The Role of Putative Brain Serotonergic and Dopaminergic Modulators in Central Fatigue During Exercise in Health and Disease

Marios Hadjicharalambous

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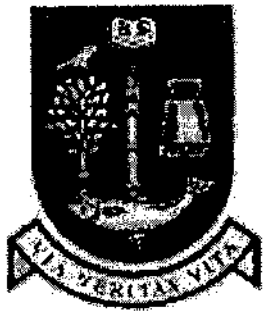
A Thesis Presented for the Degree of
Doctor of Philosophy



in

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"It is what we think we know already that prevents us from learning"

Claude Bernard (1813-1878)

To the memory of my beloved father whose grateful smile is always in my mind; to my newborn lovely son, Manos; to my charming Godson, Stefanos whose little heart I harmed all this period, at a very critical stage of his life, due to my absence (Sinxorame kale mou); and to my lovely wife Xenia, for her support, encouragement but especially disinterested love.

Declaration

I hereby declare that this thesis has been conducted by myself, that the work of which it is a record has been done by myself except where assistance and help have been acknowledged, which it has not been submitted in any previous application for a higher degree and that all sources of information have been specifically acknowledged by mean of references.

Some of the results obtained in this thesis have been presented as follows:

Hadjicharalambous, M., Georgiades, E., Kilduff, L. P., Turner, A. P., Tsofliou, F. and Pitsiladis, Y. P. (Under consideration) Influence of caffeine on perception of effort, metabolism and exercise performance following a high fat meal. *Journal of Sports Sciences.*

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Hadjicharalambous, M., Kilduff, L.P., Turner, A.P., Cathcart, A.J., Georgiades, E., Pitsiladis, Y.P. Influence of caffeine on perception of effort during exercise following a high fat meal. European College of Sports Science, Athens, 24th-28th July 2002.

Hadjicharalambous, M., LP Kilduff, E Georgiades, YP Pitsiladis. Influence of caffeine on perception of effort, metabolism and exercise performance following a high fat meal in well-trained cyclists. IOC, World Congress & Sport Science, Athens 2004, 7th-11th October 2003.

Kilduff LP, Georgiades E, James N, Minnion RH, Mitchell M, Kingsmore D, M Hadjicharalambous, Pitsiladis YP. (In press) The effects of creatine supplementation on cardiovascular, metabolic and thermoregulatory responses during exercise in the heat in endurance-trained humans. *International J Sports Nutr Exerc Metab.*

Georgiades, E., WMH Behan, A Jackson, D McCready, L Kilduff, M Hadjicharalambous, EE Mackie, SA Ward, YP Pitsiladis (2003) Chronic fatigue syndrome: A central fatigue disorder? *Clinical Science.*

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Sign here,

M. Hadjicharalambous

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Summary

The primary purpose of the present experiments was to elucidate the role of putative brain serotonergic and dopaminergic modulators and indices (markers) in 'central fatigue' during exercise in health and disease, with a concurrent examination of metabolic, cardiovascular and perceptual responses.

In experiments (EXPs) 1 and 2, the subjects underwent three main exercise trials. The first, following a pre-exercise high CHO meal (Control trial) and the remaining two after a pre-exercise high fat meal with (FC trial) and without (F trial) caffeine. The use of fat meals was employed to elevate circulating plasma free fatty acids (FFA) levels in an attempt to produce i) a similar muscle metabolic adjustment, and, ii) a parallel displacement of plasma Trp from albumin by plasma FFA and therefore similar brain serotonin (5-HT) manipulation in both F and FC trials. Differences on brain 5-HT and dopamine (DA) modulators and indices, exercise performance and perceptual (RPE), metabolic and cardiovascular responses could be examined by ingestion of caffeine. Thus, caffeine would differentiate between peripheral and central aspects affecting exercise fatigue and therefore the mechanism(s) associated with central fatigue could be evaluated.

Modulators and indices of brain 5-HT and DA functions (i.e. plasma free and total tryptophan (Trp), tyrosine (Tyr), large neutral amino acids (LNAA), Trp:LNAA ratio, free-[Trp]:[Tyr] ratio, Trp:Tyr ratio, and prolactin) and exercise performance were not different between the trials but RPE was reduced and metabolic/cardiovascular responses increased during exercise with caffeine. The first two EXPs indicate a differentiation between putative metabolic and CNS effects of caffeine during constant-load exercise (since RPE was reduced following caffeine ingestion despite an elevation in cardiopulmonary and metabolic responses). However, the first two experiments demonstrate a dissociation between i) modulators of brain 5-HT function and RPE, ii) RPE and metabolic/cardiovascular responses and iii) RPE and exercise performance. Consequently, the reduction in RPE *per se* cannot contribute to enhancing exercise performance and the mechanism of this RPE attenuation seems not to be associated with brain 5-HT modulators and function during exercise in well-trained humans. The EXP 2 showed also that during prolonged exercise in a low temperature (10°C) the primary cause of fatigue is muscle glycogen depletion. Consequently, neither

'central fatigue' can be developed nor caffeine can contribute in enhancing endurance performance by attenuating brain 5-HT function and/or counterbalancing brain 5-HT:DA ratio in well-trained humans.

The aim of EXP 3 was to examine the effects of a creatine (Cr) supplementation on putative modulators and indices of brain 5-HT and DA function and on thermal stress during prolonged exercise in the heat. The Cr was used as a 'vehicle' to increase hydration and/or reduce thermal stress-induced increase in brain 5-HT function. Cr reduced thermoregulatory (e.g. sweat rate, rectal temperature and heart rate) and perceptual responses, plasma free-[Trp] ($P = 0.001$) and free-[Trp]:[Tyr] ratio ($P = 0.001$) and enhanced endurance performance in subjects classified as 'responders' to Cr. The EXP 3 revealed that, the effectiveness of Cr to alter key modulators of brain 5-HT and DA function may have contributed to the reduced thermal stress and effort perception during exercise in the heat and to the enhancement of endurance performance in the 'responders' to Cr supplementation.

In EXP 4, chronic fatigue syndrome (CFS) patients were examined because they are reported to have an up-regulation of brain Trp uptake (e.g. Bakheit et al. 1992). The aim of the fourth EXP was to elucidate the role of putative modulators (Trp, LNAA, Tyr) of central fatigue in CFS, to evaluate the association between these modulators with perceptual and metabolic responses in CFS patients and to compare their results to those of matched sedentary controls. It was found that plasma free-[Trp], free-[Trp]:[LNAA] ratio and RPE were higher and plasma [Tyr] and exercise tolerance lower in the CFS relative to the sedentary control group. It was concluded that the significant differences between patients with CFS and healthy controls observed in several key brain 5-HT and DA modulators suggest that central neural mechanisms associated possibly with up-regulation of brain 5-HT system may contribute to the decreased central motivation (increased RPE) and impaired exercise tolerance in CFS.

Taken together, the results across studies imply that putative modulators of brain serotonergic and dopaminergic systems (and therefore brain 5-HT and DA function) play a key role in pre-exercise central motivation and also in central fatigue process of well-trained humans during exercise in the heat (EXP 3) and in the exacerbated RPE, exercise intolerance and pathogenesis of CFS (EXP 4). However, under physiological conditions this central neural drive of fatigue associated with

metabolic up-regulation of brain 5-HT does not affect exercise performance of well-trained humans (EXPs 1, 2). The exact mechanism(s) therefore for the attenuation of RPE with caffeine is unlikely to be a brain 5-HT-mediated but most likely a DA-mediated effect. Nevertheless, neither brain 5-HT nor DA systems would appear to be implicated in the fatigue process when exercise is performed without significant thermoregulatory stress thus enabling fatigue development during exercise to occur due to peripheral factors (i.e. glycogen depletion). However, the reduced RPE during exercise in the heat following Cr supplementation may be due, in part at least, to the reduction in modulators of brain 5-HT function and/or to a concomitant elevation in brain DA activation. Finally, the brain 5-HT up-regulation in conjunction with the brain DA down-regulation may have contributed in exacerbating RPE and reducing exercise tolerance in CFS.

The series of the present EXPs were the first to show:

1. The lack of central neural involvement, associated with brain 5-HT and DA modulators and function on the fatigue process during exercise in relatively thermoneutral environments in which fatigue occurs due to peripheral limiting factors (i.e. cardiovascular stress, glycogen depletion).
2. A possible contribution of Cr supplementation to the reduction in the putative modulators of brain 5-HT system and/or a direct contribution to the increased brain DA function.
3. Abnormalities in putative modulators of brain serotonergic and dopaminergic function in CFS patients, even in resting conditions, implicating the involvement of central neural mechanisms in the increased RPE and impaired exercise tolerance in CFS.

Tables of abbreviations

ADP:	Adenosine diphosphate
ANOVA:	Analysis of variance
AMP:	Adenosine monophosphate
ATP:	Adenosine triphosphate
BBB:	Blood brain barrier
BCAA:	Branched chain amino acids
BIA:	Bioelectrical impedance analyser
C:	Celsius
cAMP:	Cycle adenosine monophosphate
CHO:	Carbohydrate
CK:	Creatine kinase
Cl^-:	Chloride ions
CNS:	Central nervous system
CO_2:	Carbon dioxide
Cr:	Creatine
CrP:	Creatine Phosphate
DA:	Dopamine
DOPA:	Dihydroxyphenylalanine
DOPAC:	3,4-dixydroxyphenylacetic acid
HPA:	Hypothalamic pituitary adrenal-axis
ECW:	Extracellular water
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethylene glycol-bis (β-aminoethylether) –N,N,N`,N`- tetraacetic acid
FFA:	Free fatty acids
Free-Trp:	Free tryptophan
G-FA:	Glucose-fatty acids cycle
G-6-P:	Glucose-6-phosphate
H^+:	Hydrogen
Hb:	Haemoglobin
HCl:	Hydrochloric acid
Hct:	Haematocrit
HPLC:	High performance liquid chromatography

HR:	Heart rate
3-HAO:	3-hydroxyathralinic acid oxidase
3-OHB:	3-hydroxybutyrate
5-HT:	5-hydroxytrypatmine (serotonin)
5-HIAA:	5-hydroxyindole-3-acetic acid
5-HTP:	5-hydroxytryptophan
IQR:	Interquartile range
K_3EDTA:	Tri-potassium ethylenediamine-tetracetic acid
K^+:	Potassium ions
K_d:	Non-saturable transport constant
K_m:	Michaelis-Menton constant
LNAA:	Large neutral amino acids
LT:	Lactate threshold (or Anaerobic threshold)
MCT:	Medium chain triglycerides
Na^+:	Sodium ions
NH_3:	Ammonia
NMR:	Nuclear magnetic resonance
O_2:	Oxygen
PCr:	Phosphocreatine
Pi:	Inorganic Phosphate
Prl:	Prolactin
RER:	Respiratory exchange ratio
RPE:	Ratings of perceived exertion
RPM:	Revolution per minute
S.D.:	Standard deviation
SSRI:	Selective serotonin reuptake inhibitor
Trp:	Tryptophan
TRH:	Tryptophan hydroxylase
Tyr:	Tyrosine
TBW:	Total body water
T_{rec}:	Rectal temperature
T_{skin}:	Weighted mean skin temperature

$\dot{V}CO_2$:	Volume of carbon dioxide produced
$\dot{V}O_{2\,max}$:	Maximal oxygen uptake
$\dot{V}O_2$:	Oxygen uptake
$\dot{V}E$:	Minute ventilation
WR_{max}:	Maximum work rate

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Chapter one

General Introduction

1. General Introduction

Fatigue is physiologically characterised by the inability to maintain muscular force or power. It may have either a peripheral or a central mechanistic origin (Gandavia, 1998; Newsholme *et al.*, 1987; 1991). Under physiological conditions, peripheral fatigue refers to fatigue that has primarily a skeletal muscle cell malfunction in origin involving an inhibition of actin-myosin cross bridges which reduce muscle shortening and therefore power output (Edwards, 1981). Central fatigue implies malfunction at central nervous system (CNS) and spinal cord levels (Asmussen, 1979; Edwards 1981); predominantly, abnormalities in neurotransmitter pathways co-existing with psychological problems (Lloyd, 1998). Nevertheless, when reviewing the literature, one of the main difficulties is how to define fatigue and how to discriminate between central and peripheral fatigue. This becomes more complicated since several reports suggest that there are not only independent peripheral or central factors that contribute in fatigue but both intramuscular metabolic abnormalities and central motor-drive failure may simultaneously and proportionally contribute to developing general tiredness and muscle fatigue (Kent-Braun, 1999; Lewis and Haller, 1991; Swain, 2000). In addition, fatigue during physical activity is influenced by motivation and effort perception (RPE) (Davis and Bailey, 1997; Dugan and Frontera, 2000), and its underlying mechanism varies according to the type, duration and intensity of exercise as well as the fitness and pre-exercise nutritional status of the subjects (Brooks *et al.*, 2000). Therefore, in order to examine central fatigue during exercise, effort perception and peripheral aspects that influence tiredness and muscle fatigue, such as metabolic and cardiovascular responses, have to be taken into consideration.

According to Layzer (1998), four different types of fatigue can be distinguished in physiology and clinical settings. These include objective, subjective, systemic fatigue, and asthenia. *Objective fatigue* is an incapability to maintain a precise force and/or work rate during exercise. *Subjective fatigue* is the fatigue that can be produced by unpleasant feeling, such as muscle pain, dyspnoea and an excessive heart rate during exercise. *Systemic fatigue*, which is non-muscular exhaustion, can be formed during prolonged exercise, due in part, to dehydration, hyperthermia, hypotension, and hypoglycaemia. *Asthenia*, is the fatigue that mainly has a cerebral mediated effect without exhibiting abnormal physiological responses during

exercise. It consists of general exhaustion, weakness, tiredness and the inability to perform minor physical and mental activities.

Furthermore, fatigue can be sub-divided into i) acute, which is usually self-limited fatigue caused by identifiable aetiology, such as physical exertion, and is relieved by appropriate rest, and ii) chronic, which lasts more than six months and is not associated with exertion (usually with multiple-unknown aetiologies) (see Swain, 2000). During physical activity for example, fatigue is a time-related exercise-induced reduction in muscle power capability depending upon the type of exercise and environmental conditions and it may have a peripheral or a central origin (e.g. Bergstrom and Hultman, 1967; Coyle *et al.*, 1986; Davis and Bailey, 1997; Meeusen and De Meirleir, 1995; Nielsen *et al.*, 1993; Sahlin *et al.*, 1998; Woledge, 1998). However, under some abnormal circumstances such as chronic fatigue syndrome (CFS) the aetiology of fatigue cannot be identified easily, probably due to the multiple co-existing clinical symptoms that CFS patients may experience (Fukuda *et al.*, 1994). Although a number of studies exclude skeletal muscle dysfunction-induced fatigue in CFS suggesting a centrally mediated effect (e.g. Bakheit *et al.*, 1992; Bested *et al.*, 2001; Castell *et al.*, 1999; Lane *et al.*, 1998; Lloyd *et al.*, 1988; 1991; Sacco *et al.*, 1999; Sargent *et al.*, 2002; Sharpe *et al.*, 1997; Swain, 2000), the issue remains controversial and the mechanical origin of fatigue and physical activity intolerance in CFS remains unclear.

Some major factors that may influence exercise fatigue development include nutrition, pharmacological treatments and environmental temperature. Pre-exercise diet, caffeine and creatine (Cr) administration and heat-stress could affect peripheral and central mediated factors of exercise fatigue. Their manipulation therefore could contribute to probing, elucidating and understanding of the physiological and biochemical mechanism(s) of central fatigue development. Although the mechanisms of peripheral fatigue have been well characterised and understood the mechanism(s) of central fatigue with particular reference on putative brain serotonergic and dopaminergic modulators during exercise, have not been extensively studied and are not well understood in humans.

1.1 Peripheral fatigue

Peripheral factors contributing to muscle fatigue primarily include intracellular metabolic attenuation of the contractile process and excitation contraction-coupling failure (Baker *et al.*, 1993; Gandavia, 1998). Peripheral fatigue during exercise most likely depends upon the inability of the energy production systems to produce adenosine triphosphate (ATP) (Newsholme and Leech, 1983). This is highly associated with the mode, duration and intensity of exercise and pre-exercise nutritional status (Burke and Hawley 1999; Coyle, 2000; Horowitz and Klein 2000; Lambert *et al.*, 1997; Spriet and Peters, 1998).

During prolonged submaximal exercise for example, in relatively low or thermoneutral temperatures fatigue is due to muscle glycogen depletion (Bergstrom *et al.*, 1967; Galloway and Maughan, 1997) and in part, to a reduction in blood glucose availability and this is associated with the duration of exercise (Coyle *et al.*, 1985). These may drive excitation-contraction coupling failure resulting in a slow time-course of recovery after this type of exercise (Baker *et al.*, 1993; Edwards *et al.*, 1977). During short-duration high-intensity exercise however, glycogen depletion or blood glucose availability do not limit exercise capacity and muscle fatigue is developed by the accumulation of intramuscular metabolic end products (Newsholme and Leech, 1983). These metabolic end products presumably include: i) an increased intramuscular lactic acid, ii) an accumulation in inorganic phosphate (Pi), and iii) an accumulation of interstitial potassium (K^+). i) The intramuscular lactic acid is dissociated into lactate and hydrogen ions (H^+) (Fitts and Cellular 1994). Lactate has a minimal effect in muscle fatigue (Posterino *et al.*, 2001) but H^+ may enhance muscle acidosis by reducing intramuscular pH (Pate *et al.*, 1995). Acidosis, may affect muscle cross bridge or sarcoplasmatic reticulum (SR) function (Fitts and Cellular, 1994; Sahlin *et al.*, 1998; Westerblad and Allen, 2002). ii) The accumulation in inorganic phosphate (Pi), which is elevated during high intensity exercise due to break-down of creatine phosphate (CrP), was found to depress contractile function (Baker *et al.*, 1993; Westerblad and Allen, 2002; Westerblad *et al.*, 2002). iii) The accumulation of interstitial potassium (K^+) was found to reduce excitability of active muscle due to increased [K^+]-induced muscle acidification (Bangsbo *et al.*, 1996; Juel *et al.*, 2000; Nordsborg *et al.*, 2003).

It is likely however, that the cause of muscle fatigue during intense exercise is due to combinations of all the above biochemical reasons without having any direct relationship between each mechanism. For example, the concurrent accumulation of intracellular H^+ and extracellular K^+ which both may reduce pH separately elevating muscle acidosis (for review see Fitts and Cellular, 1994; Westerblad *et al.*, 2002). In addition, evidence suggests that muscle fatigue may be developed during high-intensity exercise due to an elevation in intracellular Ca^{2+} stores, which may become trapped within the SR as a result of precipitation with the elevated levels of phosphate, (Allen *et al.*, 2002) and/or to the depletion of intramuscular phosphocreatine (PCr) concentration [PCr] (Hultman *et al.*, 1990). These result in a reduction in ATP-resynthesis (Soderlund and Hultman, 1991) and therefore energy deficiency (Sahlin *et al.*, 1998). Whatever the situation, it is evident that the development of muscle fatigue during exercise is depending on the fine balance between hydrolysis (as shown in equation 1.2.1) and re-synthesis of ATP in order energy deficiency to be minimised and the intramuscular chemical energy to be converted to mechanical power.

$$ATP^{4-} + H_2O \dashrightarrow ADP^{3-} + Pi^{2-} + H^+ \qquad [1.2.1]$$

However, since the intracellular ATP storage within the skeletal muscle is negligible (~ 5mM/kg) (Bergstrom *et al.*, 1967; Coyle *et al.*, 1986; Hultman *et al.*, 1967), ATP re-synthesis during exercise is provided through the anaerobic alactic, anaerobic lactic and aerobic energy production systems (Newsholme and Leech, 1983). The anaerobic alactic system involves the breakdown of stored muscle PCr which then donates a high-energy phosphate to adenosine diphosphate (ADP) for ATP and Cr production, as shown in equation 1.2.2:

$$PCr + ADP + H^+ \leftrightarrow Cr + ATP \qquad [1.2.2]$$

$$\text{Glucose} + 2P_i + 2ADP \rightarrow \text{pyruvate} \leftrightarrow \text{lactate} + 2H_2O + 2ATP \qquad [1.2.3]$$

The intramuscular PCr that can be stored is at around $15mM \cdot kg^{-1}$ dry weight (250-400mg in total), and therefore provides an extremely rapid but limited supply of ATP (~ 6s for 'all out' exercise) (Hultman *et al.*, 1967; Pernow and Karlson, 1971). The anaerobic lactic system produces ATP through the oxidation of carbohydrates

(CHO) by way of glycolysis and lactate and H^+ production (Gollnick and Hermansen, 1973). Glycolysis involves a series of chemical reactions which break down glucose or glycogen to the end-product pyruvate acid. Pyruvate is then converted to lactate as shown by equation 1.2.3. This yields a low ATP in comparison to the capacity of the aerobic system, but at a more rapid rate (Alpert, 1965).

The aerobic system utilises the energy available through the oxidation of CHO, fats and to a much lesser degree protein (Newsholme and Leech, 1983). As described above, glucose or glycogen is broken down through glycolysis resulting in the production of pyruvate. Since pyruvate is then converted to acetyl coenzyme A (acetyl CoA), it is available for the Krebs cycle within the mitochondria. Fats undergo β-oxidation leading to the production of acetyl CoA. Proteins have a more complex catabolic steps compared to CHO and fats. Proteins depending on the specific amino acids enter the energy production system at the level of pyruvate, acetyl CoA or directly within the Krebs cycle (for reviews see Harper *et al.*, 1984; Wolfe 2000; Newsholme and Leech, 1983).

Acetyl CoA undergoes further chemical reactions within the Krebs cycle, which most importantly lead to the generation of nicotinamide adenine dinucleotide (NADH) and, to a lesser degree, flavin adenine dinucleotide ($FADH_2$). These reduced substrates are then available to the electron transport system and go through a series of redox reactions, with O_2 being the final electron acceptor. The energy from these electron transfers pumps protons across the mitochondrial membrane and the electrical gradient then harnesses the energy required for ATP re-synthesis. The O_2 taken up per ATP produced is higher for fat than CHO, although the CO_2 production per O_2 is less for fat than CHO metabolism as shown by equations 1.2.4 and 1.2.5, respectively (for more details see Newsholme and Leech 1983).

$$C_{16}H_{32}O_2 + 23O_2 \leftrightarrow 16CO_2 + 16H_2O + 130\ ATP \qquad [1.2.4]$$

$$C_6H_{12}O_6 + 6O_6 \leftrightarrow 6CO_2 + 6H_2O + 38\ ATP \qquad [1.2.5]$$

1.1.1 Nutritional considerations and peripheral fatigue

From a historical point of view, the pioneering studies by Krogh and Lindhard (1920) using respiratory exchange ratio (RER) measurement demonstrated the fundamental role of CHO and fat as the major sources of energy production during prolonged exercise. In addition, Levine *et al.*, (1924), in studies measuring blood glucose concentration ([glucose]) at the end of a marathon race identified the basic role of hypoglycaemia in developing muscle fatigue.

After this initial work many studies have been conducted to identify ways to delay an early onset of fatigue during exercise. Gordon *et al.*, (1925) suggested that pre-exercise CHO loading and CHO replacement were able to enhance blood [glucose] and to suppress early muscle fatigue during prolonged exercise. Similarly, Bergstrom and Hultman (1966; 1967), using the direct needle muscle biopsy technique, observed that muscle glycogen was gradually depleted if the submaximal exercise was sustained and it was almost totally depleted after an exhausted prolonged exercise bout. Bergstrom and Hultman, designed an outstanding experiment in order to produce muscle glycogen depletion in an active leg and to compare the muscle glycogen levels between the active and resting quadricep muscle the following days after consuming high CHO diet. Interestingly, they found lower and normal muscle glycogen levels on the active and resting muscles respectively at exhaustion relative to pre-exercise levels. After three days CHO loading the muscle glycogen levels of the active muscle were almost a two-fold higher compared with the inactive muscle. Consequently, they recommended the classic CHO-loading and 'glycogen supercompensation' regimen before and after a prolonged exercise respectively and stressed out the important role of the enzyme glycogen synthase in contributing to glycogen resynthesis.

Thereafter, many studies have supported that CHO loading may enhance endurance performance (Coyle *et al.*, 1983; 1986; Coggan and Coyle, 1987; 1991; Burke and Hawley, 1999; Febbraio *et al.*, 2000) by reducing the rate of glycogen utilization, sparing therefore the glycogen in type I muscle fibres (Tsintzas *et al.*, 1995) and by suppressing hepatic glucose production and increasing glucose uptake through an increased plasma glucose and insulin levels (McConell *et al.*, 1994). However, although Febbraio *et al.*, (2000) found an increase in performance after high CHO

ingestion before and during exercise they did not find a difference in the contribution of CHO and fat oxidation to skeletal muscle. In a subsequent study also, it was found that an additional $3g \cdot kg^{-1}$ (from $6g \cdot kg^{-1}$ to $9g \cdot kg^{-1}$) three days CHO loading (Hawley *et al.*, 1997), or a pre-exercise high CHO ingestion (Sparks *et al.*, 1998) did not improve endurance performance or prevent early glycogen depletion during prolonged exercise. This probably occurred because as stated earlier the liver and muscle glycogen stores capacity are relatively small, at approximately 400g and 90g respectively in a ~70kg male athlete (Bergstrom *et al.*, 1967; Hultman *et al.*, 1967; Coyle, *et al.*, 1986; Maughan, 2002).

