	29.64	48.34	**62.16**	**8.41**
40min	95.74	103.48	44.88	107.08	44.92	37.49	44.18	41.55	59.76	32.91	55.85	**60.71**	**8.36**

Plasma FFA (mmol/L))

Control

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	0.88	0.99	0.23	0.34	0.44	0.81	0.40	0.79	0.78	0.15	0.89	0.61	0.09
0min	0.65	0.35	0.37	0.38	0.48	0.83	0.38	0.70	0.76	0.20	0.86	0.54	0.07
5min	0.40	0.27	0.30	0.32	0.33	0.54	0.21	0.45	0.63	0.13	0.40	0.36	0.04
10min	0.31	0.30	0.37	0.26	0.34	0.54	0.24	0.49	0.63	0.22	0.44	0.38	0.04
20min	0.39	0.28	0.41	0.24	0.33	0.40	0.11	0.54	0.30	0.30	0.33	0.33	0.03
30min	0.27	0.31	0.41	0.22	0.42	0.39	0.13	0.60	0.29	0.32	0.31	0.33	0.04
40min	0.29	0.29	0.46	0.28	0.51	0.50	0.20	0.63	0.29	0.51	0.43	0.40	0.04

NA

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	1.07	0.49	0.21	0.29	0.48	0.21	0.12	0.73	0.98	0.55	0.23	0.49	0.10
0min	0.17	0.14	0.14	0.13	0.13	0.08	0.10	0.08	0.20	0.04	0.05	0.11	0.01
5min	0.08	0.12	0.10	0.07	0.07	0.06	0.08	0.02	0.06	0.03	0.04	0.07	0.01
10min	0.09	0.16	0.12	0.07	0.06	0.09	0.07	0.07	0.07	0.05	0.00	0.08	0.01
20min	0.10	0.11	0.11	0.06	0.05	0.08	0.05	0.02	0.03	0.06	0.06	0.07	0.01
30min	0.09	0.11	0.09	0.05	0.07	0.07	0.01	0.02	0.05	0.03	0.01	0.06	0.01
40min	0.10	0.10	0.08	0.05	0.04	0.07	0.03	0.06	0.00	0.07	0.03	0.06	0.01

Plasma Epinephrine (nmol/L)

Control

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	0.56	0.39	0.56	0.42	0.21	0.27	0.50	0.31	0.15	0.33	0.30	**0.36**	**0.04**
0min	0.56	0.39	0.56	0.42	0.21	0.27	0.50	0.31	0.15	0.33	0.30	**0.36**	**0.04**
5min	1.04	0.37	0.51	0.54	0.38	0.36	0.81	0.53	0.38	0.47	0.79	**0.56**	**0.07**
10min	0.87	0.44	0.75	0.54	0.54	0.64	0.91	0.98	0.56	1.25	1.03	**0.77**	**0.08**
20min	0.90	0.59	1.73	0.37	0.68	1.41	1.25	1.46	1.43	1.05	1.09	**1.09**	**0.13**
30min	1.05	0.59	1.78	0.48	0.79	0.62	1.07	1.44	1.36	0.83	1.04	**1.00**	**0.12**
40min	1.01	0.59	1.60	0.72	1.01	0.69	0.89	1.33	1.58	1.20	0.85	**1.04**	**0.10**

NA

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
-60min	0.37	0.29	0.68	0.35	0.26	0.43	0.48	0.37	0.28	0.47	0.11	**0.37**	**0.04**
0min	0.85	0.88	1.24	0.34	0.68	0.83	2.14	0.44	0.41	1.54	0.23	**0.87**	**0.17**
5min	1.00	1.29	2.05	0.47	1.04	0.83	1.95	1.65	0.72	1.86	0.90	**1.25**	**0.16**
10min	0.86	0.75	1.39	0.64	1.03	1.54	1.43	1.77	0.72	1.14	1.26	**1.14**	**0.11**
20min	1.35	1.17	2.60	0.61	0.91	1.50	1.03	1.11	1.24	1.06	1.26	**1.26**	**0.15**
30min	1.96	1.11	1.50	0.64	0.68	1.44	1.00	1.10	1.36	1.13	1.96	**1.26**	**0.13**
40min	2.05	1.03	2.55	0.54	0.65	1.15	1.12	1.05	1.15	1.63	1.67	**1.33**	**0.18**