On the other hand, it has been shown that pre-exercise CHO loading may result in fat sparing rather than in sparing endogenous CHO stores (Bosch *et al.*, 1993) or muscle glycogen stores during exercise (Bosch *et al.*, 1994; Coyle *et al.*, 1986). It has been also shown that even when large amount of CHO is ingested before or during exercise the amount of blood glucose disappearance (Rd) and uptake is relatively small (Febbraio *et al.*, 2000). The latter is supported by Bosch *et al.* (1994) and Wagenmakers *et al.* (1993). These authors suggested that the maximum oxidation rate of orally-ingested CHO is not more than 1.1 $g.min^{-1}$; even if CHO ingestion rates increases up to 2 $g.min^{-1}$ during exercise the glucose oxidation rate is not more than 1 $g.min^{-1}$. In addition, high rate of CHO ingestion has been found to completely block endogenous glucose production and to attenuate exogenous glucose oxidation (Jeukendrup *et al.*, 1999).

Alternative ways for delaying glycogen depletion during exercise have therefore been investigated. For example, pre-exercise high fat meal (Hawley *et al.*, 2000; Hickson *et al.*, 1977; Okano *et al.*, 1996; 1998; Pitsiladis *et al.*, 1999; Whitley *et al.*, 1998) and medium-chain-triglycerides (MCT) ingestion (Jeukendrup *et al.*, 1996; Angus *et al.*, 2000) were found to enhance circulating plasma [FFA] and fat oxidation. Hickson *et al.* (1977) using Wistar rats examined whether an increase in circulation of plasma FFA by corn oil/heparin infusion could enhance CHO sparing by slowing the rate of glycogen use during prolonged exercise. They found an increase in circulating plasma levels of FFA, blood glycerol, 3-hydroxybutyrate (3-OHB), and improvement in endurance exercise performance in the rats that were administrated fat-oil/heparin (5 ml of corn oil + 200 U sodium heparin) relative to control (5 ml of carboxymethyl cellulose + 0.9% NaCl), results that were attributed

to CHO-sparing. Similarly, Pitsiladis *et al.* (1999) examined the combined effect of CHO loading and pre-exercise high fat meal/heparin administration on metabolic responses and endurance performance during prolonged exercise to exhaustion at 10°C. Although no significant differences between trials were observed on major cardiovascular and metabolic responses ($\dot{V}O_2$, RER, HR, blood glucose and lactate) there were significant differences throughout exercise in plasma [FFA], resting blood glycerol and plasma TG levels, which all were higher in the high fat trial. Pitsiladis *et al.* (1999) concluded that such diet manipulation may improve endurance performance of well-trained cyclists by increasing circulating FFA levels, resulting in an earlier rise in the rate of fat utilisation and sparing CHO. However, although other investigators found an increase in fat oxidation and CHO sparing (Hawley *et al.*, 2000) or an enhancement in plasma [FFA] (Okano *et al.*, 1996; 1998), [glycerol] and [3-OHB] (Whitley *et al.*, 1998) following pre-exercise high fat meal manipulations they observed no improvement in endurance.

It is possible that the inconsistencies between the above studies regarding metabolism and exercise performance, (after artificial elevation in plasma [FFA]), to be due to the different experimental protocols used. For example, different i) environmental temperatures (see Galloway and Maughan, 1997), ii) fluid replacement protocols during the trials (see McConell *et al.*, 1997), iii) pre-exercise diet manipulation regimes (see Hawley and Burke, 1997; Tarnopolsky *et al.*, 1995), iv) exercise intensities, prolonged exercise to fatigue for example, between 50% to 80% of $\dot{V}O_{2\,max}$ and/or time-trials (see Angus *et al.*, 2000), and v) inadequate recovery after each experimental trial by some studies (i.e. only 24 hours recovery) (e.g. Whitley *et al.*, 1998) but not by others (more than 48 hours recovery) (see Goforth *et al.*, 1997). According to Goforth *et al.* (1997), after prolonged exercise, at least 48 hours are required under resting supercompensated condition in order to achieve a complete hepatic-glycogen store recovery.

However, some other important central neural-factors may play a role in modifying exercise performance after pre-exercise high fat meal manipulation. For example, the artificial elevation in plasma [FFA], although it may increase fat oxidation and enhance CHO-sparing in some cases (Hawley *et al.*, 2000; Hickson *et al.*, 1977; Pitsiladis *et al.*, 1999) it may reverse this metabolic benefit in some others by enhancing brain serotonin (5-HT) turnover and increasing effort perception (Young,

1986). Plasma FFA may displace tryptophan (Trp) (the brain 5-HT precursor) from plasma albumin and therefore elevate brain Trp uptake and 5-HT turnover (Curzon *et al.*, 1973) (see sections 1.2.1 and 1.2.5 for more details). Pitsiladis *et al.*, (1999) for example, observed that after a pre-exercise high fat meal/heparin administration there was an earlier rise in perceived exertion and 5 out to 6 subjects ranked the fat trial as the most difficult.

Some studies linked brain 5-HT metabolic up-regulation with elevation in effort perception during exercise (Blomstrand *et al.*, 1995; 1997; Davis *et al.*, 1993). Davis and Bailey (1997) have proposed that a high brain 5-HT:DA ratio may favour central fatigue, while a low 5-HT:DA ratio may favour increased arousal and motivation. Consuming a high fat meal prior to exercise has the potential, therefore, to increase brain 5-HT turnover elevating perception of effort. On the other hand, a recent study, has shown an enhancement in exercise performance when the brain 5-HT:DA ratio was reduced after intracerebral caffeine injection (Davis *et al.*, 2003). Some others have also demonstrated that caffeine reduced effort perception during exercise resulting in enhanced exercise performance (Cole *et al.*, 1996; Bridge *et al.*, 2000). Perception of effort during exercise may therefore be the outcome of a fine balance between the 5-HT and DA functions, with changes in effort perception resulting from a disturbance of this balance. Consequently, it could be hypothesised that if the increased effort perception after a high fat meal (Pitsiladis *et al.*, 1999) imitates an increased brain 5-HT function and the reduction in effort perception with caffeine (Bridge *et al.*, 2000; Cole *et al.*, 1996; Jacobson *et al.*, 2001) reflects a reduction in brain 5-HT:DA ratio (Davis *et al.*, 2003) this effect might be reversed if caffeine is co-ingested with the high fat meal. However, the exact mechanism in reducing effort perception after caffeine ingestion and/or whether effort perception is associated with the brain 5-HT and DA functions in humans during exercise is still unknown.

1.2 Central fatigue

Various approaches have been used to establish a definition for central fatigue, to distinguish its functional development and mechanical origin and to discriminate it from peripheral fatigue. Central fatigue can be defined as the voluntary or involuntary inhibition of motor units resulting in a reduction in motor unit firing frequency due to malfunction of neuronal-drive centres (Edwards, 1981). These CNS dysfunctions, which reduce the ability to fully activate the muscle, cannot be explained by a dysfunction within the skeletal muscle cell (Davis and Bailey, 1997). Central fatigue is characterised by a progressive decline in discharge rate of moto-neurones and therefore a progressive reduction in muscle contraction (Gandevia, 1998). CNS fatigue can be distinguished from peripheral fatigue by comparing the repeated maximal voluntary force generated with a maximal electrical (tetanic) stimulation. It has been suggested for example that when there is a greater fall in voluntary force compared to electrical stimulation force, fatigue is partly due to central factors. However, when both muscle contractions, voluntary and tetanic force decline in synchrony the fatigue has a muscular mediated effect and not a CNS origin (Asmussen, 1979; Bigland-Ritchie, 1981; Bigland-Ritchie *et al.*, 1986).

Aspects that may be implicated in producing central fatigue or central inhibition include an attenuation of motor-cortex drive information supply, increase in tremor of the exercising limb and inability of the motor neurons to recruit only the specific muscle-group associated with the muscle contraction in order to achieve an energetic task (Gandevia, 1998). However, the reduction in motor neurons charge may be due to mechanoreceptors reflex feedback or associated with skeletal muscle activation-end products that may affect group III and IV nerve endings (Bigland-Ritchie *et al.*, 1992). Therefore, it is reasonable to consider that central fatigue during exercise is perhaps associated with muscle metabolic end products. No strong body of studies has to date simultaneously examined the interaction between central components of fatigue and muscle metabolic responses during exercise.

The various techniques of comparing voluntary and involuntary muscle contraction (Twitch Interpolation Technique) to examine peripheral and central fatigue, however, preclude the examination of central physiological and biochemical parameters, which may play a role in fatigue process when many muscles groups are

involved during exercise (for review see Enoka and Stuart, 1992). Furthermore, although the exact mechanism(s) in driving to moto-neuronal failure is not clear, brain serotonergic pathway dysfunction may play a role. It was recently reported that the primary functions of brain 5-HT system is to facilitate the motor output and to concurrently restrain sensory information processing (Jacobs and Fornal, 1993; 1999). For example, it has been suggested that during metabolic up-regulation of brain 5-HT synthesis, more than the normal amount of this neurotransmitter can be released into the synaptic cleft during neuronal firing charge resulting in a higher postsynaptic stimulation in 5-HT neurons which may enhance central fatigue and lethargy (Newsholme *et al.*, 1987). However, the metabolic regulation of brain 5-HT is mainly dependent on the availability of plasma amino acids precursors, free and total Trp. Brain 5-HT does not contain an enzymatic step that is rate limiting for its own bio-synthesis and formation (Anderson, 1981; Curzon *et al.*, 1973; Sarna *et al.*, 1985).

1.2.1 Tryptophan: structure, synthesis and metabolism

Interest in the amino acid Trp (Figure 1.1) centres on its role as a precursor to brain 5-HT (Figure 1.2.), which acts as a neurotransmitter allowing neurons to transfer electrical impulses to other neurons and cells (Fernstrom, 2000) and regulating significant physiological functions such as mood, sleep, thermoregulation, pain, appetite and fatigue (Young, 1986).

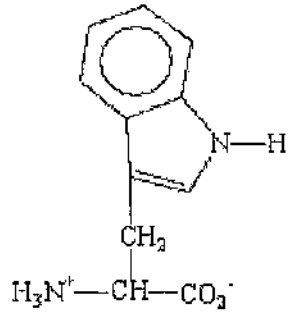


Figure 1.1 Chemical structure of the essential amino acid tryptophan

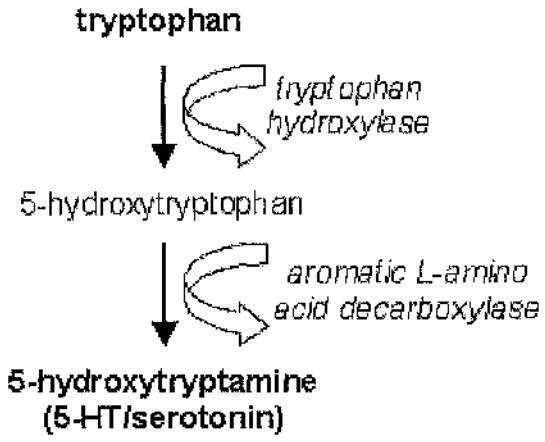


Figure 1.2 Conversion of tryptophan to brain 5-hydrohytryptamine (5-HT)

Trp is an essential amino acid which is similar to phenylalanine and tyrosine in structure with aromatic amino-acids groups (Young, 1986). Its concentration in human cells is dependent upon dietary intake and the peripheral protein synthesis (Reilly *et al.*, 1997). Rich dietary sources of Trp include poultry meats, milk, dairy, eggs, nuts, wheat germ and mainly grains and legumes such as beans (Newsholme and Leech, 1991). The daily requirement amount of Trp in an adult is around 0.25 g and dietary deficiency, which can induce a negative nitrogen balance, is rare under normal circumstances (for review see Newsholme and Leech, 1991). Nevertheless, it has been reported that the average daily Trp intake is at around 1-1.5 g and during clinical therapeutic prescription is at approximately 4 g (Reilly *et al.*, 1997).

Although little is known about chronic or excessive Trp ingestion, the majority of psychopharmacological and nutritional studies suggest that acute Trp depletion or loading significantly affects human behaviour by reducing or elevating brain 5-HT synthesis (for reviews see Young, 1986; Reilly *et al.*, 1997; Fernstrom, 2000). In short, studies carried out by Young and colleagues (1985; 1988) showed that Trp-deficient drink caused a marked plasma Trp depletion promoting depression and reducing performance on a proof-reading task. In addition, more recent evidence suggests that rapid dietary Trp depletion increases subjective and behavioural aggression in subjects with high-trait aggression (Cleare and Bond, 1995), and anxiety and fear in subjects with panic disorders (Miller *et al.*, 1995). Furthermore, acute Trp depletion increased impulsiveness and reduced stimulus-discriminating ability of normal individuals (Walderhaug *et al.*, 2002). In contrast, a high Trp diet

increased the plasma [Trp]:[LNAA] ratio, improved mood and reduced depression during acute stress in vulnerable subjects by elevating brain 5-HT synthesis indicated by measuring plasma prolactin (Prl) levels (a brain 5-HT marker) (Markus *et al.*, 2000).

After digestive processing of dietary proteins (to amino acids and di- or tripeptides) and proteolysis in the lumen of the intestine, Trp is released to the circulation and then is mainly absorbed and metabolised by the liver where is oxidised by tryptophan oxidase (Newsholme and Leech, 1991). Thereafter, Trp may be metabolised by three major metabolic pathways in mammals. i) In the periphery Trp is metabolised to formyl-kynurenine by the enzymes tryptophan-2,3-dioxygenase and indoleamine-2,3-dioxygenase and then to kynurenine by the enzyme kynurenine formylase (Lapin, 1980; Stone and Connick, 1985; Moroni, 1999). ii) In the brain Trp is metabolised to 5-HT via hydroxylation of Trp and decarboxylation of 5-hydroxytryptophan and the rate-limiting enzyme of this reaction is tryptophan hydroxylase (Newsholme *et al.*, 1987). In the brain Trp, can also be metabolised to kynurenine by the enzyme indoleamine-2,3-dioxygenase, under some abnormal circumstances such as inflammation (Saito *et al.*, 1993). Metabolism of Trp to kynurenine in the brain is believed to account for 45% of its metabolism to 5-HT (Gal and Sherman, 1980). iii) Lastly about 3% of Trp is metabolised to tryptamine in the brain (Lapin, 1981) and tryptamine is further converted to kynuramines (Stone and Darlington, 2002).

It was reported that more than 95% of dietary Trp (that is not used in protein synthesis) is metabolised to kynurenine (Stone, 1993; Stone and Darlington, 2002). It was of a great interest to note that one of the significant reactions after the conversion of Trp to kynurenine in the liver is that kynurenine can be easily carried to cerebrospinal fluid (Heyes and Quearry, 1990) or directly passed to various brain regions by the LNAA-carrier (Fukui *et al.*, 1991). Therefore, its concentration may be elevated in the brain. It was previously found for example that at around 60% of the brain [kynurenine] has a peripheral origin (Gal and Sherman, 1980). In the brain after the conversion of Trp to kynurenine the later can further be metabolised to kynurenic acid by the kynurenine aminotransferases, to quinolinate acid by 3-hydroxyathralinic acid oxidase (Stone and Darlighton, 2002) or to antharnilic acid by kynureninease (Amirkhani *et al.*, 2002). Kynurenic acid was found to be

neuroprotective but quinolinate acid highly neurotoxic and it may cause mitochondrial dysfunction, increase free-radical generation and produce neuronal damage in vivo (Stone and Darlighton, 2002). Although the normal concentration of quinolinate acid in the brain is around and seldom surpasses 1μM, in some abnormal circumstances such as in response to bacterial endotoxin it can be increased to the levels of 246μM and 66μM in the brain and extracellular space respectively, producing toxicity within a few hours (for review see Stone and Darlington, 2002). Consequently, quantifying plasma [kynurenine] and its metabolites during exercise-stress may give a better understanding of the role of plasma Trp uptake by the brain and also examine whether kynurenine metabolites play a role in central fatigue during exercise.

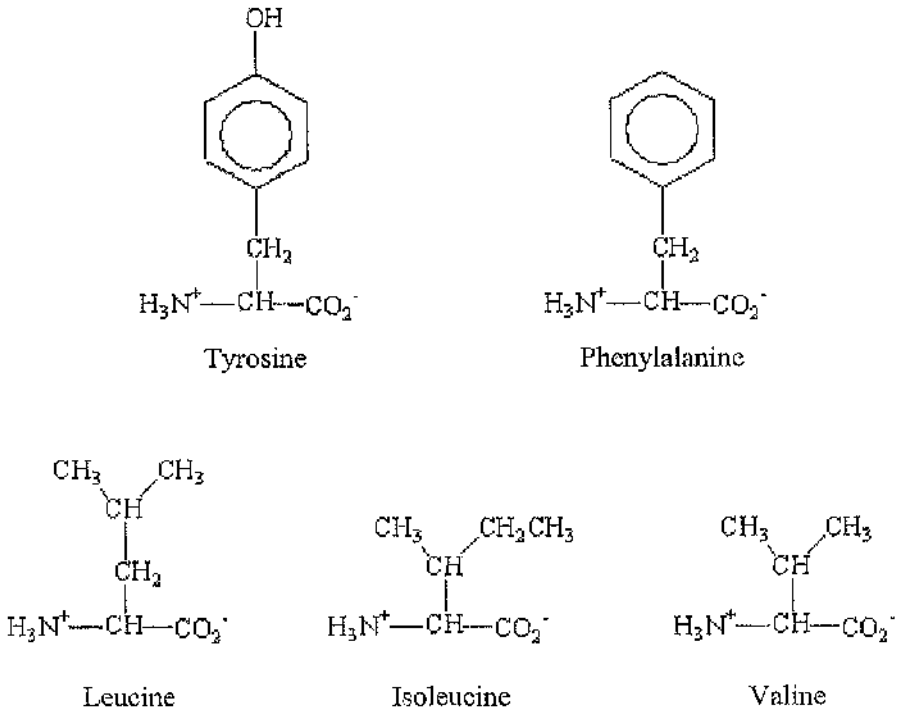


Figure 1.3 Chemical structures of the large neutral amino acids: tyrosine, phenylalanine, leucine, isoleucine, and valine

One of the main distinguishing characteristics of Trp from the other amino acids is that it is loosely bound to plasma albumin (McMenamy and Oncley, 1958) and shares, with the other LNAA including the aromatic amino acids, the same plasma transport (L-system) carrier (Fernstrom and Wurtman, 1972). The L-system carrier includes leucine, isoleucine, valine, phenylalanine, methionine, tyrosine and tryptophan (Kilberg *et al.*, 1980). The LNAA include tyrosine, phenylalanine,

leucine, isoleucine, valine; the branched chain amino acids (BCAA) include valine, leucine, and isoleucine; the aromatic amino acids include phenylalanine and tyrosine (Figure 1.3).

It was reported that 90% of Trp is loosely bounded to plasma albumin and the remaining 10% is unbound (Cangiano *et al.*, 1999). Consequently, plasma [Trp] can change proportionally to the plasma [LNAA] and easily cross the blood brain barrier (BBB) (Fernstrom and Wurtman, 1971; Pardridge, 1977). This may enhance brain 5-HT synthesis although controversy does exist. A few studies for example, suggested that brain 5-HT synthesis may increase irrespective of the plasma [Trp] and brain Trp uptake (Elks *et al.*, 1979) or Trp levels do not modify brain serotonergic neurons functional activity (Trulson, 1985).

One of the main questions regarding Trp regulation, metabolism and its role on 5-HT synthesis is whether it is the free, albumin-bound Trp or both that contribute in brain 5-HT synthesis (Bloxam *et al.*, 1980; Chaouloff *et al.*, 1986; Yuwiler *et al.*, 1977). It has been previously observed that the bound Trp molecules have the ability to be released rapidly from albumin molecules in the blood enabling all bound Trp molecules to be taken up from brain and enhance 5-HT synthesis (Pardridge and Fierer, 1990; Fernstrom and Fernstrom, 1993). However, this action may be dependent on the experimental conditions. For instance, starvation was reported to elevate brain Trp levels by increasing availability of free Trp due to an enhancement in lipolysis and plasma [FFA], which may displace Trp from albumin (Chaouloff, 1993; Curzon et al. 1973).

Some physiological parameters may affect the delivery of Trp to the brain-regions and therefore the brain 5-HT synthesis. These include i) the plasma Trp binding to albumin (Bloxam *et al.*, 1980; Sarna *et al.*, 1985; Yamamoto *et al.*, 1997); ii) The plasma LNAA that competes with Trp to cross the BBB (Fernstrom and Wurtman, 1972); iii) The plasma [FFA], which may displace Trp from albumin (Curzon *et al.*, 1973); iv) The degree that total Trp is freed from albumin when the blood passes through the brain regions (Pardridge, 1979); v) Disturbances of the L-system transporters (Yamamoto and Newsholme, 2000); vi) Alteration in peripheral or brain kynurenine pathways of Trp metabolism that may affect plasma and brain [Trp] and therefore diminish Trp metabolism to brain 5-HT (Tiihonen *et al.*, 2001); vii) In

vitro studies also showed that an increase in plasma kynurenine levels may enhance plasma free-[Trp] by reducing the binding Trp to albumin since kynurenine competes with Trp for binding to plasma albumin (Cangiano *et al.*, 1999).

1.2.2 Brain serotonergic system and behaviour

5-HT was initially discovered in the serum by Rapport and colleagues (1948) who suggested that 5-HT has the ability to elevate blood vessel tone (cited by Dinan, 1996). However, the discovery of 5-HT in the brain regions was only reported in the mid fifties by Twarog and Page (1953) and further established in the early sixties by Dahlstrom and colleagues (1965 cited by Dinan, 1996). Its significant role in regulating various neurochemical reactions in human and, more specifically, in hypothalamic and neuroendocrine regulation, was discovered only over the last two decades (for review see Dinan, 1996).

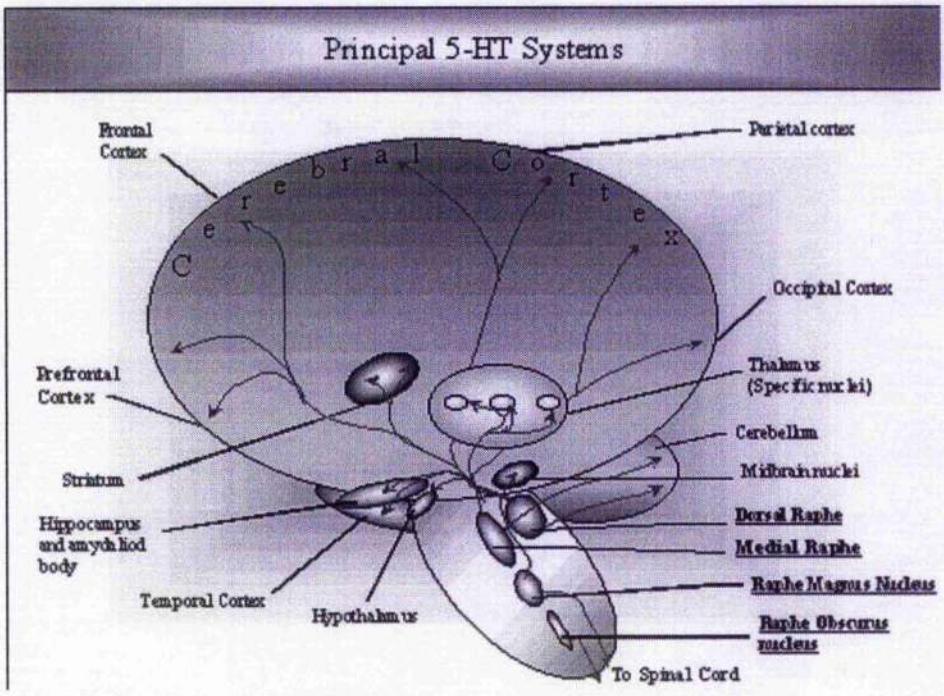


Figure 1.4 Schematic illustrations of the brain parts that accommodate the brain 5-HT synthesis and actions.

Brain 5-HT is a member of indolamines, which included with catecholamines (dopamine, epinephrine and norepinephrine) in biogenic amines. The cell bodies of serotonergic neurones are present in the mesencephalon, pons and medulla oblongata which are all mainly located in or near the brain stem raphe nuclei with projection to the hypothalamus (Jacobs and Fornal, 1999) and possibly to all areas in

CNS (Figure 1.4). For example, efferent fibres of 5-HT-containing neurons may innervate the hippocampus, cortex, nucleus caudatus and accumbens, putamen, the substantia nigra, various thalamic centres, medulla and ventral horn of the spinal cord (Meeusen and De Meirleir, 1995). As stated above, all brain 5-HT is synthesised in the brain because the rate limiting enzyme, tryptophan hydroxylase, of brain 5-HT biosynthesis is not saturated under physiological conditions (Fernstrom and Wurtman, 1972; Reilly *et al.*, 1997).

Brain 5-HT is charged at physiological pH without being able to cross the BBB or to be diffused into cells from the extracellular space (Diksic and Young, 2001). The rate at which 5-HT is released from neurons in the brain has been demonstrated to influence the rate of 5-HT turnover and synthesis (Schaechter and Wurtman, 1990). The specific brain-regions that are implicated to accommodate the 5-HT synthesis are mainly hypothalamus (Bailey *et al.*, 1993b) midbrain, hippocampus, striatum (Chaouloff *et al.*, 1989), and frontal cortex (Meeusen *et al.*, 1996) (Fig. 1.4). Brain 5-HT is believed to play an important role in various neuroendocrine, physiological and subjective behavioural functions. These include regulation of the activation of the hypothalamic-pituitary-adrenal (HPA) axis, anterior pituitary and the adrenal cortex, appetite, locomotion, thermoregulation, sleep, arousal, pain sensation, effort perception and exercise fatigue (Acworth *et al.*, 1986; Ben-Jonathan *et al.*, 1989; Dinan 1996; Newsholme *et al.*, 1987; Wilckens *et al.*, 1992; Young 1986). There is now considerable evidence to support the concept that the presence of 5-HT nerve terminals and/or receptors in different brain regions are associated with different responses to various behavioural and neuroendrocrinological stressors. For example, hippocampus, hypothalamus, brainstem and medulla are key stress-related neuroendocrine regulators regions and amygdala, striatum, part of hippocampus, cortex and periaqueductal grey are key stress-related behavioural responses regions (Chaouloff *et al.*, 1999). Consequently, the neuroendrocrinological responses intended to provide physiological adjustments, via the sympathetic nervous system and corticotropic axis, which permit suitable behavioural responses to stimulus (Chaouloff *et al.*, 1999).

The significant role of brain 5-HT on human behaviour is due to its action as a neurotransmitter. Like dopamine (DA) (see below) and nor-epinephrine, 5-HT is known to act as chemical messenger transmitting signals from one neuron to another

including chemicals by which efferent neurons communicate with effector cells (Meeusen and De Meirleir, 1995). Changes therefore, in the synthesis and concentration of one neurotransmitter may influence the activity of the other reflecting modifications in neuroendocrine, physiological and psychological conditions (Dishman, 1997; Newsholme *et al.*, 1987). For these reasons many studies examined the metabolic effect of brain 5-HT on various clinical diseases. However, the actual role of this neurotransmitter and its effect in modifying the functional status of human behaviour in health and disease during exercise remains to be determined. This probably occurs because the serotonergic neurons interact with the other monoaminergic neurons (dopaminergic, noradrenergic) (Davis and Bailey, 1997; Meeusen *et al.*, 2001) and/or act independently to modify brain function and motor output (Gu, 2002). Previously, there has been discussion about the actual role of each of the monoaminergic systems linking dopaminergic neurons mainly with motor activity and norandrenergic system primarily with autonomic function (Meeusen and De Meirleir, 1995). However, recent evidence suggests that serotonergic neurons play a vital role not only in regulating smooth and cardiac muscle activation but also in altering neuroendocrine function, facilitating skeletal-muscle motor activity (Jacobs and Fornal, 1999) and coordinating sympathetic nerve activity (Bago *et al.*, 2002).

1.2.3 Brain dopaminergic system and behaviour

The role of the neurotransmitter DA or 3,4 dihydroxyphenylethylamine in human behaviour was first reported only five decades ago (see Baumester and Francis, 2002). Thereafter, several clinical studies support the significant effect of brain DA metabolism on affective behaviour (Moore and Lookingland, 2000) and neurological diseases such as Parkinsonism, Alzheimer (Prat *et al.*, 2000; Smith and Zigmond, 2003; Sutoo and Akiyama, 2003) and schizophrenia (Baumeister and Francis 2002). In addition, brain DA system has been reported to affect mood state and cognitive behaviour (i.e. D_1 receptors) (Goldman-Rakic *et al.*, 2000). Although DA was the first neurotransmitter that has been reported to potentially determine motor activity (Freed and Yamamoto, 1985) and CNS fatigue (Bliss and Ailion, 1971) in rat, only few studies have examined the role of putative modulators of brain DA function in central fatigue during exercise in humans (e.g. Bridge *et al.*, 2003).

Dopaminergic cells group are found in the mesencephalon, the diencephalon and the telencephalon and most of the DA neurons, in the higher vertebrates, are located in the midbrain within the substantia nigra and the ventral tegmental area (for review see Goridis and Rohrer, 2002). The substantia nigra and ventral tegmental DA-neurons innervate the striatum (or limbic system) and neocortex respectively (Hynes and Rosenthal, 1999). The loss of substantia nigra DA-neurons result in the motor disorders such as Parkinsonism whereas overstimulation of ventral tegmental DA neurons has been associated with mental illnesses such as schizophrenia and drug addiction (see Hynes and Rosenthal, 1999).

DA is synthesised from the amino acid Tyr that is initially hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) by Tyr hydroxylase, the rate-limiting enzyme for brain DA biosynthesis (for DA structure, synthesis and metabolism see Fig. 1.5).

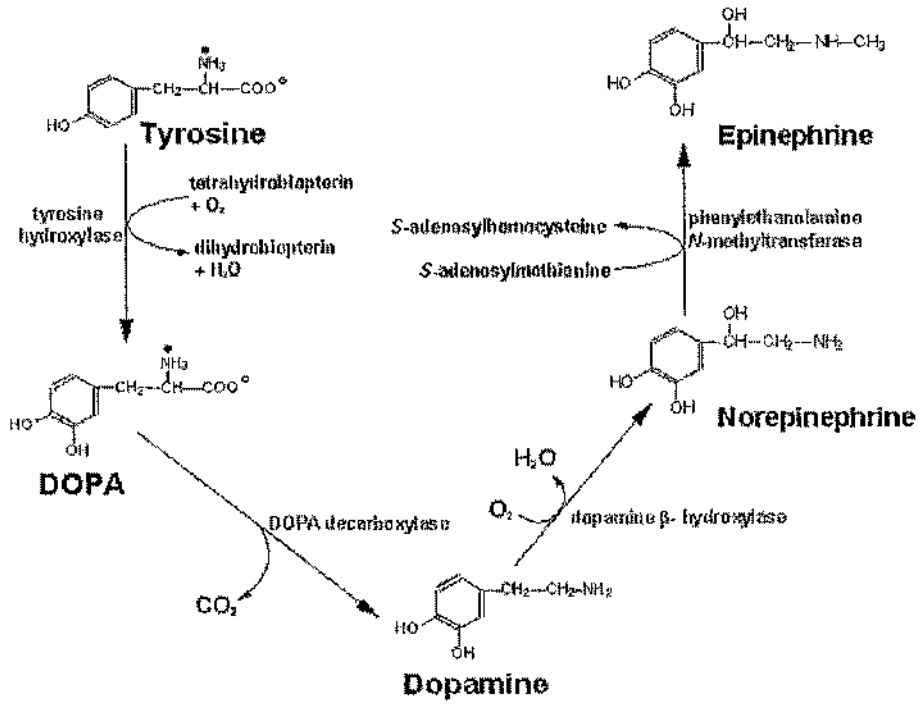


Figure 1.5: Dopamine structure, enzymatic pathways of DA synthesis and DA metabolism. Adapted from Moore and Lookingland, (2000).

The majority of this enzyme is located within the catecholamine nerve terminals. DOPA then, is decarboxylated to DA by the enzyme dopa-decarboxylase. DA, under normal physiological conditions is metabolised to 3,4-dihydroxyphenelinic acid (DOPAC) by monoamine oxidase and aldehyde oxidase. Thereafter, DOPAC is metabolised into homovallinic acid by catecho-*o*-methyltransferase (for reviews of

DA metabolism see Goridis and Rohrer, 2002; Fernstrom and Fernstrom 1994; Moore and Lookingland, 2000). However, although changes in plasma [Tyr] via nutritional effects may modify brain DA metabolism (Fernstrom and Fernstrom 1994; 1995), some studies have recently demonstrated a direct metabolic up-regulation of brain DA synthesis, through predominantly adenosine receptors-inhibition effects (e.g. Davis *et al.*, 2003; Fredholm *et al.*, 1999), irrespective of levels of peripheral [Tyr] and brain Tyr uptake (for more details see section 1.2.5.3). In addition, it has been suggested that daily exercise may increase Ca^{2+} levels and serum Ca^{2+} is transported to the brain where it activates Tyr hydroxylase which stimulates DA synthesis through a calcium/calmodium-dependent system (for review see Sutoo and Akiyama, 2003).

Some studies, using rats support but not entirely explain the role of brain dopaminergic function in modifying exercise performance and central fatigue (Bailey *et al.*, 1993a; Chaouloff *et al.*, 1986a; Davis *et al.*, 2003; For more extensive discussion associated with these studies see sections 1.2.5.2 and 1.2.6.4). Consequently, more studies are required to examine the role of brain dopaminergic modulators and function in central fatigue in humans.

1.2.4 Methods for detecting brain 5-HT and DA metabolism

Various invasive and non-invasive methods have been applied to examine brain 5-HT and DA metabolism. The most extensively used methods include i) whole brain microdialysis techniques (see Benveniste and Huttemier, 1990), ii) cerebrospinal fluid (CSF) collection for inspecting 5-hydroxyindole-3-acetic acid (5-HIAA) and 3,4-dixydroxyphenylacetic acid, the brain 5-HT and DA metabolites respectively (e.g. Chaouloff *et al.*, 1986a; Bertilsson, 1987), iii) Positron Emission Tomography (PET) (see Diksic and Young, 2001) for detecting small structures such as brain stem and brain metabolic responses (Tirelli *et al.*, 1998), iv) α-methyl-L-tryptophan method employed alone (Diksic and Grdisa, 1995) or in combination with auto-radiography or PET techniques (see Diksic and Young, 2001), v) measurement of plasma [Prl] (Ben-Jonathan *et al.*, 1989; De Meirleir *et al.*, 1985b).

Microdialysis, may be predominantly used in small animals during free movement with limited amount of brain trauma tissue in order to assess alterations in

neurotransmitters metabolisms in brain extracellular space (Benveniste and Huttemier, 1990). Autoradiography measurement is for visualising small animals' brain 5-HT synthesis under specific stressors (see Nagahiro *et al.*, 1990). α-methyl-L-tryptophan method permits the imaging of the brain trapping of labelled α-methyl-L-tryptophan, which is analogous of Trp, enabling the calculation of the brain 5-HT synthesis (see Diksic and Young, 2001).

Some methods employed however, to examine brain neurotransmission are not in general applicable when humans are tested. These include methods where a 5-HT agonist or antagonist drugs are administrated and brain microdialysis or autoradiography techniques are used for direct quantification of 5-HT metabolism. In addition, apart from not being generally applicable in humans the validity of α-methyl-L-tryptophan method has recently been extensively criticised. α-methyl-L-tryptophan for example, is a substrate for Trp hydroxylase but not for protein synthesis and its final metabolite product, which is α-methyl-5-hydroxytryptamine, accumulates in brain tissue as it is not a substrate for monoamine oxidases. Consequently the calculation of 5-HT synthesis using this method is inaccurate (see Gharib *et al.*, 1999; Shoaf *et al.*, 2000). The PET method however, although it has many advantages is economically limited (Tirelli *et al.*, 1998). Furthermore, although the CSF collection-method has been previously found useful in animal studies (Chaouloff *et al.*, 1986a), some limitations should be noted. For example, there are not matching responses between changes in brain neurotransmission (e.g. 5-HT and DA) synthesis and CSF metabolites (e.g. 5-HIAA and DOPAC) (for review see Diksic and Young, 2001). Indeed, in a study conducted by Chaouloff *et al.*, (1986a) there was an increase in brain DA synthesis and its metabolite, DOPAC, during running but there were no changes in CSF-DOPAC levels. In addition, the pain and discomfort of a lumbar puncture is unethical for human volunteers. For these reasons and for practical perspectives it is obviously difficult to collect multiple CSF samples for examining the rate responses of 5-HT synthesis during exercise. Consequently, probably the most economical and practically applicable method would be with human subjects undergoing examination and multiple blood samples collected, in order to investigate brain 5-HT and DA metabolism is the plasma [Prl] method with a simultaneous examination of the putative brain 5-HT (plasma Trp, LNAA, FFA) and DA (plasma Tyr) modulators.

1.2.4.1 Prolactin: brain 5-HT and DA metabolic interaction marker

Prl, which in human is composed of 199 amino acids, is a single-chain protein hormone secreted by the adenohypophysis (pituitary gland) (Sinha, 1995). This is an endocrine gland in the small, bony cavity at the base of the brain with projection to hypothalamus (Ben-Jonathan *et al.*, 1989). The majority of human studies are currently limited to examining the association between peripheral indices (markers) of central fatigue and exercise performance due to difficulty in directly assessing brain 5-HT and DA levels.

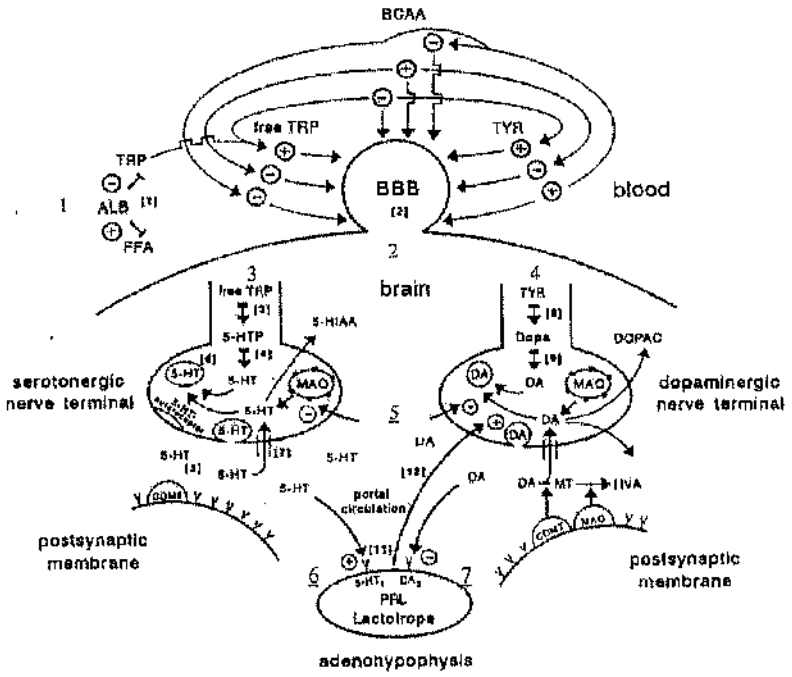


Figure 1.6 Schematic illustration of the control of hypothalamic (anterior pituitary gland) prolactin (PRL) release. 1. Augmentation of plasma FFA increases free-TRP displacement from albumin. 2. Plasma free-TRP, BCAA, and TYR compete for transport to the brain over the L-carrier at the BBB. 3. The brain free-TRP is converted to brain 5-HT at serotonergic nerve terminals. 4. The TYR is converted to dopamine at dopaminergic terminals. 5. The newly synthesised 5-HT and DA are released in the outer layer of the median eminence. Then they enter the portal circulation, where they may directly act on the mamotrophs of the anterior pituitary. 6. 5-HT which is bounded to 5-HT_1 receptors has a stimulatory effect on PRL release. 7. DA, which is bounded to DA_2 receptors, has an inhibitory effect on PRL release. Adapted by Struder *et al.* (1998) and re-modified based on Struder *et al.* (1998), Bed-Jonathan *et al.* (1989), Nagy *et al.* (1992).

However, evidence suggests that Prl release is mainly under the control of the central serotonergic system (De Meirleir *et al.*, 1985a; b) and/or under the hypothalamic 5-HT and DA metabolic interaction (for review see Ben-Jonathan *et al.*, 1989; Freeman *et al.*, 2000). For example, DA has been suggested to be the major Prl-secretion inhibitor factor (Nagy *et al.*, 1992) and 5-HT injection or its

agonist precursors and re-uptake inhibitors have been found to increase hypothalamic Prl release (e.g. Somoza *et al.*, 1983; Van de Kar *et al.*, 1996) (Figure 1.6). Confirming the regulatory control of brain serotonergic system on Prl secretion, recent clinical studies using a 5-HT_{1A}-receptors agonist drug, buspirone, observed an elevation in plasma [Prl] suggesting a metabolic up-regulation of these receptors resulting in enhancing Prl secretion (Bakheit *et al.*, 1992; Meltzer and Maes, 1994; Sharpe *et al.*, 1996).

It should be noted that some studies do not support that changes in peripheral modulators of brain 5-HT influence Prl secretion and/or brain 5-HT and DA metabolic interaction. For example, Struder *et al.* (1995) found an elevation in plasma free-[Trp] and free-[Trp]:[BCAA] ratio during tennis but there was no effect on plasma [Prl]. They suggested a dissociation between plasma free-[Trp]:[BCAA] ratio and [Prl] during exercise. However, although inconsistency does exist, strong body of evidence suggests that plasma [Prl] represents brain 5-HT metabolism and/or 5-HT and DA metabolic interaction (e.g. Ben-Jonathan *et al.*, 1989; De Meirleir *et al.*, 1985a; b; Fischer *et al.*, 1991; Lamberts and MacLeod, 1978; Nagy *et al.*, 1992; Somoza *et al.*, 1983; Struder *et al.*, 1997; Van de Kar *et al.*, 1996).

1.2.5 Brain 5-HT and DA metabolism and 'central fatigue'

The first studies to report a metabolic up-regulation of brain 5-HT synthesis during exercise were published in the early sixties and mid-seventies by Barchas and Freedman (1963) and Romanowski and Grabiec (1974). They used animal models and observed a significant elevation in the whole brain [5-HT] after prolonged swimming and running respectively. Thereafter, and based on the role of brain dopaminergic system in controlling human movement (Freed and Yamamoto, 1985), DA has been implicated in fatigue process during exercise. Heyes *et al.*, (1985; 1988) for example, suggested that manipulation of brain dopaminergic system was able to modify exercise performance. However, they did not indicate to what extent and under which mechanisms DA influence exercise performance. Subsequently, in a pioneering series of studies by Chaouloff and colleagues (1985; 1986a; b; 1987) it was demonstrated the association between i) plasma free-Trp:BCAA ratio and brain 5-HT synthesis, ii) CSF and 5-HIAA levels and iii) brain 5-HT/DA metabolic

interaction and motor activity during exercise. Chaouloff *et al.*, (1985) for example, use rats to examine the effect of prolonged submaximal running (1 h and 2 h) on lipolysis, [Trp] in plasma, liver and brain and on whole brain 5-HT and 5-HIAA. They found that prolonged running significantly increased plasma [FFA], which displaced Trp from albumin and therefore elevated brain free Trp uptake leading to accelerating brain 5-HT synthesis. It is noted that these responses to running were more potent after 2h than after 1h of activity and also they were more effective to pre-exercise fasting than non-fasting condition (Chaouloff *et al.*, 1985).

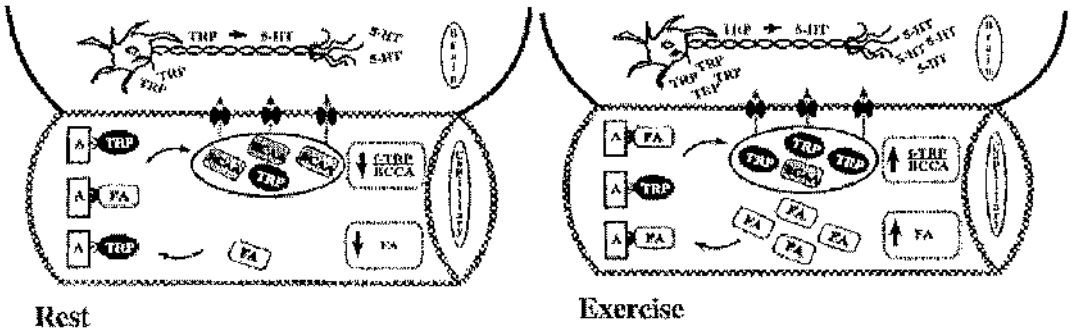


Figure 1.7 The primary components of 'central fatigue' hypothesis at rest and during prolonged exercise (see text for details). BCAA: branched-chain amino acids; FA: Fatty acids; f-TRP: free TRP; 5-HT: 5-hydroxytryptamine (serotonin); TRP: tryptophan. Adapted from Davis *et al.*, (2000).

Newsholme *et al.*, (1987) proposed the 'central fatigue' hypothesis to explain the relationship between plasma [Trp]:[LNAA] ratio and brain 5-HT turnover in light of the information on the effects of brain 5-HT on motivation, effort perception, sleepiness, lethargy, and fatigue (Figure 1.7). According to this hypothesis, during prolonged exercise, when the glycogen stores become progressively reduced there is an increase in BCAA catabolism and in adipose tissue lipolysis, in order both nutrients to contribute to muscle energy production (ATP). The increase in BCAA muscle uptake reduces plasma [BCAA] and increases plasma [Trp]:[BCAA] ratio. On the other hand, an increase in adipose tissue lipolysis and plasma [FFA] may additionally increase plasma [Trp] because plasma [FFA] may displace Trp from albumin and potentially increase [Trp]:[LNAA] ratio (Curzon *et al.*, 1973; Spector 1975). Since Trp competes with the other LNAA for transport across the BBB (Padridge *et al.*, 1977; 1986; Fernstrom, 1994) (Figure 1.7), the previous reactions may increase brain Trp uptake and 5-HT turnover, which may contribute in central fatigue (Newsholme *et al.*, 1987).

Testing the 'central fatigue' hypothesis two early studies conducted by Blomstrand, Newsholme and colleagues (1988; 1989) suggest that prolonged exercise increases plasma [Trp], [Trp]:[BCAA] ratio and therefore brain 5-HT synthesis supporting this hypothesis. Thereafter, investigators have used nutritional supplementations and 5-HT and DA agonist and antagonist drugs to test the validity of this hypothesis in experimental settings as well as the effect of nutritional supplements and drugs on central fatigue during exercise.

1.2.5.1 Central fatigue and nutritional considerations

Blomstrand and colleagues (1991a; 1991b) have supported that prolonged exercise may induce elevation in plasma [Trp]:[BCAA] ratio, which in turn may enhance brain 5-HT synthesis leading to central fatigue and this effect can be ameliorated during exercise by BCAA supplementation. However, although Blomstrand *et al.* (1991a; b) have shown a reduction in plasma [Trp]:[BCAA] ratio and an improvement in exercise and mental performance during a marathon race, a 30km running trial and soccer game, some methodological limitations should be pointed out from these reports. For example, Blomstrand *et al.* did not initially observe differences in marathon performance time between BCAA and placebo groups but only after subdivision of faster and slower athletes (according to their performance-time in the race). Since there was no a clear rational of subdividing the athletes into two groups without justifying the cut-off criteria in this selection there is a possibility for statistical bias. In addition, apart from this bias some other factors that may affect brain 5-HT synthesis and exercise performance should be considered during uncontrolled field studies. For example, the pre-race diet and caffeine consumption that both affect neurotransmission and brain 5-HT and DA modulators were not controlled (Chaouloff, 1993; Davis *et al.*, 1992; 2000; 2003; Fredholm *et al.*, 1985; 1999). Environmental temperature also and fluid replacement during the race both of which can modify muscle metabolism (Febbraio *et al.*, 1996) and CNS function and therefore exercise performance was impossible to control (Galloway and Maughan, 1997; Nielsen *et al.*, 1993; Nybo and Nielsen 2001a, b, c; Nybo *et al.*, 2003). Consequently, due to these methodological limitations the studies by Blomstrand *et al.*, (1991a, b) do not provide strong support of the central fatigue hypothesis as proposed by Newsholme *et al.*, (1987) but do not preclude the involvement of brain 5-HT system in central fatigue during exercise.

Several other laboratory studies have however, demonstrated an attenuation in central fatigue development during exercise when there is a reduction in plasma [Trp]:[LNAA] ratio through CHO supplementations. Davis *et al.* (1992) examined the effect of 6% and 12% of CHO drinks on peripheral modulators and indices of brain 5-HT function during prolonged exercise. They observed a reversion of plasma free-[Trp]:[BCAA] ratio and an enhancement in endurance performance without observing any peripheral metabolic benefit following CHO feeding. They attributed their findings to the action of CHO supplementation in delaying an early elevation in FFA mobilisation and plasma [FFA] which displaces Trp from albumin increasing [Trp]:[LNAA] ratio, brain Trp uptake and 5-HT turnover. However, Davis *et al.* (1992) may have overestimated their performance results because several of the subjects in the CHO trials were stopped, prior to volitional fatigue. Consequently, it was not possible to determine the actual cause of fatigue between 6% and 12% CHO-treatments and whether this could be associated with the plasma regulators of Trp transport to the brain.

Contradictory results concerning the effect of CHO feeding on plasma [Trp]:[BCAA] ratio but without precluding the implication of brain 5-HT metabolic up-regulation during exercise, were obtained by Farris *et al.* (1998). Using racing horses, they found that Trp infusion has diminished time to fatigue and elevated plasma [Prl] relative to placebo and glucose infusions trials. They suggested a metabolic up-regulation of brain 5-HT synthesis, without observing muscle glycogen depletion. However, there was no effect of glucose infusion on plasma [FFA], free-[Trp] and free-[Trp]:[BCAA] ratio results that dispute the metabolic theory of central fatigue during prolonged exercise as proposed by Newsholme *et al.*, (1987). Moreover, these results do not preclude a central fatigue development since Prl secretion was elevated and endurance performance diminished after Trp infusion (Farris *et al.*, 1998). These results, regarding the role Trp in brain 5-HT synthesis and central fatigue, are consistent with those obtained by Soares *et al.* (2003) who suggested that intracerobroventricular Trp injection reduced mechanical efficiency and time to fatigue in rats. Gomez-Marino *et al.* (2001). Using brain microdialysis technique they observed that the direct brain administration of the amino acids L-valine attenuated the extracellular elevation in hippocampus 5-HT

release and thus central fatigue development during exercise by reducing brain Trp uptake.

In contrast, van Hall *et al.* (1995) found a reduction (8-12%) and an elevation (7- to 20 fold) in brain Trp uptake after a BCAA supplementation and Trp ingestion respectively but no performance improvement in well-trained endurance athletes. In addition, Madsen *et al.* (1996) supported van Hall *et al.* (1995) results but in disagreement with Davis *et al.* (1992) and Farris *et al.* (1998), who both did not observe any improvement in endurance performance of well-trained athletes after BCAA or glucose supplements. Although free-[Trp]:[BCAA] ratio was significantly lower with BCAA relative to glucose or placebo. Finally, Verger *et al.* (1994) found that BCAA supplementation even reduced exercise performance relative to glucose or placebo supplements adding more confusion to this debate.