MUSCLE ENZYMES AND METABOLITES

HSLa (mmol/min/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	1.65	2.03	1.54	2.85	1.99	3.00	2.29	2.37	1.92	2.26	2.82	**2.25**	**0.15**
C-5min	2.64	1.93	3.20	3.42	2.42	2.30	3.07	3.40	3.19	2.53	4.23	**2.94**	**0.20**
C-20min	2.10	2.53	1.84	2.58	3.02	2.68	2.37	3.68	2.61	3.03	3.22	**2.70**	**0.16**
C-40min	2.68	1.77	2.55	1.16	2.87	3.76	2.62	4.42	3.07	2.14	2.50	**2.69**	**0.27**
NA-0min	1.05	2.46	1.22	0.82	2.25	1.99	2.74	3.65	2.97	1.81	3.23	**2.20**	**0.28**
NA-5min	1.96	3.22	3.12	3.96	2.35	2.53	2.83	3.70	2.85	1.77	3.31	**2.88**	**0.21**
NA-20min	1.53	2.66	3.67	1.89	2.47	2.60	2.89	3.03	2.32	1.99	2.04	**2.46**	**0.18**
NA-40min	2.25	2.49	3.00	2.96	2.17	2.19	2.67	2.60	4.04	1.74	4.59	**2.79**	**0.25**

PCr (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	74.21	66.59	73.12	83.91	78.29	87.59	84.25	73.65	66.83	94.40	78.29	**78.3**	**2.6**
C-5min	47.13	13.93	62.75	58.80	47.22	37.87	44.93	45.67	13.17	75.70	47.07	**44.9**	**5.6**
C-20min	51.89	17.52	67.67	70.40	58.41	55.62	69.98	57.74	20.84	68.96	42.63	**52.9**	**5.7**
C-40min	55.09	27.99	53.42	62.28	49.33	61.51	58.77	60.36	38.01	76.71	44.18	**53.4**	**4.0**
NA-0min	71.98	59.73	70.59	76.43	76.79	85.02	83.81	71.59	58.37	95.90	83.12	**75.8**	**3.3**
NA-5min	46.57	24.60	54.49	65.99	53.28	60.65	77.66	52.64	14.37	74.20	40.70	**51.4**	**5.8**
NA-20min	54.45	25.57	61.30	51.85	45.43	65.76	38.89	48.61	33.11	72.98	40.66	**49.0**	**4.3**
NA-40min	44.76	29.27	60.42	57.18	45.03	71.93	61.37	52.58	30.79	80.87	39.00	**52.1**	**4.9**

Creatine (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	45.81	40.46	31.72	25.87	31.58	36.84	52.74	27.95	35.09	35.97	36.40	**36.40**	**2.34**
C-5min	72.90	93.12	42.09	50.98	51.55	86.57	66.41	55.93	88.75	54.68	67.49	**66.41**	**5.19**
C-20min	68.14	89.53	37.18	39.38	35.11	68.82	67.00	43.86	81.08	61.42	71.92	**60.31**	**5.62**
C-40min	64.94	79.06	60.61	47.51	44.19	62.93	78.22	41.24	63.91	53.67	70.38	**60.61**	**3.87**
NA-0min	48.05	47.32	34.26	33.35	34.80	39.42	53.17	30.01	43.55	34.47	31.43	**39.08**	**2.34**
NA-5min	73.46	82.45	50.35	43.80	40.24	63.79	59.33	48.96	87.55	56.17	73.86	**61.81**	**4.76**
NA-20min	65.58	81.48	43.54	57.94	48.09	58.68	98.09	52.99	68.81	57.40	90.97	**65.78**	**5.30**
NA-40min	75.27	77.77	44.42	52.61	48.49	52.50	75.61	49.03	71.13	49.50	75.55	**61.08**	**4.11**