According to Davis *et al.* (2000) one possible reason for the discrepancies among studies used BCAA supplementations is the amount of BCAA given and that large dose may be required in order to produce attenuation in brain 5-HT synthesis during exercise. However, when large doses of BCAA were administrated, although there was a reduction in brain Trp uptake, there was an elevation in ammonia production due to metabolic deamination of amino acids during exercise (Wagenmakers, 1992). It is noted for example, that in the studies by van Hall *et al.* (1995) and Madsen *et al.* (1996) the ammonia production was significantly higher during exercise after BCAA relative to glucose and placebo treatments.

Ammonia was found to be noxious to the muscles and brain and to reduce performance by attenuating motor control and motor coordination (Banister and Cameron, 1990; MacLean and Graham, 1993; Wagenmakers *et al.*, 1989). In addition, ammonia production was found to be more pronounced when the prior to exercise glycogen levels were low causing a net rate of protein uptake and oxidation from muscles (Blomstrand and Saltin, 1999; Lemon and Mullin, 1980). Therefore, considering that many studies have examined the effect of pre-exercise BCAA supplementation on exercise performance and central fatigue after an overnight fast it was possible this manipulation would negatively affect performance. It was also found that even when muscle glycogen levels are normal before exercise ammonia

production was higher after BCAA supplementation trial compared with placebo group (MacLean and Graham, 1993).

Summary and interpretation

It is difficult to explain the contradictory findings regarding the effect of nutritional supplements (BCAA, CHO, Trp) on 'central fatigue'. It is possible that changes in peripheral modulators of brain 5-HT function via BCAA, CHO and Trp interventions do not always reflect changes in brain 5-HT synthesis. Alternatively, Trp infusion *per se* may directly enhance brain 5-HT synthesis without significantly influencing putative plasma modulators of 5-HT synthesis and this is more prominent in animals models relative to human subjects. Although, after BCAA supplementation, ammonia production seems to be a reason for reducing exercise performance due to its toxicity in muscle and brain (e.g., Banister and Cameron, 1990), several other studies do not support an elevation in ammonia production after BCAA supplementation (e.g. Blomstrand *et al.*, 1997). Others have reported that even when ammonia production is elevated this did not diminish exercise performance (e.g., Calders *et al.*, 1997; 1999). It is also possible that BCAA and CHO supplements enhance muscle metabolism and therefore diminish peripheral fatigue during exercise rather than attenuating plasma [Trp]:[BCAA] ratio, brain 5-HT synthesis and central fatigue. Blomstrand *et al.* (1996) for example, using muscle biopsy technique found that BCAA supplementation contributed in maintaining muscle glycogen levels during prolonged exercise relative to placebo trial suggesting an increased supply of BCAA prevents muscle glycogen degradation during exercise.

Some major factors therefore that may contribute in discrepancies among the above studies are i) different experimental protocols, ii) the fitness level of the subjects used and iii) the environmental temperature during the experiments. In Blomstrand *et al.* (1991a; b) studies for example, the BCAA supplementation improved the physical and mental performance of the slower-runners who were considered to be less-trained than the faster-runners. On the other hand, in subsequent control laboratory studies where well-trained endurance athletes were examined after BCAA supplementation, exercise performance was not improved (Blomstrand *et al.*, 1997; van Hall *et al.*, 1995). Acworth *et al.* (1986) for example, demonstrated that brain Trp uptake and therefore 5-HT turnover increased significantly during

prolonged submaximal exercise only in untrained rats. In addition, recent results indicate that well-trained individuals have different responses to Trp and cortisol regulation and 5-HT_{2A} and 5-HT_{2C} receptors during exercise than non-fit individuals (Brooks *et al.*, 2001; Dwyer and Browning, 2000; Weicker and Struder, 2001). It is possible that the chronic endurance training (> 6 weeks) (Dwyer and Flynn, 2002; Jakeman *et al.*, 1994) increases the sensitivity of 5-HT post-synaptic receptors resulting in metabolic down-regulation in brain 5-HT synthesis and therefore attenuation in central fatigue (Newsholme and Blomstrand, 1996). In addition, it has been shown that differences in environmental temperature (from 11°C to 20°C or 20°C to 30°C) can significantly influence thermoregulatory responses, substrate oxidation and fatigue development during prolonged exercise (Galloway and Maughan, 1997). Consequently, since environmental temperature cannot be controlled during field studies and the ambient temperature used from many previous laboratory studies could affect exercise fatigue for reasons other than glycogen depletion (e.g. thermophysiological stress), so that the implication of brain serotonergic system in the fatigue process cannot be easily interpreted.

Based on these studies it could be suggested that BCAA and CHO supplements and Trp infusion may not be efficient nutritional manipulation methods to study central fatigue during exercise. Considering that 8 to 16-fold elevations of [Trp]:[LNAA] ratio are needed through diet manipulation to produce changes in brain 5-HT turnover (Leathwood and Fernstrom, 1990) other strategies should be used in order to elicit changes in brain 5-HT synthesis and therefore study central fatigue development. Since plasma FFA may displace Trp from albumin (e.g. Curzon *et al.*, 1973) an artificial elevation in circulating plasma [FFA] should be an alternative physiological strategy in order to elicit changes in brain 5-HT metabolism.

1.2.5.2 Central fatigue and pharmacological considerations

The association between brain 5-HT metabolism and central fatigue was further examined by pharmacological-intervention studies. Wilson and Maughan (1992) examined the effect of Paroxetine (20 mg), a selective serotonin reuptake inhibitor (SSRI) drug, on exercise performance in humans. SSRI drugs block the re-entry of 5-HT in the pre-synaptic nerve ending increasing extracellular [5-HT] (Meeusen *et al.*, 2001). Wilson and Maughan, found that SSRI diminished endurance exercise performance without affecting peripheral metabolic and physiological responses,

results that were attributed to the increased synaptosomal [5-HT] and therefore central fatigue. However, Wilson and Maughan did not measure several important blood metabolite variables of the brain 5-HT and DA function, such as peripheral indices (e.g. plasma Prl) and modulators (plasma Trp, LNAA, Tyr and FFA). It is therefore, not clear whether the inhibition in exercise performance observed after a SSRI drug is accounted for by an up-regulation of brain 5-HT synthesis and if so, what mechanism is responsible for this inhibition in exercise performance.

More controlled studies, however, suggested that up-regulation of brain 5-HT synthesis or a down-regulation of brain DA metabolism may develop central fatigue during exercise. Bailey *et al.* (1993a and b), using rats found that exercise performance deteriorated or improved after administration of brain 5-HT agonist (quipazine dimaleate or m-chlorophenyl piperazine) and antagonist (LY 53,857) drugs respectively. Interestingly, the results obtained by Bailey *et al.* (1993b), which are consistent with the results obtained by Chaouloff *et al.* (1986), demonstrated a strong association between brain 5-HT and DA metabolic interaction and central fatigue during exercise. Bailey *et al.* (1993b) suggested that it is not only the elevation in brain 5-HT synthesis that plays a role in central fatigue development but the reduction in brain [DA] or elevation in brain [5-HT]:[DA] ratio is important. However, although animal models studying the effect of drugs on brain 5-HT metabolism seems to be relative consistent, there is not the same consistency when humans are examined.

Davis *et al.* (1993) using Fluoxetine (SSRI drug) suggested that endurance performance deteriorated and effort perception elevated without observing significant differences on various metabolic and cardiovascular responses in humans. In addition, Struder *et al.* (1998) examined the effect of Paroxetine (20 mg), BCAA, tyrosine and placebo interventions on brain monoamines and central fatigue during prolonged exercise in humans. They found that Paroxetine diminished endurance performance relative to placebo, BCAA and Tyr trials. However, plasma [Prl] was not influenced by the SSRI drug but it was significantly higher after 20 g Tyr administration relative to the three other trials. In addition, although BCAA treatment reduced plasma free-[Trp]:[BCAA] ratio, free-[Trp] was not different between the trials and endurance performance was not improved relative to placebo and Tyr trials. These results suggest that if plasma or brain [Trp] is not acutely

elevated after diet or SSRI manipulation, changes in brain 5-HT synthesis and endurance performance should not be expected.

Other human studies, however, have failed to observe influences on exercise performance and brain monoaminergic system after pharmacological manipulation (e.g. Meeusen *et al.*, 1997; 2001). Meeusen *et al.* (2001) administrated capsules containing 20 mg-Fluoxetine or placebo in eight well-trained cyclists and they found no differences on exercise performance, various blood metabolites and effort perception. However, they found an increase in plasma [Prl] and β-endorphins levels after placebo relative to SSRI treatment suggesting no increase in hypothalamic 5-HT function with SSRI drug. In a subsequent study Meeusen *et al.* (1997) using a 5-HT antagonist drug, Ritanserin, and a DA agonist, L-DOPA, examined the brain 5-HT and DA metabolic interaction and central fatigue during prolonged submaximal exercise. They demonstrated that neither the DA agonist nor the 5-HT antagonist drugs when given in two single doses, influenced exercise performance.

Summary and interpretation

The inconsistencies among pharmacological studies examining central fatigue during exercise in humans may be due to various factors: i) the dosages of the drugs used relative to the fitness levels and body weight of the subjects. For example, Wilson and Maughan (1992), Struder *et al.* (1998) and Meeusen *et al.* (1997; 2001) used recreationally active, moderate fit and very well-trained endurance groups respectively but the same dose (20 mg) of the drugs, although the mean body weight varies between the studies; ii) the different drugs used, Fluoxetine or Paroxetine. Although, both drugs have SRRI action, the effectiveness of the drugs to influence brain 5-HT receptors is not the same, with Fluoxetine being relatively more potent SSRI (5-HT_3 receptors) than Paroxetine. Probably because Fluoxetine does not interfere with the re-uptake of DA and nor-epinephrine (Bowsher *et al.*, 1988); iii) the different exercise protocols in association with the fitness levels of the subjects. After giving the same drug for example, Wilson and Maughan (1992) found an increased exercise performance in moderately fit subjects and Meeusen *et al.* (1997; 2001) found no difference in endurance performance in well-trained subjects; iv) the inability of some subjects within the study groups to tolerate the drugs causing individual variation in response and therefore interferes with the mean results (Bridge *et al.*, 2001); v) the inability of the SSRI drugs to influence 5-HT receptors

due to single or double doses given prior to the experiments. Some studies for example, observed a delayed increase in some aspect of brain 5-HT synthesis after single administration of SSRI drugs (e.g. Rigdon and Wang, 1991). This occurs because a single or double dose of SSRI drug may initially decrease 5-HT cells firing since the subjects are not desensitised to the drugs action (Chaouloff, 1993; Rigdon and Wang, 1991). Recent neuroendocrine-challenge studies for example, have failed to observe changes in brain 5-HT receptors after single doses of agonist or antagonist to 5-HT drugs (Montgomery *et al.*, 2001; Bhagwagar *et al.*, 2003); and finally; vi) the genetically determined differences on the behavioural, physiological and biochemical responses to monoamine agonist and antagonist drugs and to exercise-stress between the rat species (Martin *et al.*, 2000a; b; Pollier *et al.*, 2000). For example, SSRI response was found to differ among spontaneously-hypertensive, Lewis and Wistar-Kyoto rats after administration of the same amount of a 5-HT metabolic inhibitor drug: citalopram. (Pollier *et al.*, 2000).

In addition, most of the above-referred studies did not concurrently examine aspects affecting both peripheral and central components affecting fatigue and effort perception during exercise. It is difficult therefore to interpret the actual mechanism in diminishing or enhancing exercise performance after nutritional and pharmacological interventions. These factors make the existing results unclear and more studies using alternative strategies are warranted to examine central fatigue in humans.

1.2.6 Caffeine and exercise fatigue: focused on central effect

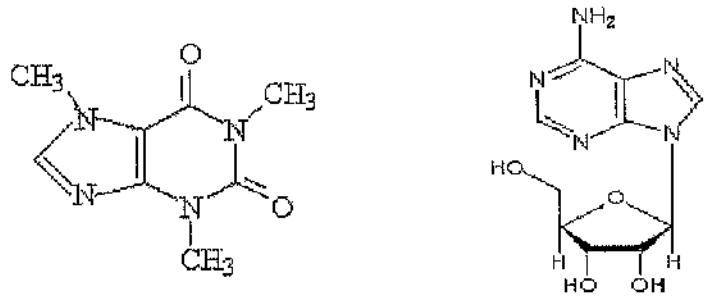
Caffeine is probably the most widely consumed 'drug' in modern societies all over the world (Fredholm *et al.*, 1999). Dietary sources of caffeine come from foods and beverages such as chocolate bars, cacao products and soft drinks but especially from coffee and tea (Fredholm *et al.*, 1999). It has been generally agreed that caffeine has the potential to improve mood-state and cognitive performance and stimulate CNS resulting in attenuating tiredness and sleepiness (Hofer and Battig, 1994; Hogervorst *et al.*, 1999; Kamimori *et al.*, 2000). Medically, caffeine has been suggested to be useful as a cardiac stimulant, mild diuretic and a headache inhibitor (George, 2000). Although caffeine has extensively been investigated, the question of what is the actual mechanism(s) by which it affects human tissues, especially CNS, is still

remained unanswered. Pharmacological studies into caffeine have proposed many theories, most of them however inconclusive, none entirely accepted or well characterised and understood. The problem arises from the multiple and simultaneous actions of caffeine on several tissues such as neuroendocrine, cardiovascular, respiratory, renal, and central and peripheral nervous systems (Fredholm *et al.*, 1985; Zhang and Wells, 1990).

Although, evidence suggests that caffeine may be a potential drug of abuse (Holtzman *et al.*, 1990) there are no strong side effects after caffeine consumption or any important negative consequences on human health. There are no strict medical restrictions on the amount of caffeine consumed by humans (see Fredholm *et al.*, 1999). However, excessive and acute caffeine intake (e.g. 40 cups of coffee which may achieve more than 0.5 mM plasma [caffeine]) has been shown to be a toxic dose and it can negatively affect physiological function and psychological state (Fredholm, 1980). In addition, some reports suggest that caffeine should not be ignored as a possible addictive drug because it has a similar mechanistic action such as other psychoactive CNS-stimulant drugs including amphetamines, cocaine and heroin (Holtzman *et al.*, 1990). It should be noted however, that caffeine has a much milder effects than amphetamines, cocaine and heroin (see George, 2000). Caffeine does not have any nutritional value but the last three decades it was commonly and extensively used by sports competitors as a nutritional ergogenic aid (Dodd *et al.*, 1993; Graham, 2000a; b). For this reason the international Olympic Committee (IOC) were concerned about research relating to the ergogenic effect of caffeine and defined levels of more than 12 mg/l of caffeine in urine as illegal doping, adding it to the list of band substances (Spriet, 1995).

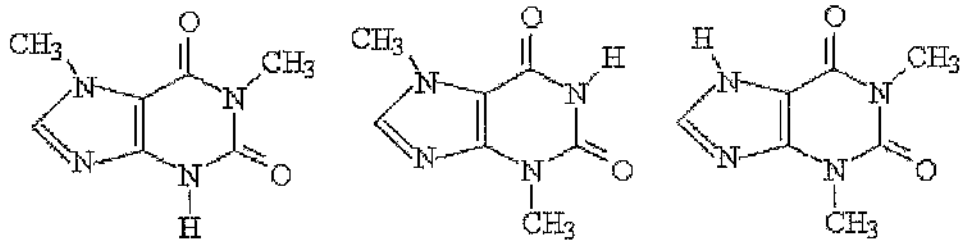
1.2.6.1 Biochemistry of caffeine

Caffeine ($C_8H_{10}N_4O_2$) or 1,3,7-trimethylxanthine, is a member of a group of purine alkaloids (organic compound found in plants whose name derives from its chemical basis) known medically as the xanthines or methyl-xanthines (Figure 1.8a). Other chemical members of this group are theophylline, theobromine, and paraxanthine (Figure 1.8b) (Arnaud, 1987). Theophylline and theobromine are predominantly found in tea (0.3% by mass) and cacao beans (1.5% by mass) respectively as well as in human metabolites of caffeine. Around 75% of paraxantine is derived from caffeine metabolism (Arnaud, 1993).



Caffeine Adenosine

Figure 1.8a Chemical structure of caffeine (1,3,7-trimethylxanthine) and adenosine



Paraxanthine Theobromine Theophylline

Figure 1.8b Chemical structure of caffeine's primary metabolic breakdown products, the dimethylxanthines: paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) adapted from Hawke *et al.*, (2000).

1.2.6.2 Metabolism of Caffeine

After caffeine ingestion, caffeine can be spread rapidly to almost all cells of the human body by passing through the cell membranes, including BBB due to its hydrophobic properties compounds (Fredholm *et al.*, 1999). The blood-to-plasma ratio of caffeine is close to unity indicating limited plasma protein binding and free passage into blood cells (McCall *et al.*, 1982). Absorption of caffeine from tea and coffee is much faster than from soft drinks; for hot drinks the caffeine levels are highest about one hour after consumption (Morgan *et al.*, 1982; Arnaud and Welsch, 1982). Generally, caffeine absorption from gastrointestinal tract is rapid reaching at around 99% in both animal and humans about 45 min after intake (Bonati *et al.*, 1982). However, evidence suggests that caffeine absorption is not complete when the compound is taken orally from coffee (Arnaud, 1993; Morgan *et al.*, 1982).

After circulating the body, most caffeine molecules will eventually be taken up by the liver, where they are metabolised. The principal mechanism is the demethylation and/or C-8 oxidation of caffeine, a trimethylxanthine, into dimethylxanthines and

monomethylxanthines (Arnaud, 1993). This produces dimethylxanthines and as we saw before the primary caffeine metabolites: paraxanthine, theophylline and theobromine. In addition, in the liver, takes place the biotransformation of caffeine by oxidisation and conversion to uric acids and water-soluble caffeine can be directly lost in urine. There is no accumulation of caffeine in body fat or specific organs and the same permeability that enables caffeine to enter into the cells, also facilitates its rapid exertion from the cells (Fredholm *et al.*, 1999).

For physiological doses (less than 10 $mg{\cdot}kg^{-1}$), caffeine half-life (the time taken for the body to eliminate one half of a given amount of a compound), ranges between 2.5 to 4.5 hours in healthy human-adults and it is relatively similar with monkeys' ranging from 3 to 5 hours (Bonati *et al.*, 1984-5). However, it is not the same in small animals such as rat and mouse (0.7 to 1.2 hours) (Arnaud, 1987). The primary metabolic difference between small animal and humans is that in small animals around 40% of caffeine is metabolised to trimethyl (3-metyl) derivatives as opposed to 6% in humans (Arnaud, 1993). In addition, caffeine metabolism in human leads to paraxantine (~ 75% of caffeine intake) but in animals this leads to theophylline formation (Fredholm *et al.*, 1999). However, theophylline is a more potent inhibitory factor of brain adenosine receptors than caffeine *itself* or paraxanthine (Benowitz *et al.*, 1995). It is noted that several studies suggested that women could metabolise caffeine at around a 25% faster rate than men (see Fredholm *et al.*, 1999). Consequently, this in association with the different caffeine half-lives between animals and human may be a reason of the inconsistencies between studies into the actions of caffeine.

1.2.6.3 Actions of caffeine

Caffeine has been suggested to have four particular actions in vitro, although the relationship between these actions and the drug effects in vivo are not well established. These actions include: i) adenosine A_{2A} and A_1 receptors blockade, ii) inhibition of the enzyme phosphodiesterase resulting in attenuation of cyclic nucleotide breakdown, iii) enhancement in Ca^{2+} release and action in periphery, and iv) $GABA_A$ receptors blockade (Fredholm, 1980). However, the actions of caffeine on human physiological tissues are depended upon its' dose. For example, in order to achieve an inhibition of the enzyme phosphodiesterase, to block $GABA_A$ receptors and to enhance Ca^{2+} release at around 20, 40, and 100 cups of coffee (~

0.5 to 4 mM of plasma [caffeine]) respectively are required (Fredholm, 1985). However, these doses of caffeine intake were found to produce severe toxicity with sometimes fatal consequences in humans; therefore these excessive caffeine intake levels should be avoided (Fredholm *et al.*, 1999). Only adenosine receptor inhibition can be achieved in human with physiological caffeine doses (less than 10 $mg \cdot kg^{-1}$, equivalent to 1-6 cups of coffee) antagonising almost all the actions of adenosine in human tissues, especially in CNS (see Fredholm, 1999). This probably occurs because caffeine has a distinctive molecular structure, which is biochemically similar to that of adenosine (Figure 1.8a) (Holtzman *et al.*, 1991). For this reason, caffeine molecules have the advantage of binding to adenosine receptors sites and deactivating and/or antagonising adenosine functions (Zhang and Wells, 1990).

Adenosine is created by the human body (mainly by brain tissue) and adenosine molecules are attached to their receptors sites (A_1, A_{2A}, A_{2B} A_3) (Latini and Pedata, 2001). These are located at various tissues through-out the body including brain, cardiac and smooth muscles, adrenal gland and adipose tissue (Van Soeren and Graham, 1998). Adenosine plays an important role in biochemical processes, such as energy transfer - as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) - and in signal transduction as cyclic adenosine monophosphate (cAMP) (Latini and Pedata, 2001). At cellular level adenosine receptors may inhibit (A_1) or stimulate (A_{2A}) adenylate cyclase respectively which catalyses the formation of cAMP from ATP (Olah and Stiles, 2000).

Adenosine is probably the most important neuromodulator in the central and peripheral nervous system meaning that its molecules can be diffused easily through the synaptic cleft and control nerve impulsiveness (Latini and Pedata, 2001). Consequently, adenosine has the potential to decrease the rate of spontaneous nerve cell firing and inhibits the release of several neurotransmitters, including DA, which controls the neuronal excitability. When adenosine molecules bind the adenosine receptors (mainly to A_1, A_{2A} receptors) in the brain contribute i) to dilating blood vessels enabling more oxygen to be up taken by the CNS and sometimes causing vascular headaches (Latini and Pedata, 2001), and ii) to enhancing the release of neurochemical signals implicated to contribute in body relaxation and promote mood-depressing, drowsiness and lethargy (Fredholm *et al.*, 1995). For this reason some headache medications involve caffeine in order to counterbalance adenosine

activation (Fredholm *et al.*, 1985; 1999). In humans, for example, the A_1 and A_{2A} receptors are blocked with caffeine with a K_D of less than 50 μM (Fredholm *et al.*, 1999). Consequently, even after small caffeine doses (1-2 cups of coffee), caffeine may antagonise this drowsiness effect of adenosine enabling human cells to function slightly faster than normal and facilitating brain alertness; even when adenosine levels are high in the body (Fredholm *et al.*, 1999; Garrett and Griffiths, 1997).

Additionally, due to the increase in cell function and to the alertness effects of caffeine on CNS, there is a stimulation of the pituitary gland which enhances epinephrine production that it may further antagonise the drowsiness (Graham, 2000a) and also increase adipose tissue lipolysis, fat oxidation and muscle blood flow (Costill *et al.*, 1978; Essig *et al.*, 1980; Ivy *et al.*, 1979, see section 1.2.4.6 for more details). However, the most significant action of caffeine as an adenosine receptors' inhibitor is likely to be the promotion of brain dopaminergic activation, which has been found to regulate several basic neurological functions including integrative control of muscle movement, motor coordination and balance (Davis and Bailey, 1997; Meeusen and De Meirleir, 1995), body temperature (Hasegawa *et al.*, 2000), and hormonal secretion (Ben-Jonathan *et al.*, 1989). Apart from these effects, dopaminergic system has the potential to stimulate the 'pleasure centre' in the CNS (Ferre *et al.*, 1992; Fredholm *et al.*, 1995), and reduce perception of exertion and/or physical and mental fatigue sensation (Davis and Bailey, 1997). Consequently, antagonism of adenosine activation in the CNS through physiological amount of caffeine ingestion may have the potential to attenuate central fatigue during exercise by enhancing brain DA:5-HT ratio.

Even though the primary action of caffeine may be to block adenosine receptors leading to inhibition of brain DA release this leads to some secondary effects on the CNS, autonomic NS and cardiovascular system (Fredholm *et al.*, 1999). Caffeine for example, has been shown to increase spontaneous electrical activity, enhance convulsant activity, stimulate locomotor activity and augment operant response rates in CNS (Daly, 1993). Caffeine also has been shown to relax bronchial smooth muscle (Garrett and Griffiths, 1997) and enhance inspiratory muscle contraction (assessed by changes in the power spectral density of the diaphragm electromyogram during loaded breathing) which lead to a reduction in inspiratory muscle fatigue (Supinski *et al.*, 1986). Caffeine may enhance respiration by

blocking brain adenosine receptors (Kawai *et al.*, 1995), which act to depress ventilation by inhibiting respiratory motor centres (Runold *et al.*, 1989). However, the exact mechanism of the effect of caffeine on respiratory muscles remains elusive. It has been shown for example that methylxanthines enhance $\dot{V}_E$ and attenuate a CNS-fatigue involvement associated with respiratory muscles, by stimulating the respiratory motorneurones in medulla (Eldridge *et al.*, 1983). It was also found that caffeine ($10mg \cdot kg^{-1}$) increases the amplitude of the neuromuscular inspiratory output (Mazzarelli *et al.*, 1986). An alternative mechanism indicates that caffeine ($10mg \cdot kg^{-1}$) stimulates carotid sinus nerve discharge through an alteration of carotid chemoreceptor blood flow linked with the change in blood pressure (Bairam *et al.*, 1997). It has been previously demonstrated that methylxantines are effective bronchodilators useful in the treatment of asthma (Fredholm, 1985).

Furthermore, as it stated above, many studies have observed changes in plasma [catecholamines] after caffeine ingestion suggesting an enhancement in sympathetic nervous system activation (e.g. Van-Soeren and Graham1 1998; Graham *et al.*, 2000). It has been suggested that caffeine influences heart muscle function by causing positive inotropic/chronotropic effects (Garrett and Griffiths, 1997). Others measuring the heart-rate variability in humans following caffeine ingestion observed an enhancement in the activity of both parasympathetic and sympathetic nervous systems at rest (Hibino *et al.*, 1997) and during exercise (Nishijima *et al.*, 2002). In addition, it has been suggested that high coffee intake may cause tachycardia, palpitations and a rapid rise in blood pressure via an elevation in norepinephrine release (Fredholm *et al.*, 1999). The release of norepinephrine from sympathetic nerves could be regulated by methylxanthines by a presynaptic mechanism at the sympathetic nerve terminal and this depends on the antagonism of adenosine acting at A_1 receptors (Hedqvist *et al.*, 1978). Consequently, it is possible that the most important mechanism underlying increases in catecholamines release is a rise in the sympathetic outflow and that this is a centrally regulated phenomenon. (Fredholm *et al.*, 1999).

1.2.6.4 Caffeine and exercise performance

The effect of caffeine on exercise performance was first examined by Costill *et al.* (1978), Ivy *et al.* (1979), Essig *et al.* (1980) based on the early observation that i) caffeine may enhance epinephrine release and therefore elevate plasma [FFA] by

stimulating adipose tissue lipolysis (Bellet *et al.*, 1968). And ii) elevating circulating plasma FFA may enhance fat oxidation and therefore reduce muscle glycogen use contributing in CHO sparing during prolonged exercise (Costill *et al.*, 1977; Rennie *et al.*, 1976). After a series of studies using animal and human models Costill *et al.* (1978), Ivy *et al.* (1979) and Essig *et al.* (1980) observed that caffeine enhanced epinephrine release and contributed to enhancing adipose tissue lipolysis, increasing plasma FFA uptake and fat oxidation and reducing muscle glycogen use. This resulted in enhanced CHO sparing and improved endurance performance. Thereafter, several reports provided supporting evidence for the 'metabolic theory' effect of caffeine on exercise performance (e.g. Chesley *et al.*, 1998; Dodd *et al.*, 1993; Spriet *et al.*, 1992).

However, many studies have recently disputed the metabolic theory effect of caffeine on endurance performance (Chesley *et al.*, 1995; Cox *et al.*, 2002; Graham *et al.*, 2000; Graham and Spriet 1991; Graham and Spriet 1995; Greer *et al.*, 2000; Laurent *et al.*, 2000; Mohr *et al.*, 1998; Roy *et al.*, 2001; Van Soeren *et al.*, 1996; 1998). In addition, improved high intensity exercise performance has also been demonstrated following caffeine ingestion (e.g. McNaughton, 1986; Flinn *et al.*, 1990; Jackman *et al.*, 1996), where muscle glycogen depletion is clearly not the primary cause of fatigue. It is likely that caffeine may enhance endurance performance, not by sparing muscle glycogen but through other, possibly CNS effects. Caffeine for example, has been reported to i) reduce effort perception (e.g. Cole *et al.*, 1996), ii) attenuate brain 5-HT turnover, through an inhibition of the enzyme tryptophan hydroxylase (Lim *et al.*, 2001), and iii) inhibit central adenosine receptor activation, thereby attenuating 'central fatigue' by increasing DA:5-HT ratio in the brain (Davis *et al.*, 2003). However, the exact mechanism of these possible CNS actions of caffeine on exercise performance is still under discussion and no studies have examined the effect of caffeine on putative modulators of brain 5-HT and DA function during exercise in humans.

1.2.7 Heat stress and exercise: focused on central impairment

It has been well established that exercise capacity is impaired in the heat relative to thermoneutral or mildly-cold environments (e.g. Bruck and Olschewski, 1987; Febbraio *et al.*, 1994; Galloway and Maughan, 1997; Nielsen *et al.*, 1993) but the

precise mechanisms remain to be determined. Classic studies attributed the decrement in exercise capacity in the heat to reductions in muscle blood flow and a fall in blood and plasma volume (Nadel *et al.*, 1979; 1980; Rowell *et al.*, 1966) and to dehydration (Buskirk *et al.*, 1958; Cadarette *et al.*, 1984). Hyperthermia can also limit exercise performance by increasing muscle lactate production and accelerating the rate of muscle glycogen depletion (Fink *et al.*, 1975) even during prolonged exercise in thermoneutral environment (20°C) (Kozlowski *et al.*, 1985).

On the other hand, other studies have recently attributed the decline in exercise performance in the heat to dehydration induced reduction in blood supply to exercising muscle by lowering perfusion pressure and systemic blood flow (e.g. Gonzalez-Alonso *et al.*, 1998). In a subsequent study Gonzalez-Alonso *et al.* (1999) examined whether reduction in muscle blood flow with heat stress-induced dehydration would decrease substrate delivery and metabolism and heat removal to and from exercising muscle during prolonged exercise. They found that there was no impairment of substrate metabolite or lactate removal but heat and dehydration elevated CHO oxidation and lactate production. They also found that most of the metabolic heat produced was released out of the active muscle to the surrounding areas suggesting that hyperthermia *per se* rather than a dehydration-induced alteration in muscle metabolism contributes in an early fatigue during exercise in the heat. In addition, although glycogen depletion was found to limit exercise capacity during prolonged exercise in 11°C (relative to exercise in 4°C, 21°C, or 31°C) in the heat some other factors probably CNS in origin should be considered to limit endurance capacity (Galloway and Maughan 1997).

Nielsen and colleagues (1993; 1997) have extensively investigated the role of core and brain temperature on exercise performance impairment in the heat. They initially proposed that when core temperature increases, at approximately 39.6°C, is a critical factor to inhibit exercise performance by reducing central motivation to exercise. In subsequent studies changes in electroencephalogram of the brain's frontal area have been observed during exercise with hyperthermia suggesting that fatigue may occur at a critical brain temperature (Nielsen *et al.*, 2001); which may cause a cerebral blood flow dysfunction (Nybo *et al.*, 2002) or brain glucose uptake abnormality (Nybo *et al.*, 2003). However, the exact mechanism(s) of this dysfunction and/or whether increased core and brain temperatures may have

secondary affect brain monoamines, especially increased 5-HT metabolism or reduced brain DA function, during prolonged exercise in the heat is not known yet (Nielsen and Nybo, 2003).

1.2.7.1 Exercise in the heat and brain 5-HT metabolism

Several studies link exercise in the heat with a metabolic up-regulation of brain 5-HT synthesis, which was found to contribute in elevating body-heat storage (e.g. Feldberg and Myers, 1964; Lin *et al.*, 1998; Sugimoto *et al.*, 2000; Mills and Robertshaw, 1981; Laatikainen *et al.*, 1988). Feldberg and Myers, (1964) for example, using animal models and continuous recording examined core body temperature regulation after intraventricular injection of 5-HT and catecholamines. They observed that rectal temperature rose significantly, above the baseline levels after the injection of 5-HT but fall to normal levels after the injection of catecholamines. They suggested that high levels of injected 5-HT cause an increase in core body temperature and high levels of catecholamines have an antagonistic effect. The hypothesis of increased brain [5-HT] elevates core temperature is later supported by Sugimoto *et al.* (2000) and Lin *et al.* (1998). For example, when hypothalamic [5-HT] was increased in the rat by administration of Fluoxetine (i.e. 5-HT reuptake inhibitor) and 5-hydoxytryptophan (i.e. a 5-HT precursor), there was an increase in metabolic heat production with a concomitant reduction in heat loss (Lin *et al.* 1998). This is also supported by Sugimoto et al. (2000) who found that after administration of 5-HT agonist (*p*-Chloroamphetamine) the mice body temperature was increased, while it was decreased after administration of 5-HT_{2A} antagonist.

On the other hand, Brisson *et al.* (1986) observed a positive correlation between increased core temperature and plasma [Prl]. In a subsequent study Brisson *et al.* (1991) compared plasma [Prl] during prolonged exercise (67% of $\dot{V}O_{2\,max}$) in 41°C or 10°C. They found that during exercise in the heat either exogenous or endogenous heat loading increased blood [Prl] while no changes in [Prl] were observed during exercise in the cold condition. They concluded that changes in plasma [Prl] are highly regulated by exogenous or endogenous thermal-stress. Pitsiladis *et al.* (2002) also examined the effect of environmental temperature on peripheral markers of brain serotonergic function and their association with exercise performance during prolonged exercise in the heat and in the cold after low and high CHO diets. They

found that serum [Prl] was significantly higher and exercise performance lower during the two trials at 30°C (low and high CHO trials) relative to the two trials at 10°C. They concluded that exercise in the heat, inducing thermal stress may up-regulate brain 5-HT metabolism independently of pre-exercise diet manipulation and peripheral modulators of brain 5-HT synthesis (plasma total and free [Trp] and free [Trp]:[BCAA] ratio). Consequently, based on the above results the central fatigue development during exercise in the heat is evident but the mechanisms are still unknown.

Some strategies, such as face/head cooling (e.g. Brisson *et al.*, 1989) or pre-exercise whole body cooling (e.g. Kay *et al.*, 1999) were employed in an attempt to reduce thermal-stress and/or attenuate the elevation in Prl secretion. Gonzalea-Alonse *et al.* (1999) for example, examined whether fatigue development during exercise in the heat in trained athletes occurs at the same critical levels of core and muscle temperature, despite differences in starting core temperatures and its rate of rise. They designed an experiment where core, muscle (vastus lateralis) and skin temperatures were manipulated prior to exercise by immersing the subjects in water at 17 °C (pre-cooling trial), 36 °C (control trial) and 40 °C (pre-heating trial) respectively. They found that in all three trials, fatigue during moderate exercise at 40 °C occurred at the same high levels of internal body (oesophageal temperature ~ 40.1 °C) and muscle (~ 40.7 °C) temperatures when the initial value or the rate of increased body temperature were altered. Despite the same core and muscle temperatures observed at fatigue in all trials the shortest time to fatigue was found on the pre-heating trial relative to pre-cooling and control trials. These results may suggest that the initial core temperature plays an important role in the fatigue process during exercise in the heat and in the increase in the rate in body heat storage which contribute to the development of exercise fatigue.

However, although whole body pre-cooling may be beneficial during short-term intense exercise (i.e. 1000 m time-trial) (Maresh and Sleivert, 1999), the effectiveness of pre-cooling strategies in attenuating brain temperature (e.g. Brengelmann, 1993), body heat storage and enhancing exercise performance in the heat is insignificant when exercise lasts longer than 20-30 min (e.g. Drust *et al.*, 2000). Consequently, alternative approaches for reducing body heat storage and thermal stress during exercise in the heat (in an attempt to restrain a possibly brain

5-HT synthesis and 'central fatigue') are required. It is possible that this can be achieved by Cr supplementation through its putative water-retention effect or via its positive effect on brain DA function (see Chapter 5). However, to date no studies have examined this particular hypothesis in humans.

1.2.7.2 Creatine supplementation and exercise in the heat

Creatine (Cr) or as chemically known, α-methylguanidino-acetic acid was first discovered by Chevreul, a French scientist, in 1832 from a meat extract. Its name is derived from the Greek word Kreas, which means flesh or meat from skeletal muscles. It was not until 1847 however, that Lieberg confirmed its appearance as a regular constituent of flesh extracted from mammals (see Demant and Rhodes, 1999). A significant observation by Lieberg was that the flesh of wild foxes involved ten-fold as much Cr as that of captive creatures suggesting a strong association between Cr accumulation and muscular exercise (Demant and Rhodes, 1999). Thereafter, the studies by Denis (1912) and Folin (1914) (cited by Demant and Rhodes, 1999) demonstrated a 70% elevation in muscle [Cr] after Cr ingestion, and the studies by Fiske and Subbarow (1927, 1929) (cited by Demant and Rhodes, 1999) reported the presence of labile phosphorus in resting muscle and its reduction in electrically active cat skeletal muscle which was called phosphorcreatine (PCr). In addition, these early experiments from Fiske and Subbarow, using electrical stimulation on skeletal muscle, demonstrated the role of Cr and PCr in skeletal muscle metabolism. However, it was only within the last two decades that systematic research into the effects of Cr supplementation on muscle metabolism and exercise performance has been undertaken (Kreider, 2003).

1.2.7.2.1 Biochemistry and actions of creatine

Cr (α-methylguanidino-acetic acid) is a naturally occurring compound synthesised predominantly in the liver, kidney and pancreas from three amino acids: arginine, glycine and methionine and it is converted to creatinine before removal by the kidneys (see Lemon, 2002). The transamidination process starts with the transfer of an amidine group from arginine to glycine, which leads to the formation of guanidinoacetate and ornithine, and is a reversible reaction catalysed by the enzyme glycine-amidine-transamidinase in kidney (Devlin, 1992) (Figure 1.9). Cr is then formed by the addition of a methyl group from (S)-adenosylmethionine

(transmethylation), which requires the enzyme methytransferase for the irreversible chemical reaction in liver. Thereafter, Cr is converted to Cr phosphate (CrP) by the enzyme creatine phosphokinase and then under a non-enzymatic process, CrP is metabolised to creatinine (Devlin, 1992) (Figure 1.9).

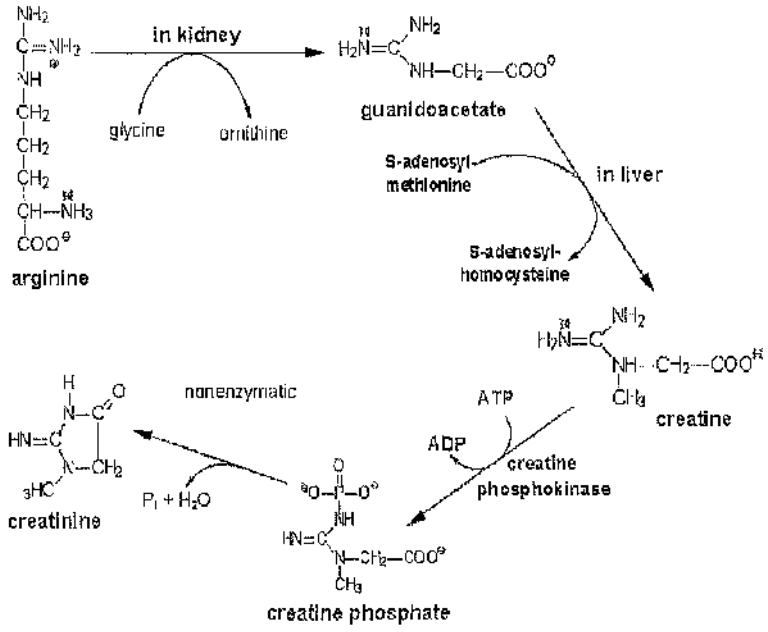


Figure 1.9 Chemical structure of creatine, the transamidination procedure of Cr in kidney and biosynthesis process of Cr in liver. Adapted from King (1998).

The normal plasma [Cr] ranges from 50 to 100 μmol/L (Harris *et al.*, 1992) and the vast majority of the body Cr/PCr pool, proportionally, is located in the muscle and neural tissues (Guimbal and Kilimann, 1993). Approximately 95% of the total body's [Cr] is found in skeletal muscle; the remaining 5% found mostly in the brain, heart and testes (Demant and Rhodes, 1999). Since the main site of Cr utilisation is skeletal muscle, Cr must be transported from its site of synthesis to skeletal muscle via the blood-stream (Demant and Rhodes, 1999). After arriving at the skeletal muscle, uptake of Cr occurs against a concentration gradient and it enters a number of cell types via a Na^+ dependent neurotransmitter transporter family associated to taurine and the members of the of γ-aminobutyric acid/betaine transporters (Guimbal and Kilimann, 1993). It has been also found that the presence of insulin and deficiency of vitamin E appears to enhance and reduce the Cr uptake respectively (Gerber *et al.*, 1965; Haugland and Chang, 1975).

In muscle and neural tissue, most of the Cr is phosphorylated to PCr in a reaction that is catalysed by the enzyme creatine kinase (CK) (Wallimann *et al.*, 1992). CK has four main isoforms (isoenzymes); i) Cytosolic or CK-MM, which is the skeletal muscle isoform, ii) CK-BB which is the brain isoform, iii) CK-MB which is the isoform found in cardiac muscle, and iv) Mt-CK, the specific mitochondrial isoform (Brdiczka *et al.*, 1994; O' Gorman *et al.*, 1996; Wallimann *et al.*, 1992). CK is a key enzyme involved in cellular energy homeostasis and it reversibly catalyses the transfer of the high-energy phosphate bond in PCr to ADP to form ATP, and it catalyses the transfer of the high-energy phosphate bond in ATP to Cr to form PCr.

1.2.7.2.2 Creatine and exercise performance

As stated above, during high-intensity exercise (~10 sec) the availability of PCr is the limiting factor of skeletal muscle performance. Consequently, Cr supplementation may enhance maximal-intensity exercise performance by increasing intramuscular Cr and PCr levels (Harris *et al.*, 1992; Kreider *et al.*, 1998) and accelerate the resynthesis rate of PCr (Greenhaff *et al.*, 1994). Thus, increased intracellular [PCr] contributes in transporting high-energy phosphates from mitochondria to cytosol and supplying high-energy phosphate into adenosine ATP preventing ATP depletion (Guerrero-Ontiveros and Wallimann, 1998). However, there are inconsistencies in whether Cr enhances muscle contraction and therefore high-intensity exercise performance (see Lemon, 2002).

Only few studies however, have examined Cr supplementation during prolonged exercise with some positive (Kern *et al.*, 2001; Volek *et al.*, 2001) or not significant (Vogel *et al.*, 2000) results on exercise performance, metabolic and thermoregulatory responses. However, none of the above studies has examined endurance performance using a prolonged time trial exercise protocol to fatigue. Vogel *et al.* (2000) for example, examined power output (5 x 5 sec maximal cycling) before and after 75 min prolonged exercise at 32°C. In the study by Kern *et al.* (2001) although thermal-stress was attenuated through water retention effect of Cr, the endurance performance was not evaluated because subjects were examined only for a fixed time period (60 min). In the study by Volek *et al.* (2001), although exercise performance (35 min prolonged exercise followed by 5 x 5 sec repeated-sprints) in the heat was improved, the cardiovascular, thermoregulatory and hormonal responses were unaltered. However, in this study there were no available

blood, cardiovascular, and perceptual responses to Cr supplementation data for more than 35 min of submaximal exercise and there was no examination of endurance performance. Consequently, whether Cr supplementation improved exercise performance through an enhancement in muscle contraction, body water maintenance or via any central effect is not known.

Studies have observed that Cr supplementation may enhance total body water (TBW) (e.g. Hultman *et al.*, 1996; Ziegenfuss *et al.*, 1998). This effect of Cr could prevent dehydration and body heat storage during prolonged exercise in the heat (Kern *et al.*, 2001). Dehydration and exercise in the heat were found to modify brain thermoregulatory centres, control evaporative heat loss (Turlejska and Lyszczart, 1982), elevate hypothalamic 5-HT synthesis (Scacchi *et al.*, 1989; Mahaparta *et al.*, 1991; Bridge *et al.*, 2003), increase effort perception and diminish exercise performance (Armstrong *et al.*, 1985; Nybo and Nielsen 2001c; Bridge *et al.*, 2000). The exact mechanism(s) however of the potential effect of Cr supplementation to attenuate body heat storage remains to be determined.

1.2.7.2.3 Creatine supplementation and brain effects

Studies on cerebral metabolism following oral Cr supplementation have been carried out in brain slices and animal models in order to assess the putative neuroprotective potential ascribed to the excess availability of Cr. Cr may exert neuroprotection by increasing or at least maintenance ATP/ADP and PCr/Cr levels in brain tissue and thereby providing extra energy for ion homeostasis and for the functional and structural integrity of mitochondria which lead to a protection against MPTP-induced dopamine depletion (Klivenyi *et al.*, 2004). Oral Cr supplementation for example, was found to increase brain [PCr] and to improve neuronal survival in rats administered malonate or 3-nitropropionic acid to deplete succinate dehydrogenase and mimic the pathophysiology of Huntington's disease (Matthews *et al.*, 1998). In addition, oral Cr supplementation increased brain DA synthesis in the substantia nigra of Parkinsonian mice, produced by n-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure, by increasing the number of surviving tyrosine hydroxylasa(TH)-positive neurons (Klivenyi et al., 2004) and by enhancing tyrosine hydroxylase activation (the rate-limiting enzyme of brain DA biosynthesis) (Klivenyi et al., 1999; Matthews et al., 1999). The exact mechanisms however of the potential effect of Cr to enhance TH-positive neurons remain elusive.

1.2.8 Chronic fatigue syndrome

A general fatigue sensation or even exhaustion with unknown actiology characterise most of the neurological diseases such as Parkinson, multiple sclerosis, post-polio fatigue, fibromyalgia, neurasthenia, depression and chronic fatigue syndrome (CFS) (Chaudhuri and Behan, 2000; Lloyd 1998). In CFS, several studies imply both peripheral (Lane *et al.*, 1994; 1995) but predominantly central abnormalities responsible for the development and persistence of the general drowsiness characterising the illness (e.g. Bakheit *et al.*, 1992; Clearc *et al.*, 1995; Demitrack *et al.*, 1991; Swain, 2000). However, the precise cause of the pathogenesis of the disease remains to be determined.

CFS has become relatively prominent and recognisable disorder only in the 1980s (e.g. Barnes, 1986; Holmes *et al.*, 1988). Although some outbreaks of the disease have been reported in the late 1930s by the US Public Health organisation (Lee, 1998) and in late 1950s by some medical reports, leading to the term 'epidemic neuromyasthenia' (Henderson and Shelokov, 1958; Poskanzer *et al.*, 1957). The prevalence of CFS has been recorded at 75-200 per 100,000 in preliminary community surveys, using criteria that excluded those with prior depression (Jason *et al.*, 1995). However, when major depression criteria have been included 740 cases per 100,000 has been recorded (Wessely *et al.*, 1997). Generally, CFS incidence, in primary care, is now estimated at 5 in 1000 individuals (Wessely *et al.*, 1995) and the prevalence of the illness was found to be significantly higher among women (373 per 100,000 persons) than men (83 per 100,000 persons) (Reyes *et al.*, 2003).

It is noted also that although a previous approach considered CFS to present more commonly in young adults, between 20 and 40 years (Levine, 1998), recent demographic and psychiatric based surveys reported CFS incidence in 5-15 years old children (e.g. Jordan *et al.*, 1998; Chalder *et al.*, 2003). In a community survey in USA for example, 2% of 4000 children were found to have CFS-like disease (Jordan *et al.*, 2000). In a recent clinical study, the incidences in CFS in children were found to be related to an abnormal brain function involving higher blood flow in the left basal ganglia and thalamus (Tomoda *et al.*, 2000).

1.2.8.1 Diagnosis of CFS

The clinical diagnosis of CFS is not simple because of the absence of the specific biochemical markers of the disease (Lange *et al.*, 1998). In addition, because several major symptoms of this illness are common and co-exist with the symptoms of other clinical diseases such as fibromyalgia, irritable-bowel syndrome, thyroid disease, anaemia but especially major depression (Chaudhuri and Behan, 2000) definitive diagnosis is difficult. For this reason, regularly CFS patients are diagnosed and treated as depressed patients (Layzer, 1998).

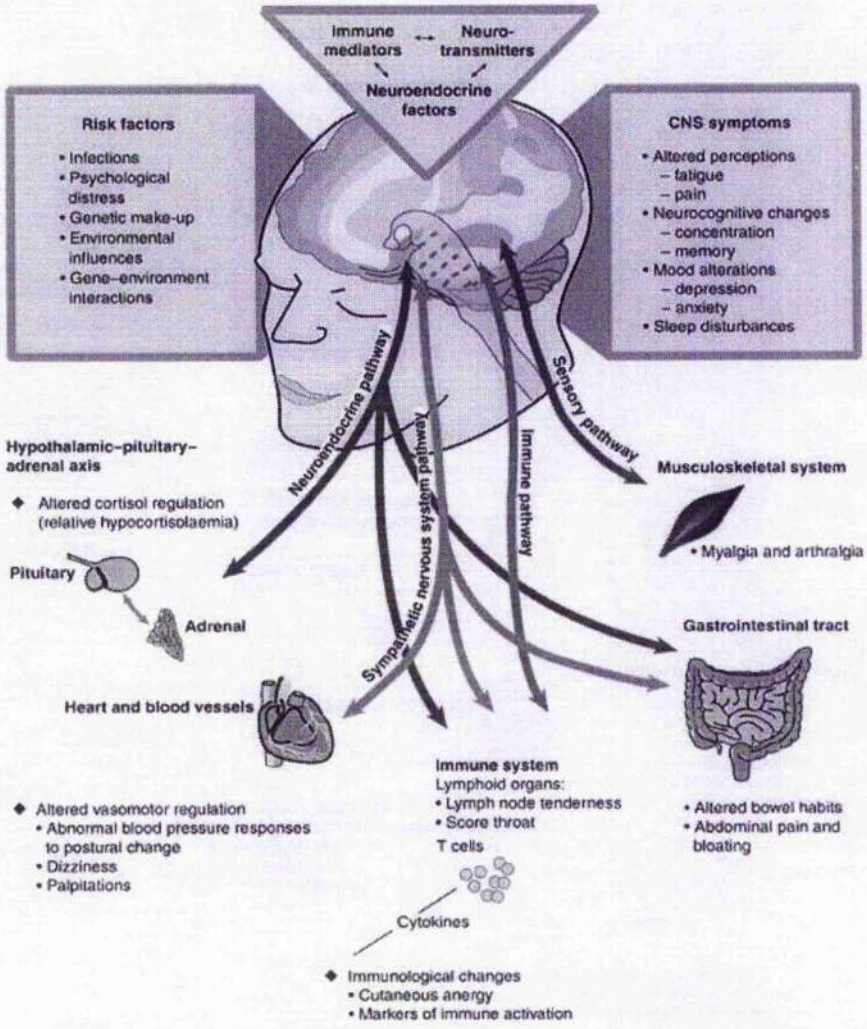


Figure 1.10 Immune mediators, neurotransmitters implicated and neuroendocrine factors (triangle) may modify neurological responses and enhance the risk factors (left square) and CNS symptoms (right square) associated with the pathogenesis of CFS. The neuroendorcrine pathway (1st reversed arrow from left) associated with hypothalamic-pituitary-adrenal axis, immune system and gastrointestinal tract dysregulation. The sympathetic NS pathway (2nd reversed arrow from left) associated with heart and blood vessels, immune system and gastrointestinal tract dysfunction. The immune pathway (3rd reversed arrow from left) related with the immune system, lymphoid organs responses. The sensory pathway (1st reversed arrow from right) associated with the possible musculoskeletal system dysregulation in CFS. Adapted from Royal Australasian College *of* Physicians (2002).