ATP (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	27.22	22.73	22.62	27.17	24.57	21.35	25.84	22.22	26.59	28.52	24.88	**24.9**	**0.72**
C-5min	23.13	19.87	27.25	27.54	22.32	20.16	25.35	22.22	28.93	29.33	23.01	**24.5**	**1.02**
C-20min	27.52	17.93	25.45	26.19	26.45	21.66	28.62	23.24	28.11	30.43	19.62	**25.0**	**1.19**
C-40min	23.21	21.54	24.40	26.54	24.83	21.97	25.53	25.58	29.17	25.33	20.29	**24.4**	**0.76**
NA-0min	25.19	19.61	23.19	23.45	23.67	23.06	21.62	23.89	30.79	26.99	24.08	**24.1**	**0.87**
NA-5min	25.14	20.70	23.85	25.28	21.08	22.93	29.34	22.34	29.80	23.83	24.91	**24.5**	**0.89**
NA-20min	24.82	18.30	26.14	23.14	22.04	23.47	24.43	24.24	27.87	27.96	24.92	**24.3**	**0.82**
NA-40min	27.90	18.53	25.86	24.92	20.89	21.11	30.08	21.79	28.04	27.57	24.08	**24.6**	**1.10**

ADPf (umol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	92.04	79.08	47.15	48.08	55.73	47.72	90.48	47.95	80.17	62.40	64.05	**65.0**	**5.3**
C-5min	172.01	429.07	87.63	126.03	111.34	195.47	174.77	128.32	824.60	118.91	141.74	**228.2**	**65.8**
C-20min	178.44	308.83	71.71	80.32	80.01	124.72	152.84	96.00	477.35	138.11	178.76	**171.6**	**36.7**
C-40min	136.18	251.81	143.11	114.57	121.27	107.10	175.03	94.73	268.63	99.43	169.53	**152.9**	**17.9**
NA-0min	88.32	78.00	62.16	58.48	59.12	59.38	78.72	57.44	125.90	55.71	49.56	**70.3**	**6.6**
NA-5min	192.71	210.15	104.11	80.47	84.06	119.20	108.76	100.63	842.40	94.37	163.52	**190.9**	**66.5**
NA-20min	145.96	220.67	88.66	136.50	119.15	108.79	273.96	140.86	291.69	118.53	261.35	**173.3**	**22.3**
NA-40min	229.36	226.05	93.63	125.05	124.18	81.66	163.14	110.09	340.88	93.86	209.23	**163.4**	**24.1**

AMPf (umol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	0.30	0.26	0.09	0.08	0.12	0.10	0.30	0.10	0.23	0.13	0.16	**0.17**	**0.03**
C-5min	1.22	8.82	0.27	0.55	0.53	1.80	1.15	0.71	22.39	0.46	0.83	**3.52**	**2.02**
C-20min	1.10	5.07	0.19	0.23	0.23	0.68	0.78	0.38	7.72	0.60	1.55	**1.68**	**0.73**
C-40min	0.76	2.80	0.80	0.47	0.56	0.50	1.14	0.33	2.36	0.37	1.35	**1.04**	**0.25**
NA-0min	0.29	0.30	0.16	0.14	0.14	0.15	0.27	0.13	0.49	0.11	0.10	**0.21**	**0.04**
NA-5min	1.41	2.03	0.43	0.24	0.32	0.59	0.38	0.43	22.68	0.36	1.02	**2.72**	**2.00**
NA-20min	0.82	2.53	0.29	0.77	0.61	0.48	2.93	0.78	2.91	0.48	2.61	**1.38**	**0.33**
NA-40min	1.80	2.63	0.32	0.60	0.70	0.30	0.84	0.53	3.95	0.30	1.73	**1.25**	**0.35**

Pif (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min												**10.80**	
C-5min	36.21	61.17	19.55	36.20	41.12	58.22	48.41	38.17	62.78	28.61	40.59	**42.82**	**4.12**
C-20min	32.45	59.18	15.27	23.55	30.11	41.46	24.69	26.05	55.44	35.80	45.42	**35.40**	**4.14**
C-40min	29.52	47.69	29.80	33.05	39.67	35.43	36.20	24.33	39.91	28.23	44.25	**35.28**	**2.16**
NA-0min												**10.80**	
NA-5min	35.34	42.95	25.86	20.78	34.04	32.45	16.74	29.38	53.09	32.26	51.44	**34.03**	**3.46**
NA-20min	28.34	44.07	19.26	34.69	41.18	27.91	54.93	32.81	35.57	32.52	51.16	**36.58**	**3.17**
NA-40min	38.41	40.25	20.78	30.07	42.89	22.35	32.42	29.42	38.20	26.16	52.97	**33.99**	**2.90**

Pyruvate (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	0.19	0.29	0.24	0.24	0.17	0.19	0.18	0.24	0.22	0.20	0.22	**0.22**	**0.01**
C-5min	0.39	0.40	0.45	0.22	0.28	0.54	0.48	0.37	0.44	0.29	0.43	**0.39**	**0.03**
C-20min	0.44	0.37	0.35	0.25	0.32	0.60	0.32	0.44	0.57	0.46	0.52	**0.42**	**0.03**
C-40min	0.39	0.48	0.39	0.19	0.35	0.61	0.24	0.47	0.60	0.26	0.34	**0.39**	**0.04**
NA-0min	0.26	0.41	0.18	0.17	0.19	0.23	0.23	0.31	0.47	0.19	0.24	**0.26**	**0.03**
NA-5min	0.44	0.57	0.32	0.33	0.23	0.39	0.36	0.42	0.46	0.21	0.48	**0.38**	**0.03**
NA-20min	0.29	0.65	0.50	0.17	0.30	0.28	0.39	0.41	0.34	0.24	0.41	**0.36**	**0.04**
NA-40min	0.41	0.54	0.31	0.23	0.24	0.33	0.42	0.23	0.41	0.22	0.69	**0.37**	**0.04**

Glucose-6-Phosphate (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	0.53	0.55	0.28	0.65	0.84	0.59	0.38	0.46	0.84	0.90	0.60	**0.60**	**0.06**
C-5min	2.21	2.84	1.91	0.36	1.60	2.89	2.10	1.07	2.52	1.80	2.04	**1.94**	**0.22**
C-20min	1.20	1.24	1.27	1.41	1.40	1.90	0.75	1.12	2.20	1.35	1.63	**1.41**	**0.12**
C-40min	0.93	2.26	0.99	0.04	0.94	2.05	0.46	0.22	0.56	1.18	1.26	**0.99**	**0.21**
NA-0min	0.74	0.83	0.50	0.16	1.25	0.51	0.60	0.22	0.56	0.56	0.56	**0.59**	**0.09**
NA-5min	1.61	3.82	1.54	0.62	1.52	3.23	0.82	0.59	2.27	0.80	2.34	**1.74**	**0.32**
NA-20min	0.72	1.72	1.33	0.85	2.22	2.67	1.39	1.19	1.04	1.77	2.66	**1.60**	**0.21**
NA-40min	0.34	1.83	0.69	0.14	0.92	2.05	1.41	0.62	0.74	0.22	2.51	**1.04**	**0.24**

Lactate (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-0min	5.30	0.25	19.80	0.00	2.36	8.68	2.96	1.05	0.00	0.00	4.04	**4.04**	**1.78**
C-5min	19.63	63.89	19.85	9.37	25.37	33.40	22.86	21.79	33.80	2.46	32.07	**25.86**	**4.79**
C-20min	16.58	59.14	12.46	5.11	14.59	23.01	3.12	5.84	30.19	13.03	6.51	**17.23**	**4.86**
C-40min	15.75	36.21	11.52	1.67	5.66	20.35	12.06	6.14	4.91	2.52	9.95	**11.52**	**3.01**
NA-0min	9.87	14.76	4.36	0.53	4.60	3.69	0.00	0.00	4.94	0.00	5.91	**4.42**	**1.39**
NA-5min	18.37	70.86	21.60	19.94	9.31	16.50	18.61	18.75	23.48	10.36	51.22	**25.36**	**5.63**
NA-20min	17.99	46.00	20.23	9.42	12.96	11.09	28.28	8.08	14.49	7.02	22.38	**17.99**	**3.43**
NA-40min	17.68	24.57	16.99	5.71	4.37	8.80	29.39	6.47	9.52	3.54	27.03	**14.01**	**2.89**

Glycogen (mmol/kg dm)

	1	2	3	4	5	6	7	8	9	10	11	MEAN	SE
C-(0 or 5min)	688.85	305.98	393.89	462.05	488.81	533.46	481.30	358.60	442.50	394.30	459.50	**455.39**	**30.42**
C-40min	330.47	172.85	307.98	338.56	460.27	294.86	373.10	214.10	248.00	237.80	337.40	**301.40**	**24.31**
NA-(0 or 5min)	706.97	577.22	520.65	530.77	470.25	652.90	430.10	348.20	407.30	371.30	438.0	**495.79**	**34.53**
NA-40min	349.49	466.46	331.50	377.71	376.09	325.95	306.10	180.20	181.40	111.80	280.90	**298.87**	**31.32**