The primary diagnosis of CFS is depended upon which symptoms are the most dominant but usually the diagnosis of CFS is accompanied with concurrent depression diagnosis which most of the CFS patients experienced (Royal Australasian College *of* Physicians, 2002). Due to the multiple clinical and neurological symptoms that characterise CFS (see Figure 1.10), it has been suggested that CFS cannot be simply defined as a specific disease and the term illness or asthenia are used (Layzer, 1998). The diagnosis of CFS is based on eight clinical symptoms. These include 1) impaired memory or concentration, 2) sore throat, 3) tender cervical or axillary lymph nodes, 4) muscle pain, 5) multi-joint pain without arthritis, 6) headaches of a new type, pattern, or severity, 7) unrefreshing sleep, and 8) post-exertional malaise lasting more than 24 h (Fukuda *et al.*, 1994; Lee, 1998; Lloyd, 1998). If four or more ($\geq$ 4) of these symptoms, which are completely unrelated to physical exertion, are consistent and concurrently appear for more that six months CFS should be considered (Fukuda *et al.*, 1994).

1.2.8.2 Chronic Fatigue Syndrome and central neural mechanism

The first report that demonstrated an implication of CNS dysfunction in chronic fatigue diseases conducted by Poteliakhoff (1981) who showed a significant lower baseline cortisol levels in chronic fatigue patients relative to control. However, this novel study by Poteliakhoff did not specifically include CFS patients since the disease was not recognisable until late 1980s. Subsequently, two reports by Lloyd *et al.* (1988; 1991) precluded muscle contractile failure to be associated with the pathogenesis of the disease implying a central mediated effect. Similarly, a study measuring cortisol and glucocorticoid levels between CFS patient and control group implicated the hypothalamic-pituitary-adrenal (HPA) axis dysfunction being responsible for the development of the disease precluding therefore the primary immune system disturbances in the pathogenesis of the CFS (Demitrack *et al.*, 1991). Thereafter, several studies focused on the role of CNS in the pathogenesis and pathophysiology of CFS. Neuro-imaging studies for example, have shown that CFS patients had significant hypo-metabolic responses in right medio-frontal cortex and brainstem in comparison with the healthy control group (e.g. Tirelli *et al.*, 1998; Lange *et al.*, 1998). In addition, the serotonergic system has received considerable attention, because of its association with 'central fatigue' (e.g. Newsholme *et al.*,

1987) and its involvement in the control of HPA axis function (Chaouloff, 1993; Komaroff and Buchward, 1998).

Central serotonergic function in CFS patients has been assessed using neuroendocrine challenge tests and measuring circulating levels of 5-HT modulators, such as plasma [Trp] (e.g. Castell *et al.*, 1998) and other indices like Prl secretion and cortisol response (Bakheit *et al.*, 1992; Cleare *et al.*, 2000; Sharpe *et al.*, 1997). However, a recent neuroendocrine challenge and physical stress study suggested an insignificant effect of the potential lower cortisol secretion on the pathogenesis of CFS (Gaab *et al.*, 2002). Additionally, some other studies measured GH responses to neuroendocrine challenge since GH deficiency or HPA dysfunction-induced GH release attenuation may deteriorate muscle contraction (Parker *et al.*, 2001). However, a recent, large and well-designed study that examined GH responses to neuroendocrine challenge (hydrocortisone treatment) in CFS did not support GH deficiency in CFS (Cleare *et al.*, 2000). Consequently, the lack of significant implication of GH and cortisol observed by neuroendocrine challenge studies may enable brain serotonergic system dysfunction as an important factor in the development of CFS.

Up-regulation of hypothalamic 5-HT_{1A} post-synaptic receptors, indicated by measuring hypothalamic Prl secretion, in CFS patients has been reported at rest after a 5-HT agonist drug, buspirone (Bakheit *et al.*, 1992; Sharpe *et al.*, 1996). Enhanced 5-HT activity has been shown following administration of the selective 5-HT releasing drug, *d*-fenfluramine (Cleare *et al.*, 1995; Sharpe *et al.*, 1997). As with peripheral indices, however, reports of an association between abnormal 5-HT function and CFS have not been without controversy. Two studies recorded enhanced 5-HT mediated responses to *d*-fenfluramine in patients with CFS (i.e., as reflected in raised levels of cortisol and Prl) (Cleare *et al.*, 1995; Sharpe *et al.*, 1997) while another investigation found no differences between CFS patients and healthy controls in these indices of serotonergic function (Beam *et al.*, 1995). However, buspirone and *d*-fenfluramine were found not only to enhance 5-HT activation but also to bind to DA-D_2 receptors and affect catecholamines respectively (Sharpe *et al.*, 1996). Their action therefore in enhancing hypothalamic Prl secretion may be due to its ability in blocking D_2 receptors' activation. This is the primary Prl secretion inhibitor factor (Parker *et al.*, 2001).

Consequently, the extent to which the inconsistencies might reflect heterogeneity of patient responses or to neuroendocrine challenge tests limitations is presently unclear.

1.2.8.3 Chronic Fatigue Syndrome and exercise tolerance

It has been well established that CFS is a disorder associated with persistent, often debilitating, physical and mental fatigue, that can be exacerbated by even mild degree of physical activity (Komaroff and Buchwald, 1998; Fukuda *et al.*, 1994). A number of studies have therefore examined whether the pathogenesis of the disease is associated with skeletal muscle dysfunction and/or cardiovascular system abnormalities. The results however, obtained from those studies are conflicting. Early reports for example indicated abnormalities of muscle metabolism (Wong *et al.*, 1992) and/or histology in CFS (Behan *et al.*, 1992). Wong *et al.* (1992) for example, using magnetic resonance spectroscopy (MRS) examined metabolic responses of the gastronemius muscles during graded exercise to exhaustion and at recovery. They found a higher acceleration rate in muscle glycolysis during exercise and a lower intracellular ATP concentration at exhaustion in CFS patients relative to sedentary control group. They concluded that a deficiency in oxidative metabolism with a secondary acceleration of glycolysis in the skeletal muscle causes a reduction in physical endurance during exercise in CFS. In addition, Behan *et al.* (1991) using muscle biopsy examined muscle characteristics in postviral fatigue syndrome patients and they found mild to severe atrophy of type II fibres and mitochondrial abnormalities in 39 out of 50 patients.

De Becker *et al.* (2000) also examined cardiovascular responses in CFS female patients at rest and after graded exercise to maximum. They found that CFS patient had lower maximum oxidative capacity and achieved lower maximum work-load. CFS patients also had higher resting HR but lower HR at maximum relative to control. De Becker *et al.* suggested that autonomic nervous system disturbances associated with peripheral incapability being responsible for the pathogenesis of CFS. Fulle *et al.* (2000) also, examined whether the pathogenesis of CFS is explained by oxidative damage to DNA and lipids in skeletal muscle sample comparing CFS patients with matched sedentary control. Consistent with Behan *et al.*, (1991), Fulle *et al.* (2000) suggested that mitochondrial dysfunction and oxidative damage may be important factors in the pathogenesis of CFS. Similarly, in a subsequent study Fulle *et al.* (2003) investigated whether excitation-contraction-

coupling abnormalities could be involved in the development and sustaining nature of CFS. They suggested that CFS patients had significant abnormalities in Na^+/K^+ pump function, sarcoplasmatic reticulum network and Ca^{2+} transporters to muscle dysfunction relative to control. However, it is noted that the studies by Fulle *et al.* (2000; 2003) are characterised by a small-unpaired sample size, only six subjects in each group, and therefore the generalisation of the results should be considered with caution.

Inconsistencies however, in the literature suggest that other mechanisms may be involved. For example, initial indications of premature blood lactate accumulation during exercise in CFS patients (Lane *et al.*, 1994) were followed by subsequent studies showing considerable variation in the blood lactate response to exercise (Lane *et al.*, 1995). In addition, Lloyd *et al.*, (1988 and 1991) examined whether peripheral and subjective fatigue are responsible for the pathogenesis of CFS. They found that peripheral responses to physical activity such as muscle strength and fatigability were normal in CFS when compared with healthy control group but the results concerning effort perception between the two studies were inconsistent. They suggested that muscle contractile failure and poor motivation are not important factors in the pathogenesis of CFS. More-recent studies also suggested that lactate metabolism and $\dot{V}O_{2\,max}$ were normal in CFS (Sargent *et al.*, 2002). In a subsequent study, it was found that although there was a partial restriction in muscle blood flow in CFS patients, oxygen delivery, (measured with near infrared spectroscopy) and oxidative muscle metabolism, (measured with MRS) there were no differences between CFS and sedentary control groups during exercise and recovery (McCully *et al.*, 2004). Consequently, these results may suggest no muscle metabolic or histological abnormalities nor cardiovascular incapability in CFS patients indicating possible a central mediated effect.

It is possible that some peripheral abnormalities and the characteristic exercise intolerance of CFS, observed from some studies, are due to deconditioning (e.g Fulcher and White, 2000; Montague *et al.*, 1989). Deconditioning may be of primary importance in the pathogenesis of the disease (Riley *et al.*, 1990) and contributes in exacerbating the symptoms and maintenance of the physical disability in CFS (Gaab *et al.*, 2002). Alternatively, some other studies support the hypothesis that CFS is a heterogeneous disorder with some patients influenced by peripheral

abnormalities but some others not susceptable (Barnes *et al.*, 1993; Lane *et al.*, 1998). Barnes *et al.* (1993) for example, measured muscle bioenergetic and intracellular pH in CFS patients using phosphorus MRS. Although, they found abnormal muscle metabolic responses in 12 out of 46 patients, overall there were no significant abnormalities in glycolysis, mitochondrial metabolism and pH regulation in CFS patient when compared with sedentary control. However, although several lines of evidence exist to suggest an abnormal hypothalamic response (e.g. Demitrack *et al.*, 1991), particularly central neurotransmitter dysfunctions in CFS (e.g. Bekheit *et al.*, 1992; Cleare *et al.*, 1995), the modulators of brain serotonergic and dopaminergic functions in association with perceptual and metabolic responses have yet to be studied in this patient population during exercise.

1.3 Objectives

The primary purpose of the present research is i) to clarify and improve the current understanding of the role of putative brain 5-HT and DA modulators (free and total Trp, Tyr, LNAA) and therefore of the brain serotonergic and dopaminergic function in exercise fatigue ii) to elucidate the relationship with the brain 5-HT and DA modulators and perceptual and metabolic responses in health and exercise intolerant patients and iii) to differentiate the mechanism between peripheral and central fatigue development in health and disease during exercise. The four separate experiments (Chapters 3 and 4: experiments 1 and 2, Chapter 5: experiment 3 and Chapter 6: experiment 4) share these common objectives, although the specific aim of each separate experimental chapter is summarised below:

A. Experiments 1 and 2. To simultaneously examine physiological and biochemical aspects affecting central and peripheral fatigue in well trained humans, using caffeine co-ingested with a pre-exercise high fat meal. This examination was intended i) to elucidate the relationship between putative modulators of brain 5-HT and DA function and perceptual and exercise fatigue development and ii) to clarify whether the actions of caffeine are associated with brain 5-HT and DA activation or with putative altered substrate utilisation effects dynamic exercise in well-trained humans.

B. Experiment 3. To examine the effects of oral Cr supplementation induced hyperhydration on putative modulators and indices of brain serotonergic and dopaminergic function, perceptual and thermoregulatory responses during exercise in the heat in well-trained humans.

C. Experiment 4. To investigate the role of putative plasma modulators of brain 5-HT and DA function (Trp, Tyr, LNAA) in possible central fatigue development in CFS patients at rest and during graded exercise-stress and to evaluate the association between these modulators and perceptual, metabolic and cardiovascular responses in CFS patients.

Chapter two

General Methods

2. General Methods

This chapter describes the general methodology used throughout this thesis. The thesis presents four main experiments. Specific methods to each particular experiment are described in the relevant chapters.

2.1 Subjects and study approval

All experiments (EXPs) described in the present thesis involved human volunteers. The subjects for experiments 1 (n = 8), 2, (n = 10) and 3 (n = 21) were all well-trained male athletes. For EXP 4, subjects were chronic fatigue syndrome (CFS) patients (male: n = 2, female: n = 10) and healthy inactive sedentary control (male: n = 2 and female: n = 9).

Prior to each EXP, all subjects were informed of the nature and the purpose of the EXP and they made fully aware of the known risks associated with each experimental trial. Subjects in EXPs 1, 2, 3 and 4 (sedentary control) underwent a general medical assessment including completion of a questionnaire relating to subjects individual and family medical history and physical activity profile (Appendix A). All patients who took part in EXP 4 fulfilled the Centres for Disease Control Criteria for CFS (Fukuda *et al.*, 1994) and were clinically examined in order to exclude any other medical condition. Primarily office based workers were recruited as control subjects and individually matched to CFS patients for physical activity. All subjects provided written informed consent prior to their participation in an EXP (Appendix B). It was emphasised that subjects should only take part in the EXP if they agreed to fulfil all requirements and were fully committed. All EXPs were approved by the University of Glasgow Research Ethics Committee.

2.2 Determination of $\dot{V}O_{2\,max}$, lactate threshold and exercise work-rate

Prior to EXPs 1, 2 and 3, all subjects underwent a $\dot{V}O_{2\,max}$ test using a ramp incremental protocol (15-20 $W \cdot min^{-1}$) to the limit of tolerance (Figure 2.1) on an electrically braked cycle ergometer (EXP 1: Excalibur Sport, Lode, The Netherlands; EXPs 2 and 3: Bosch Erg-551 Forckenbecksti, Berlin, Germany). The two different cycle-ergometers were methodologically demanded since the

Excalibur Sport ergometer used in EXP 1 could not be used in EXPs 2 and 3 as in these EXPs the 10 °C and 30 °C ambient temperature respectively with 70% relative humidity employed (during the familiarisations and main trails) could damage the Excalibur Sport ergometer. The primary criteria for indicating $\dot{V}O_{2\,max}$ were the reaching plateau of $\dot{V}O_2$ with an increasing work-load (i.e. EXP 1) in conjunction with achieving near maximum heart rate (HR) (220 beats·min^{-1} – years of age) (i.e. EXPs 2 and 3) (Astrand and Rodalh, 1977; Lamb, 1984).

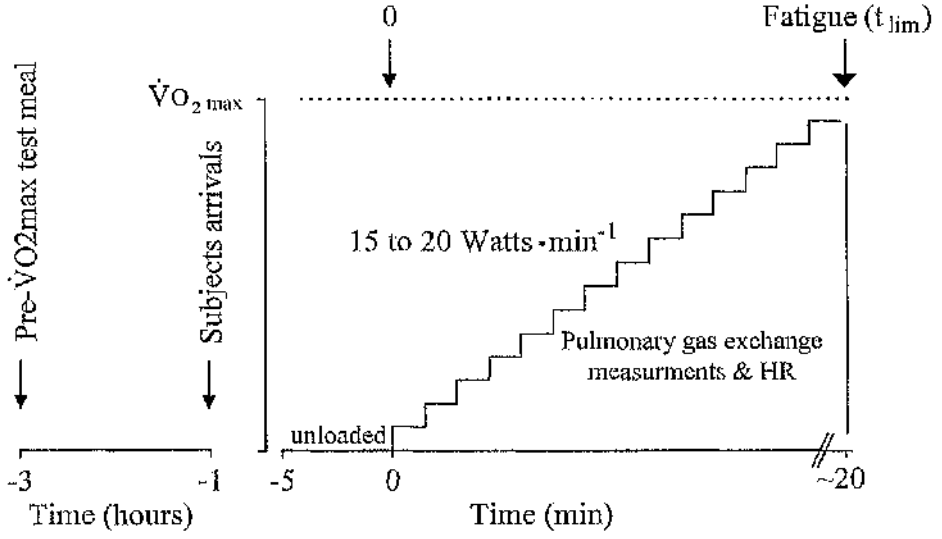


Figure 2.1 Schematic of the protocol used during incremental cycle tests to the limit of tolerance (t_{lim}) for experiments 1-3.

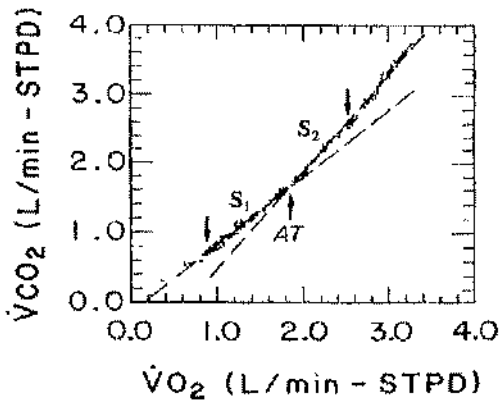


Figure 2.2 Non-invasive estimation of LT (or AT) using V-slope technique. Best-fit lines (S1 & S2) are plotted though the sub- and supra-LT data respectively, LT determined as the point of intersection of the two lines. Adapted from Beaver *et al.*, (1986).

Lactate threshold (LT) or anaerobic threshold (AT) was estimated non-invasively as the $\dot{V}O_2$ at which: (a) the break-point in the relationship between CO_2 output ($\dot{V}CO_2$) and $\dot{V}O_2$ {"V-slope" technique, (Beaver *et al.*, 1986)} occurred and (b) the ventilatory equivalent for O_2 ($\dot{V}E/\dot{V}O_2$) started to increase systematically without a concomitant increase in the ventilatory equivalent for CO_2 ($\dot{V}E/\dot{V}CO_2$) (Whipp *et al.*, 1986) (Figure 2.2). The purpose of measuring the LT was to deliberately normalise exercise intensity across the individuals, not only relative to $\dot{V}O_{2\,max}$, but also the LT. This allows a more valid standardisation of exercise intensity during the main exercise trials in EXPs 1, 2, and 3 since not everyone's LT will occur at the same percentage of $\dot{V}O_{2\,max}$. Therefore, the term Δ (see method sections chapters 3, 4 and 5) can describe exercise intensity more accurately, as initially proposed by Rausch *et al.* (1991).

2.3 Gas exchange measurements: $\dot{V}O_{2\,max}$ and constant-load protocols

In EXPs 1 and 4, gas exchange variables were determined breath-by-breath using previously derived algorithms (Beaver *et al.*, 1973). Respired volumes were measured using a bi-directional turbine volume transducer (VMM, Alpha Technologies, Laguna Niguel, USA), calibrated using a high precision three litre syringe (Hans Rudolph, Kansas City, MO, USA). Respired gas concentrations were measured every 20 ms by a quadrupole mass spectrometer (QP9000, Morgan Medical, Gillingham, Kent, UK), which was calibrated against two precision-analysed gas mixtures. In EXPs 2 and 3 expired gas collections were made into Douglas bags at rest for 5 min, during unloaded pedalling for 2 min, and every minute during the incremental test. Expired gases were analysed for [O_2] (Servomex 570A, East Sussex, UK) and [CO_2] (Servomex 1400 B4, East Sussex, UK), volume (dry gas meter, Harvard Apparatus Ltd., Hertfordshire, UK) and temperature (C6600 10-Channel Microprocessor, Comark, Hertfordshire, UK). All gas volumes were corrected to STPD. The gas analysers were calibrated before each test using a two-point calibration with precision gases (CO_2 7.5%, O_2 16%, N_2 balance, certified standard gas). Barometric pressure for all EXPs (1-4) was measured using a standard mercury barometer. The results from the incremental tests were used to define the relevant work-rates for each EXPs (see individual Chapters).

The breath-by-breath gas exchange analysis of the response to exercise is typically associated with 'noise', which has been extensively characterised (Lamarra *et al.*, 1987; Rossiter *et al.*, 2000; Puente-Maestu *et al.*, 2001). These authors demonstrated the noise as an uncorrelated Gaussian distribution and that this confounding noise can prevent accurate characterisation of the underlying physiological response if not considered. In EXPs 1 and 4, the breathing pattern was monitored carefully for irregularities such as mis-triggered breaths, which can be caused by swallowing, sighing or coughing. Such breaths, which are clearly not indicative of the underlying physiological response, were identified by examining the chart tracing of the volume and gas concentration raw signals as well as the on-line individual breath characteristics, such as tidal volume, the duration of inspiration and expiration, and end-tidal gas concentrations. Individual breaths were compared with preceding and following breaths to determine whether the breath generated by the software was indeed a 'real physiological' breath. Breaths which were clearly not part of the underlying physiological response were excluded from subsequent data analysis, although dubious breaths were not removed. In tests where the response could be justifiably mathematically modelled, as linear the exclusion of such breaths was carried out by removing breaths which lie ± 4 s.d. outside the mean response as previously described by Lamarra *et al.* (1987).

The raw breath-by-breath data during the constant-load phase in EXP 1 was initially examined for outliers ('noise'). After removal of outliers (i.e., ± 4 s.d.), 2 min averages for $\dot{V}O_2$, $\dot{V}CO_2$, RER, $\dot{V}E$ were calculated for the following time points: 8-10, 18-20 and 28-30 min. In EXP 2 and EXP 3 expired gases were collected into Douglas bags at rest for 5 min and thereafter for 1 min every 15 min in EXP 2 and every 5 min throughout exercise in EXP 3. $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$ and RER were subsequently used to determine energy expenditure (EXP 1-3) (Ravussin *et al.*, 1985) and the rates of CHO and fat utilisation (Jansson and Kaijser, 1982) (EXP 1-2). Energy expenditure (EE) was calculated for each time-point using the following equation: EE (kcal·min^{-1}) = [4.686 + (RER - 0.707 / 0.293) 0.361] $\dot{V}O_2$.

2.4. Heart rate and Ratings of Perceived Exertion

In EXPs 1-3 HR was recorded continuously using Polar Sport Tester (Polar Electro Oy, Finland) and in EXP 4 using online electrodes. In EXPs 1, 2, and 3, subjective ratings of perceived leg tiredness and breathlessness (RPE) were recorded at rest, regularly during the constant-load exercise phase (every 10 min, 15 min, and 5 min in EXPs 1, 2, and 3 respectively) and at exhaustion using the Borg category scale (Borg 1982). In EXP 4, RPE was obtained at rest (sitting on the bicycle ergometer), during unloading pedalling, every 3 min during the ramp phase and at exhaustion using a visual-analogue scale where "0" represented 'nothing at all' and '100' represented 'maximum'.

2.5 Experimental designs

All performance trials and treatments and dietary interventions carried out following a Latin-square block design. In EXPs 1 and 2, where the same group of subject have been examined under performing all the different treatments, considerable care was given in order to ensure that there was no order effect on performance by involving a series of familiarisation trials in the experimental design. The primary scope of these trials was to familiarise the subjects with the main exercise trials and experimental procedures. In EXPs 3 and 4, two different groups of subjects performed the different treatments. In EXP 3, a full familiarisation scale was performed in order to familiarise the subjects with exercise in the heat and humid environment.

2.6 Blood sampling and analytical procedures

Arterialised-venous blood samples (Foster *et al.*, 1972) were obtained in all EXPs by introducing either an 18 G or a 21 G cannula into a superficial vein on the dorsal surface of the subjects' heated hand. In some subjects where blood sampling from the hand proved difficult, a superficial vein in the fore-arm was used. Subjects were comfortably seated with their forearm immersed in water at 42-44 °C for at least 10 min before a resting blood sample was obtained. The cannula was kept patent by a slow (ca. 0.5 $ml \cdot min^{-1}$) infusion of isotonic saline between samples. Arterialisation

of the venous blood was maintained throughout exercise by heating the hand via an infrared heating lamp (EXPs 1, 2, 4). In all EXPs room temperature during resting blood sampling was maintained between 20-22 °C.

Blood (10 ml) was drawn into dry syringes and dispensed (8 ml) into tubes containing K_3EDTA (all EXPs) and the remaining into tubes containing no anticoagulant for prolactin (Prl) analysis (see below). Blood metabolites were analysed by a spectrophotometric adaptation based of the methods of Maughan (1982). Duplicate aliquots (400 µl) of whole blood from the K_3EDTA tubes were rapidly deproteinised in 800 µl of ice-cold 0.3 mol·l^{-1} perchloric acid (PCA); following centrifugation the supernatant was used for the measurement of glucose, lactate (all EXPs), pyruvate (EXPs 2 and 4) and glycerol (EXPs 1, 2, 4) (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK). In order to validate the blood analysis methods were tested on a) samples with 1:3 dilution (400 µl, 800 µl 0.3M PCA); b) 1:6 dilution (200 µl, 800 µl 0.3M PCA) and c) 1:10 dilution (400 µl, 800 µl 1.2 M PCA as described by Maughan, 1982) and found no statistically significant differences in blood metabolites. A further aliquot of blood (3 ml) was centrifuged and the plasma obtained was separated and immediately placed into liquid nitrogen. The plasma was then used for the measurements of FFA (colorimetric method, Roche Diagnostics GmbH, Germany) (all EXPs) and amino acids including free tryptophan (free-Trp). For enzymatic methods regarding blood glucose, lactate and pyruvate and plasma glycerol and FFA analyses see Appendices D1, D2, D3, D4, and D5 respectively.

The analysis of plasma amino acids including free-Trp have been performed by High Performance Liquid Chromatography (HPLC) using fluorescence detection and pre-column derivatisation with 18 *o*-phthalaldehyde (Hypersel Amino acid method, ThermoHypersil-Keystone, Runcorn, UK). In summary, thawed plasma samples were spun for 10 min at 3000g at 4 °C and 80µl dispensed into eppendorf-tubes containing 20µl of internal standard (1.375mM- L-Methionine Sulfine) and 10µl of PCA (3.3M). Samples were vortexed for 1min then spun at 3000g at 4°C for an additional 10min. The supernatant was removed and placed into HPLC-tubes for reverse-phase HPLC analysis using a Gilson Gradient HPLC system (Gilson, Middleton, USA). A volume of 20µl was typically auto-injected (234 Gilson

Autoinjector, Gilson, Middleton, USA) onto an amino-acids column (250 x 4.6mm I.D., particle size 5μm, ThermoHypersil-Keystone, Runcorn, UK) at 30°C. The mobile phase pumped at a flow rate of 2.5ml·min^{-1}. Amino acids were determined by UV detection (Gilson 121 Fluorometer, Gilson, Middleton, USA) using 250nm excitation and 450nm emission. For the analytical preparation of the HPLC buffers (A and B) and mixed-reagent (C) used as well as calibration standard and mixed reagent preparations see Appendix C. For the analysis of free-Trp, Trp was separated from protein-bound Trp by filtering plasma (250μl after manually inverting-re-inverting samples for 5 min) through 10,000 NMWL, nominal molecular weight limit, cellulose filters (Ultrfree-MC filters, Millipore Corporation, USA) during centrifugation at 5000g for 60min at 4°C. Filters were filled with a 95% O_2 – 5% CO_2 mixture during centrifugation in order to stabilise pH.

Some of the uncoagulated blood was also used for the measurements of haemoglobin (cyanmethemoglobin method, Sigma Chemical Company Ltd., Dorset, UK) and packed cell volume (PCV) (conventional micro-haematocrit method) in all EXPs. Haematocrit was determined in triplicate from aliquots of EDTA blood dispensed into micro-haematocrit tubes (Hawskley and Sons Ltd, Lancing, UK), centrifuged for 12 min, and determined using a micro haematocrit reader (Hawskley and Sons Ltd, Lancing, UK). Plasma volume changes were calculated from changes in haemoglobin and PCV relative to initial resting values (Dill and Costill, 1974).

The blood in tubes without anticoagulant was allowed to clot and then centrifuged; the serum collected was used for the measurement of Prl (EXPs 1-3). Prl was measured on the Bayer ADVIA Centaur immunoassay analyser (Technicon Immuno-1 System, Bayer plc, Bayer House, Newbury, UK). The assay is a two-site sandwich immunoassay using direct chemiluminometric technology. The first antibody is a polyclonal anti-prolactin antibody labeled with acridinium ester. The second antibody is a monoclonal mouse anti-prolactin antibody which is covalently bound to paramagnetic particles. The assay is standardised against the World Health Organization 3rd IRP 84/500 reference material and measures prolactin in 25ul of serum. The whole procedure is automated and the within batch and between batch precision (CV) are < 3 and < 6% over the concentration range for the samples in the current study.

2.7 Statistical analysis

Data from all experiments are expressed as the mean ± s.d. or median (inter-quartile range: IQR) as appropriate following a test for the normality of distribution (Shapiro statistical normality test). In EXPs 1 and 2 since all subjects completed the Control trial first and were subsequently assigned to the two fat trials in randomised order, statistical analysis was carried out on the two fat trials. However, the results for all trials are shown in all particular figures and tables.

Statistical analysis of the data from EXPs 1 and 2 was carried out using a two-factor ANOVA (Treatment x Time) for repeated measures followed by a Student's *t*-test when a significant main treatment, interaction or time effect was observed. Time to exhaustion (in EXP 2) was not normally distributed and was therefore analysed using Wilcoxon signed-ranks test. In EXP 3, statistical analysis was carried out using a mixed 3-way ANOVA (Group x Pre- and Post- supplementation x Time) with repeated measures on the last two factors. A subsequent 2-way ANOVA with repeated measures was performed when there was a main effect on Group, Pre-Post supplementation, or interaction. Two-sample *t*-test (between treatment effect, i.e., magnitude of change (Δ) in the Cr group vs. Δ in the placebo group) and Student *t*-test (within treatment effect, i.e., Pre- vs. Post-supplementation) were performed if a main treatment or interaction effect was observed. For non-parametric data (all plasma amino acids, FFA and Prl results), Friedman two-way ANOVA (followed by Wilcoxon test) and Mann-Whitney tests were used for paired and unpaired data, respectively. Statistical analysis regarding plasma amino acids variables for EXP 3, includes eighteen subjects due to the lack of collecting appropriate amount of blood from three subjects for either pre- or post supplementation trials. In EXP 4 statistical analysis was carried out using independent Student's *t*-tests and Mann-Whitney tests for parametric and non-parametric data to assess differences for the measured variables between the two groups for each time point, as appropriate.

In all EXPs, Pearson's product moment r and Spearman's rho correlation analyses, for parametric and non-parametric data respectively were used to assess the relationship between selected variables. Correlation analyses performed for each time point separately. Statistical significance for all experiments was declared when $P < 0.05$.

Coefficient of Variation (C.V.): C.V. is the s.d. expressed as a proportion or percentage of the mean (Bolton, 1997). Consequently, the intra-assay C.V. was calculated (see equation 2. 1. below) from the s.d. of the difference between double measurements of the sample expressed as a percentage of the total mean sample (Table 2.1). When the cost of the duplicate analysis exceeded £1 per sample, the C.V. was determined from at least 10 aliquots of the same sample (Table 2.1).

$$C.V.\% = (s.d./mean) \chi\ 100 \qquad \text{Equation 2.1.}$$

Table 2.1: Coefficient of Variation of blood and plasma assays

Assay	Method	n	C.V.
Blood glucose	Maughan 1982	50	4.3
Blood lactate	Maughan 1982	50	1.9
Blood pyruvate	Maughan 1982	50	3.9
Blood Hb	Cyanmethemoglobin method	50	0.3
PCV	Micro-haematocrit method	50	13.1
Plasma glycerol	Boobis and Maughan 1983	12	3.5
Plasma FFA	Colorimetric method, Roche Diagnostics	10	3.4
Plasma Valine	Hypersel Amino acid method (HPLC)	10	5.7
Plasma Isoleucine	Hypersel Amino acid method (HPLC)	10	4.5
Plasma Leucine	Hypersel Amino acid method (HPLC)	10	5.2
Plasma Tyrosine	Hypersel Amino acid method (HPLC)	10	2.5
Plasma Phenylalanine	Hypersel Amino acid method (HPLC)	10	5.9
Plasma Tryptophan	Hypersel Amino acid method (HPLC)	10	4.1
Plasma free-Tryptophan	Hypersel Amino acid method (HPLC)	15	4.5

Chapter three

(Experiments 1 and 2)

A differentiation attempt between central and peripheral components affecting fatigue: the effect of caffeine co-ingested with a high fat meal

3.1 Introduction

Previous attempts to investigate central fatigue during exercise have not concurrently examined physiological and biochemical variables that may play a role in both peripheral and central fatigue development. Consequently, when 'central fatigue' has previously been studied in humans (e.g. Blomstrand, 1988; 1989; 1991b) and animals (e.g. Bailey *et al.*, 1993a) there was not always an inclusive assessment of whether metabolic, cardiovascular and perceptual responses had played a role in the fatigue process.

Caffeine has been reported to increase exercise performance by enhancing fat oxidation and, therefore, sparing glycogen (e.g. Costill *et al.*, 1978), particularly during the early stages of prolonged high intensity exercise (Spriet *et al.*, 1992) (see Chapter 1, section 1.2.6.4). A number of mechanisms have been proposed to explain this caffeine-induced sparing of muscle glycogen: i) caffeine may reduce muscle glycogenolytic rate by inhibiting glycogen phosphorylase α (Phos α) activity (the flux-generating step for muscle glycogenolysis) by decreasing the sensitivity of Phos α kinetics with respect to Pi which may play a major role in the control of skeletal muscle glycogenolysis in vivo (Rush and Spriet, 2001); ii) caffeine can enhance free fatty acid (FFA) mobilisation by stimulating the release of epinephrine and, hence, increase the potential for fat oxidation (Spriet *et al.*, 1992); and iii) caffeine may indirectly promote fat oxidation and carbohydrate (CHO) sparing by inhibiting adenosine receptors in adipose tissue, which otherwise inhibit FFA mobilisation from adipocytes (Spriet 1995).

Recently however, several studies observed an increase in endurance performance without supporting the 'metabolic theory' effect of caffeine and others found an improvement in high intensity exercise performance, following caffeine ingestion, where muscle glycogen depletion is clearly not the primary cause of fatigue (see Chapter 1, section 1.2.6.4). Consequently, caffeine may enhance endurance performance, not by sparing muscle glycogen as often suggested, but through other effect(s). For example, caffeine has been reported to i) reduce effort perception (RPE) (e.g. Cole *et al.*, 1996), ii) attenuate 'central fatigue' by reducing brain serotonin turnover, through an inhibition of tryptophan hydroxylase expression (Lim *et al.*, 2001), and iii) inhibit central adenosine receptors activation, thereby attenuating 'central fatigue' by increasing the DA:5-HT ratio in the brain (Davis *et*

al., 2003). Alternatively, caffeine or one of its by-products could directly affect skeletal muscle and/or influence the propagation of neural signals in regions between the brain and neuromuscular junction (see Spriet, 1995 for review). The contradictions reported in the literature may be due to the multiple sites of action of caffeine, within both the central nervous system (CNS) and peripheral tissues (e.g. Fredholm *et al.*, 1999). Based on the above information it could be hypothesised that caffeine may have at least two significant actions, a peripheral (metabolic) action and/or a CNS effect. Consequently, an alternative approach, which attempts to distinguish primary from secondary effects of caffeine is required before its effect on brain neurotransmission during exercise is considered.

An acute increase in plasma [FFA] similar to that reported following caffeine ingestion can be induced by giving subjects a high fat meal with or without intralipid-heparin infusion (Romijn *et al.*, 1995; Okano *et al.*, 1996; 1998; Whitley *et al.*, 1998), with consequent increases in the rate of fat utilisation (Rennie *et al.*, 1976; Costill *et al.*, 1977; Dyck *et al.*, 1993; Vukovich *et al.*, 1993; Hawley *et al.*, 2000) and, therefore, improvement in exercise performance (Hickson *et al.*, 1977; Pitsiladis *et al.*, 1999). Consequently, the aim of the first two experiments (EXPs) was to differentiate between central and peripheral aspects affecting fatigue and to examine perceptual and metabolic responses during constant-load submaximal exercise, and on incremental and endurance exercise performance after caffeine co-ingested with a high fat meal. The high fat meal was employed in an attempt to increase plasma [FFA] and fat metabolism (Pitsiladis *et al.*, 1999) so that any action of caffeine on adipose tissue lipolysis would be insignificant. Caffeine co-ingested with a high fat meal regimen was employed in order the mechanism(s) associated with central and peripheral fatigue could be evaluated. It was hypothesised that co-ingestion of caffeine with a high fat meal could differentiate between putative substrate utilisation (metabolic) effects and CNS actions of caffeine.

3.2 Methods

Subjects

Eight male endurance-trained subjects (mean ± s.d.) (age 27 ± 4 years; height 178 ± 6 cm; body mass 73.7 ± 7.8 kg; maximal oxygen uptake ($\dot{V}O_{2\,max}$) 57 ± 5 ml·kg^{-}

^{1}·min^{-1}), and ten endurance-trained male cyclists (age 25 ± 6 years; height 182 ± 7 cm; body mass 74.3 ± 8.6 kg; $\dot{V}O_{2\,max}$ 62 ± 5 ml·kg^{-1}·min^{-1}) volunteered to participate in EXP 1 and EXP 2, respectively. For more information regarding subjects medical screening and study approval see Chapter 2.

Experimental design

The determination of $\dot{V}O_{2\,max}$, LT and test workloads are described in chapter 2. The schematic illustrations of the experimental designs for both EXP 1 and 2 are presented in Figure 3.1 and 3.2 respectively.

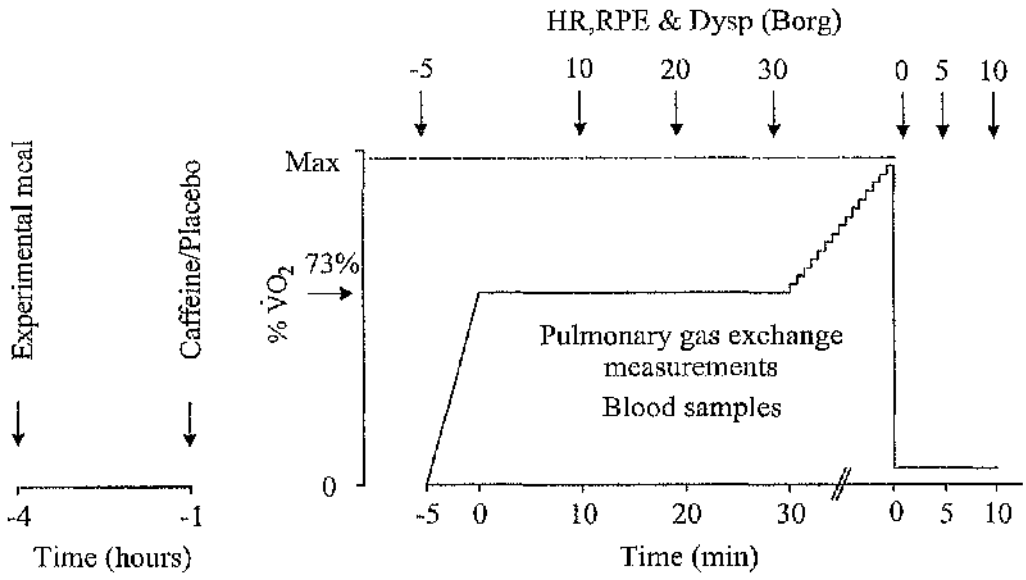


Figure 3.1 Schematic illustration of the experimental design in experiment 1

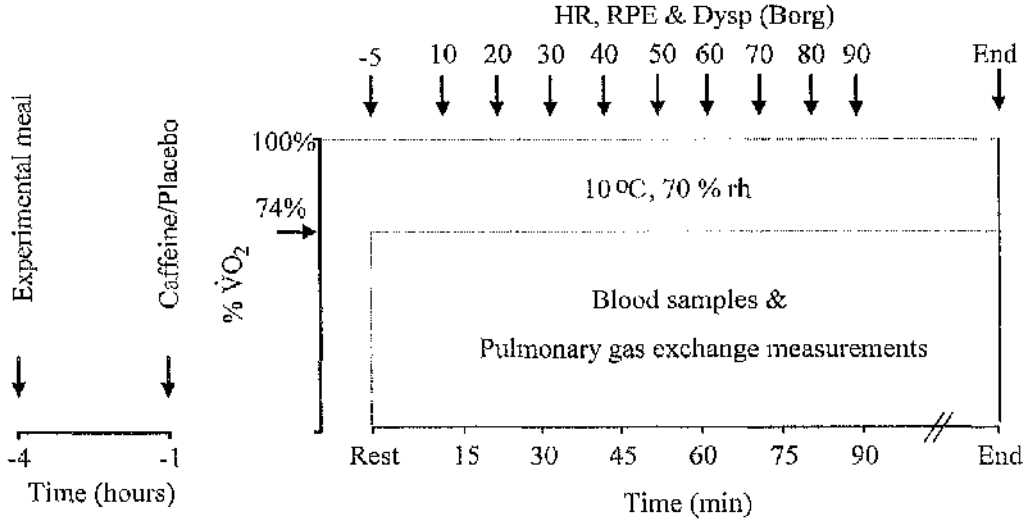


Figure 3.2 Schematic illustration of the experimental design in experiment 2

In both EXPs subjects performed three experimental trials, the first one after a high CHO meal (Control trial) and the remaining two after a pre-exercise high fat meal with (FC trial) and without (F trial) caffeine ingestion. In EXP 1, subjects cycled at a work-rate equivalent to 63 % of each subject's peak power output [i.e., 73 ± 5 % $\dot{V}O_{2\,max}$ or 46 ± 4 % Δ, which is the difference between the $\dot{V}O_2$ at the LT and $\dot{V}O_{2\,max}$, as proposed by Rausch *et al.*, 1991], which was immediately followed by a 15 W·min^{-1} ramp to the limit of tolerance. In EXP 1, each consecutive test was separated by at least one week from the previous one. In EXP 2, subjects cycled to exhaustion at a similar work-rate [i.e., 74 ± 5 % $\dot{V}O_{2\,max}$ or 42 ± 3 % Δ].

Subjects underwent at least two familiarisation trials prior to the three exercise tests, in order to become familiarised with the exercise protocol and experimental procedures and to adjust the desired exercise intensity. In EXP 2, During the familiarisation period (i.e., 3 days prior to the second familiarisation trial), each subject's normal energy intake and diet composition were determined from weighed dietary intake data, using a computerised version of the food composition tables of McCance and Widdowson (revised by Holland *et al.*, 1991). Based on this information, subjects were prescribed a 70 % CHO diet throughout the study period, intended to increase and maintain muscle and liver glycogen content (Bergstrom *et al.*, 1967) before each of the main exercise trials. The 70 % CHO diet was isoenergetic with each subject's normal daily energy intake, and food items prescribed were based predominantly on each subject's normal diet. In EXP 1, subjects were provided with a dietary record book and weight scale and they were advised to record and keep their normal daily energy intake throughout the experimental period and to try to consume items that involve mostly CHO nutritional contents. A particular nutritional list was given to each subject. The subjects were advised to consume the same items with a similar weighted amount for three days prior to each experimental trial.

Four hrs prior to the first exercise test in both EXP 1 and EXP 2, subjects consumed a standardised high CHO meal (90 % of energy intake in the form of CHO; Control trial). Since the Control trial was always performed first, it was not included into the randomisation hence into the statistical analysis. Four hrs prior to the second and third exercise tests, subjects consumed a standardised high fat meal (1 g fat·kg^{-1}

body mass; 90 % of energy intake in the form of fat). The standardised high fat meal was consistent with a commercial double fresh cream (Fat: 96.1 %; CHO: 2.2 %; Proteins: 1.6 %) mixed with 100g fresh strawberries (9 kcal; Fat 0.17g; CHO 2g; Proteins 0.1g) in order to improve the palatability of the meal (Pitsiladis *et al.*, 1999). The composition and nutrition values of the fresh double-cream consistent: Per 100g: energy 445 kcal (or 1813KJ), Fat 47.5g (saturated 32.21g; unsaturated 13.8g; polysaturated 1.49g) or 427.5 kcal, CHO 2.5g or 10 kcal, Protein 1.79g or 7.16 kcal, Fibre 0g, Sodium trace salt 0.1g. All experimental meals were isoenergetic and prepared by the same investigator. One hr prior to exercise following the high fat meals, subjects ingested, in a cross-over double blind manner, capsules containing caffeine (EXP 1: 7 $mg \cdot kg^{-1}$ body mass, EXP 2: 7.5 $mg \cdot kg^{-1}$ body mass; FC trial) or an equivalent amount of placebo (calcium carbonate; F trial). Caffeine was ingested one hr prior to exercise in an attempt to elicit peak plasma [caffeine] (Burg, 1975; Robertson *et al.*, 1978). Subjects were advised to avoid consuming items that contained caffeine or alcohol for at least 48 hours prior to each experiment or to ingest any vitamins supplements or any medication throughout the experimental period. Subjects required to avoid any strenuous activity for at least 72 hours prior to each exercise trial.

Procedures

All exercise tests were carried out between 16:00-21:00 hrs following a 4 hr fast, where water was allowed *ad libitum*. Subjects reported to the laboratory 1.5 hrs prior to the start of exercise, and on the two fat trials consumed capsules containing caffeine or placebo, 3 hrs after consuming the fat meal. Body mass was measured and, subsequently, subjects were seated comfortably with their right hand and forearm immersed for 15 min in warm water. For cannulation, blood sampling at rest and during exercise, HR and RPE recording see Chapter 2.

Briefly, in both experiments initial blood samples were obtained at rest. For EXP 1, further blood samples were obtained at 10 min intervals during the constant-load phase, at the end of the ramp, and at 5 and 10 min post-exercise and for EXP 2, at 15 min intervals throughout exercise until the 90 min time-point and at exhaustion. In EXP 1 ambient temperature during the trials was 20 °C. In EXP 2, the subjects were transferred to the climatic chamber (ambient temperature of 10.2 ± 0.2 °C, relative

humidity 69.8 ± 1.0% and air velocity of approximately 3.6 $m \cdot sec^{-1}$) and began exercise within 1 min of entering the chamber room. The exercise intensity and ambient temperature in EXP 2 were chosen to induce fatigue that would be most likely of muscle glycogen depletion, rather than the result of some failure in the thermoregulatory system (Galloway and Maughan, 1997). In EXP 2, subjects ingested 7.14 $g \cdot kg^{-1}$ and 2.14 $g \cdot kg^{-1}$ body weight of water at rest and every 15 min throughout exercise, respectively in order to minimise dehydration (McConell *et al.*, 1997). The subjects were asked to maintain a pedal cadence of 60-80 rpm throughout the test; exhaustion was defined as the point at which the subject could no longer maintain the pedal cadence above 60 rpm.

Following exercise in EXP 2, subjects were weighed and loss of body mass was calculated, after correcting for water consumed during exercise and the pre and post-exercise differences in body mass were calculated. Time to exhaustion was recorded, but withheld from the subject until all trials had been completed and the subject had answered the post-intervention questionnaire. Subjects were asked to predict the order of treatments received during the study, and to nominate the treatment they perceived produced their best performance. The experimental protocol for the removal, treatments and analyses of blood and plasma as well as gas exchange measurements and statistical methods used are described in chapter 2.

3.3 Results

Dietary analysis

Analysis of the subjects' normal diet and average daily energy intake, in addition to diet composition during the experimental period (including the three meals for EXP 2) are shown in Table 3.1. There were no differences in energy intake or diet composition during the three days prior to each exercise test. No change in body mass occurred during the entire study-period in both experiments; body mass prior to the Control, F and FC trials was 74.2 ± 9.7 kg, 74.4 ± 9.5 kg, and 74.4 ± 9.3 kg in EXP 1 ($P = 0.9$) and 74.1 ± 7.8 kg, 74.1 ± 7.7 kg, and 73.9 ± 7.6 kg in EXP 2 ($P = 0.4$), respectively.

Table 3.1: Average energy intake and diet composition before, and during the 12-day weighted intake, and each of the experimental meals in EXP 2. Values are given as mean ± s.d. or median (range).

	Normal diet	CHO diet (day 1-3)	CHO meal (day 4)	CHO diet (day 5-7)	Fat meal (day 8)	CHO diet (day 9-11)	Fat meal (day 12)
Energy intake	13.6±4.0 ($MJ \cdot day^{-1}$)	14.1±3.4 ($MJ \cdot day^{-1}$)	3.2±0.3 (MJ)	14.3±3.3 ($MJ \cdot day^{-1}$)	3.0±0.7 (MJ)	14.4±3.4 ($MJ \cdot day^{-1}$)	3.0±0.7 (MJ)
CHO (%)	54.5±8.6	72.1±3.5	87.0±1.6	73.2±4.3	8.4±0.6	73.1±4	8.4±0.6
Fat (%)	30.1±8.5	15.6±3.4	3.2±0.5	14.2±3.7	89.3±0.7	14.1±3.9	89.3±0.7
Protein (%)	14.2±3.3	12.1±2.3	9.8±1.5	12.6±2.1	2.3±0.1	12.7±2.5	2.3±0.1
Alcohol (%)	0(7.18)	0.17	-	-	-	0.1	-

Performance

Time to fatigue during maximal incremental exercise (EXP 1: Control trial: 4.9 ± 1.8 min; F trial: 5.0 ± 2.2 min; FC trial: 5.0 ± 2.2 min, $P = 0.9$) and sub-maximal constant-load exercise (EXP 2: Control trial: 116(88-145) min; F trial: 122(96-144) min; FC trial: 127(107-176) min, $P = 0.2$) was similar between the two fat trials (Fig. 3.3). In EXP 1, peak power output (Control trial: 346 ± 33 W; F trial: 340 ± 33 W, FC trial: 342 ± 32 W) and $\dot{V}O_{2\,peak}$ (Control trial: 3.74 ± 0.24 $L \cdot min^{-1}$; F trial: 3.67 ± 0.24 $L \cdot min^{-1}$; FC trial: 3.76 ± 0.28 $L \cdot min^{-1}$) were not different between fat trials. However, $\dot{V}O_{2\,max}$ determined during the initial incremental test (4.17 ± 0.32 $L \cdot min^{-1}$) was significantly higher than $\dot{V}O_{2\,peak}$ during the three experimental trials.

Perception of effort

In both experiments, ratings of perceived leg exertion were significantly lower during constant-load exercise following FC compared to the F trial (EXP 1: $F_{(1,7)} = 70.875$, $P = 0.0001$; EXP 2: $F_{(1,9)} = 11.985$, $P = 0.007$) (Fig. 3.4). Ratings of perceived breathlessness were lower during constant-load exercise following FC in both experiments, significantly so at 10 min and 30 min time-points in EXP 1 ($F_{(1,7)} = 15.996$, $P = 0.005$). In EXP 1, six out of eight subjects ranked the F trial as the most difficult trial (the remaining two subjects being unsure) and in EXP 2, six out of ten subjects ranked the FC as the easiest trial (one subject was unsure).

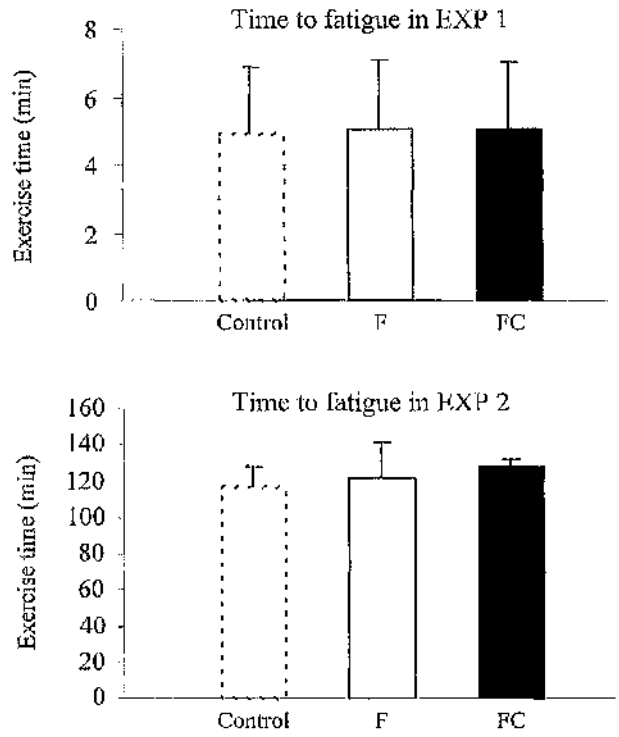


Fig. 3.3. Incremental exercise performance in EXP 1 (top panel), mean (s.d.) and Endurance exercise performance in EXP 2 (bottom panel), median (Interquartile range: IQR).

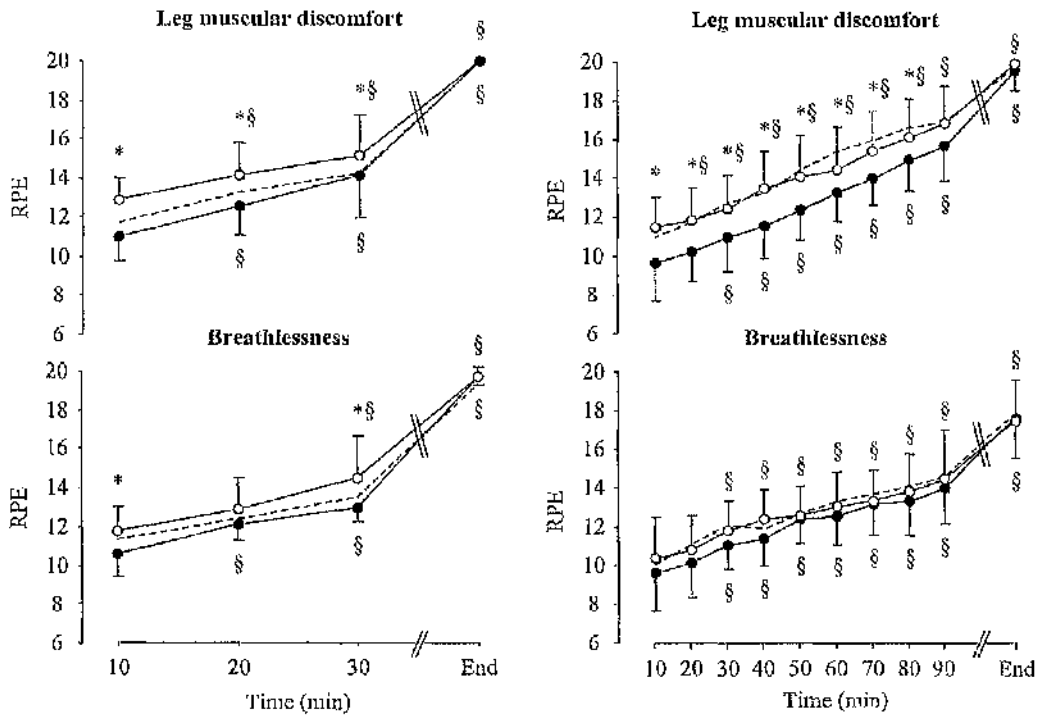


Fig. 3.4. Ratings of perceived exertion, for leg muscular discomfort (top panels) and breathlessness (bottom panels) (mean ± s.d.) in EXP 1 (left side) and EXP 2 (right side). *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time in both trials. The dash line indicates the Control trial.

Cardiopulmonary variables, fuel oxidation

HR increased over time on all trials in both experiments (Fig. 3.5). No difference between trials was found in EXP 1 ($F_{(1,7)} = 1.710$; $P = 0.232$). In EXP 2, there was a significant interaction (treatment x time) on HR being significantly higher throughout exercise on the FC compared with the F trial ($F_{(1,10)} = 3.690$; $P = 0.001$).

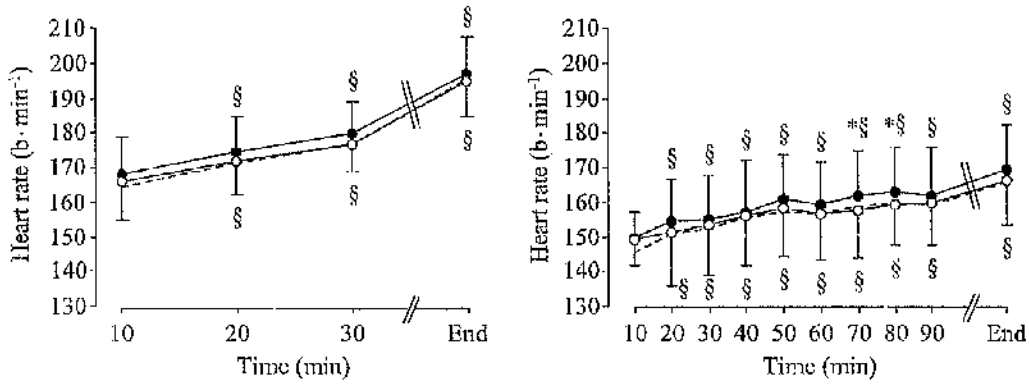


Fig. 3.5. Heart rate responses (mean ± s.d.) in EXP 1 (left side) and EXP 2 (right side). *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time in both trials. The dash line indicates the Control trial.

$\dot{V}O_2$ increased over time during constant-load exercise on the FC trial in EXP 1, and was higher compared with F trial at the 30 min time point ($F_{(1,2)} = 4.771$; $P = 0.026$) (Table 3.2). In EXP 2, $\dot{V}O_2$ increased over time on both trials and was higher on the FC trial compared with the F trial ($F_{(1,9)} = 9.980$; $P = 0.02$); statistical significance was achieved at the 75 min time point (Table 3.3). $\dot{V}CO_2$ increased similarly on the two fat trials in both EXP 1 (Table 3.2) and 2 (Table 3.3) but it was not significantly different between the trials (EXP 1: $F_{(1,7)} = 2.243$; $P = 0.178$, EXP 2: $F_{(1,9)} = 0.326$; $P = 0.582$). There was a progressive increase in $\dot{V}E$ over time on both trials in the two experiments. $\dot{V}E$ tented to be higher, during constant-load exercise, on the FC compared with the F trial in EXP 1 ($F_{(1,7)} = 3.276$; $P = 0.068$) (Table 3.2). In EXP 2, $\dot{V}E$ was significantly higher on the FC compared with F trial ($F_{(1,9)} = 10.917$; $P = 0.009$); statistical significance was reached at 30, 45, 60 and 75 min (Table 3.3). There was a reduction in RER over time in both experiments, and no differences were found between F and FC trials (Tables 3.2 and 3.3) (EXP 1: $F_{(1,7)} = 0.334$; $P = 0.582$, EXP 2: $F_{(1,9)} = 3.246$; $P = 0.105$).

In both experiments, the rate of CHO (EXP 1: $F_{(1,7)} = 0.888$; $P = 0.377$, EXP 2: $F_{(1,9)} = 2.089$; $P = 0.182$) and fat oxidation (EXP 1: $F_{(1,7)} = 0.241$; $P = 0.638$, EXP 2: $F_{(1,9)} = 3.372$; $P = 0.104$) was not different between trials; the rate of fat oxidation increased and CHO oxidation decreased over time (Tables 3.2 and 3.3 respectively). Total CHO and fat oxidation during 30 min constant-load exercise (EXP 1) and exercise to exhaustion (EXP 2) were not different between fat trials: FC trial: 84 ± 8g CHO, 14 ± 2 g fat; F trial: 78 ± 19 g CHO, 15 ± 6 g fat; Control trial: 90 ± 18 g CHO, 10 ± 6 g fat in EXP 1, and FC trial: 371 ± 82 g CHO, 77 ± 50 g fat; F trial: 388 ± 90 g CHO, 52 ± 23 g fat; Control trial: 367 ± 87 g CHO, 39 ± 23 g fat in EXP 2. EE was higher on the FC compared with the F trial and it reached statistical significant at 30 min time-point in EXP 1 (Table 3.2) and at 30 min and 75 min time-points in EXP 2 (Table 3.3). EE progressively increased over time on both trials in EXP 2, and on the FC trial in EXP 1.

Table 3.2: Cardiopulmonary variables for each of the three trials in EXP 1. Values are given as mean ± s.d..

Variables	Trials	Exercise time (min) 10	20	30	End
$\dot{V}O_2$ (L·min^{-1})	Control	3.0±0.2	3.1±0.2	3.1±0.1	3.7±0.2
	F	3.0±0.2	3.1±0.2	3.0±0.2	3.7±0.2§
	FC	3.1±0.2	3.2±0.2§	3.2±0.2*§	3.8±0.3§
$\dot{V}CO_2$ (L·min^{-1})	Control	2.9±0.2	2.9±0.2	2.9±0.2	4.0±0.4
	F	2.8±0.3	2.8±0.2	2.8±0.2	3.9±0.7§
	FC	2.9±0.2	2.9±0.2	2.9±0.2	3.8±0.5§
$\dot{V}E$ (L·min^{-1})	Control	72±11	79±16	84±17	136±16
	F	75±15	80±15	83±18§	136±19§
	FC	76±12	83±14§	89±14§	147±17§
RER	Control	0.94±0.03	0.94±0.04	0.93±0.03	1.07±0.06
	F	0.92±0.04	0.90±0.04§	0.89±0.04§	1.06±0.08§
	FC	0.92±0.01	0.91±0.02	0.90±0.01§	1.07±0.06§
Energy Expenditure (kcal·min^{-1})	Control	12.7±0.8	12.8±0.8	12.8±0.6	-
	F	12.6±0.8	12.7±0.7	12.8±0.7	-
	FC	12.9±0.9	13.1±0.9§	13.3±0.8*§	-
CHO oxidation (g·min^{-1})	Control	3.0±0.5	3.0±0.6	2.9±0.6	-
	F	2.8±0.6	2.6±0.6§	2.4±0.7§	-
	FC	2.9±0.2	2.8±0.3	2.7±0.3	-
Fat oxidation (g·min^{-1})	Control	0.3±0.1	0.3±0.1	0.4±0.2	-
	F	0.4±0.2	0.5±0.2§	0.6±0.2§	-
	FC	0.4±0.1	0.4±0.1	0.5±0.1§	-

*: Indicates a significant difference between F and FC trials

§: Indicates a significant difference over time compared with the 10 min time-point

Table 3.3: Cardiopulmonary variables for each of the three trials in EXP 2. Values are given as mean ± s.d.

Variables	Trials	Exercise Time (min)						
		Rest	15	30	45	60	75	90
$\dot{V}O_2$ (L·min^{-1})	Control	0.3±0.04	3.2±0.4)	3.2±0.4	3.4±0.5	3.4±0.5	3.5±0.6	3.4±0.4
	F	0.3±0.03	3.1±0.4	3.2±0.4§	3.2±0.4	3.4±0.4§	3.4±0.5§	3.5±0.5§
	FC	0.4±0.07	3.3±0.3	3.4±0.4	3.4±0.5§	3.5±0.5§	3.6±0.5*§	3.6±0.5§
$\dot{V}CO_2$ (L·min^{-1})	Control	0.3±0.04	3.0±0.5	3.0±0.5	3.1±0.5	3.1±0.5	3.2±0.7	3.1±0.5
	F	0.3±.003	3.0±0.4	3.1±0.4	3.1±0.4	3.2±0.4§	3.2±0.4§	3.3±0.5§
	FC	0.3±0.05	3.0±0.3	3.1±0.4	3.1±0.4	3.2±0.4	3.3±0.5§	3.2±0.4
$\dot{V}E$ (L·min^{-1})	Control	8.0±2	66±10	69±10	73±10	74±12	78±15	76±9.0
	F	8.0±1	66±10	68±11	70±12§	73±11§	76±11§	78±14§
	FC	10±2	70±6	73±8*§	75±10*§	79±11*§	81±11*§	81±10§
RER	Control	0.89±0.08	0.95±0.03	0.95±0.03	0.94±0.05	0.94±0.03	0.93±0.04	0.93±0.02
	F	0.87±0.10	0.95±0.03	0.94±0.03	0.93±0.04	0.93±0.03§	0.93±0.02	0.91±0.03§
	FC	0.87±0.07	0.93±0.04	0.91±0.03§	0.91±0.05	0.91±0.05	0.90±0.06	0.88±0.05§
Energy Expenditure (kcal·min^{-1})	Control	-	13.4±1.7	13.6±1.9	14.1±2.1	14.2±2.3	14.6±2.7	14.9±2.7
	F	-	13.1±1.7	13.4±1.6§	13.6±1.6	14.1±1.9§	14.3±2.0§	14.6±2.2§
	FC	-	13.5±1.3	13.9±1.7*	14.3±2.0§	14.6±2.1§	15.1±2.2*§	14.9±2.1§
CHO oxidation (g·min^{-1})	Control	-	3.3±0.5	3.3±0.5	3.8±0.7	3.3±0.4	3.4±0.5	3.2±0.5
	F	-	3.2±0.6	3.2±0.6	3.2±0.8	3.2±0.6	3.3±0.5	3.2±0.7
	FC	-	3.1±0.4	2.9±0.5	2.9±0.8	3.0±0.8	3.0±0.9	2.7±0.7
Fat oxidation (g·min^{-1})	Control	-	0.4±0.5	0.4±0.5	0.5±0.6	0.4±0.6	0.5±0.6	0.6±0.6
	F	-	0.3±0.1	0.3±0.1§	0.4±0.1§	0.4±0.2	0.4±0.2	0.4±0.2§
	FC	-	0.4±0.2	0.4±0.2	0.5±0.3§	0.5±0.3§	0.6±0.4	0.7±0.4§

*: Indicates a significant difference between F and FC trial

§: Indicates a significant difference over time compared with the 15 min time-point

Blood metabolites

Plasma [FFA] was not different between fat trials in EXP 1 and remained largely unchanged during exercise ($F_{(1,7)} = 0.139$; $P = 0.721$) (Fig. 3.6). However, in EXP 2, a higher [FFA] was found on the FC trial compared with the F trial at rest, and from 15 min to 90 min during exercise ($F_{(1,9)} = 10.959$; $P = 0.009$). [FFA] decreased at the 15 min time-point, compared to resting levels, in both fat trials. Thereafter, there was a progressive increase over time in both trials. Plasma [glycerol] was greater during the FC trial compared with the F trial, in both EXPs 1 ($F_{(1,7)} = 5.928$; $P = 0.045$) and 2 ($F_{(1,9)} = 25.921$; $P = 0.001$). In both experiments, plasma [glycerol] increased significantly over time in both fat trials compared with the baseline values (Fig. 3.6).

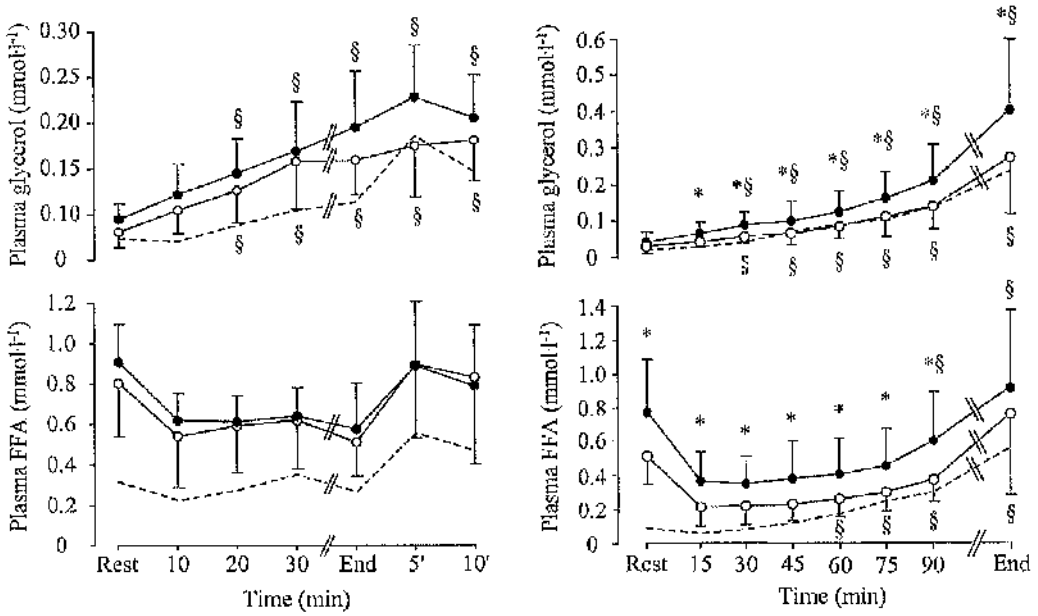


Fig 3. 6. Plasma FFA (bottom panels) and plasma glycerol (top panels) responses (mean ± s.d.) in EXP 1 (left side) and EXP 2 (right side). *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time in both trials. The dash line indicates the Control trial.

Higher blood [glucose] was found on the FC trial compared with the F trial in both EXP 1 ($F_{(1,7)} = 16.350$; $P = 0.007$) and EXP 2 ($F_{(1,9)} = 23.329$; $P = 0.001$). In EXP 1, [glucose] was higher after 20 min of constant-load exercise, at the end of the ramp phase, and during the 10 min recovery period on the FC trial compared with the F trial (Fig. 3.7). [Glucose] increased progressively over time on the FC trial from 20 min of exercise onwards in comparison with the baseline, whereas on the F trial, an

increase in [glucose] was observed only at exhaustion and during recovery. Similarly, in EXP 2, [glucose] was significantly higher on the FC compared with the F trial from the 30 min time point until exhaustion. In the FC trial, [glucose] decreased after 90 min of exercise relative to the baseline levels, whereas no such reduction was found on the F trial (3.7).

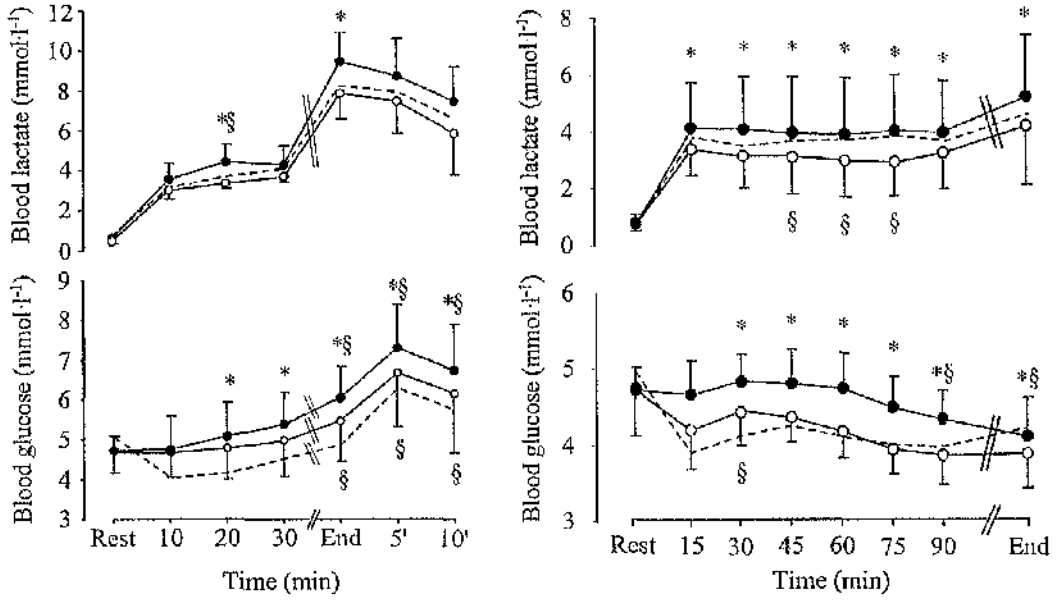


Fig 3.7. Blood glucose (bottom panels) and blood lactate (top panels) responses (mean ± s.d.) in EXP 1 (left side) and EXP 2 (right side). *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time in both trials. The dash line indicates the Control trial.

In both experiments, blood [lactate] was significantly higher on the FC trials compared with the F trials (EXP 1: $F_{(\text{treatment x time: } 1,6)} = 2.382$; $P = 0.049$; EXP 2: $F_{(1,9)} = 13.823$; $P = 0.005$). In EXP 1, [lactate] was significantly higher after 20 min of constant-load exercise, at the end of the ramp phase and during recovery on the FC trial compared with the F trial (Fig. 3.7). There was an exercise-induced increase in [lactate] at the 10 min time point (both fat trials) and at the 20 min time point relative to the 10 min time point (FC trial only). Peak [lactate] was measured at the end of the ramp phase on the two fat trials, with no differences between trials. In EXP 2, higher [lactate] was found throughout exercise on the FC compared to F trial. In this experiment, [lactate] increased significantly at the 15 min time point in both trials relative to baseline. No further increases in [lactate] were found during the FC trial, whereas on the F trial, [lactate] decreased significantly at 45, 60, and 75 min compared to the 15 min time point. Blood [pyruvte] was measured and the

[lactate]:[pyruvate] (L:P) ratio subsequently determined only in EXP 2. [Pyruvte] (from 30-60 min) and the (L:P) ratio (15 min and 75 min) were higher during exercise on the FC compared with the F trial ($F_{(1,9)} = 35.262$; $P = 0.001$, and $F_{(1,9)} = 6.173$; $P = 0.042$ respectively) (Fig. 3.8). After an initial elevation in [pyruvte] and (L:P) ratio from rest to exercise in both fat trials, both remained fairly constant thereafter.

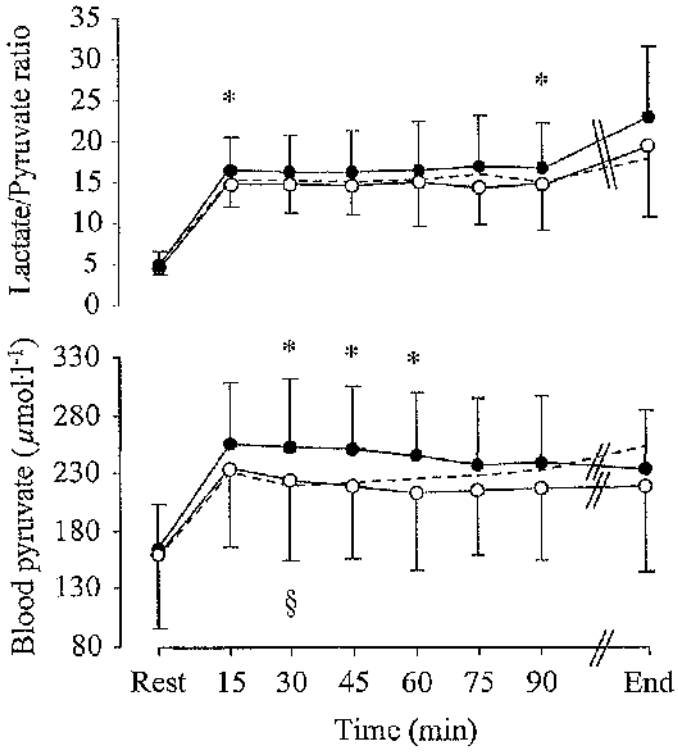


Fig 3.8. Blood pyruvate (bottom panel) and lactate to pyruvate ratio (top panel) responses (mean ± s.d.) in EXP 2. *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time in both trials. The dash line indicates the Control trial.

Reported side effects

Four out of the ten subjects in EXP 2 experienced slight gastrointestinal discomfort; three following the high fat meal co-ingested with caffeine and one following the high fat meal alone. One subject experienced more severe side effects following the high fat meal and caffeine ingestion 30 min following exercise. These effects included loss of consciousness, dizziness, abdominal pain, nausea and vomiting. These effects disappeared shortly after the experience.

3.4 Discussion

The present study was designed to differentiate between central and peripheral aspects affecting fatigue during exercise and to distinguish between the putative metabolic and CNS effects of pre-exercise caffeine ingestion. This was achieved by having the subjects exercise after elevating their circulating plasma [FFA] with a high fat meal and subsequently co-ingesting caffeine or placebo. The use of fat meal was employed in an attempt to produce a similar muscle metabolic adjustment at rest and during exercise in both F and FC trials and based on the substrate utilisation results which were not different between the trials this attempt has been successful. In addition, Although smoking, oral contraceptive, ingestion of cruciferous vegetable and physical fitness level all were found to influence the metabolic pathway of caffeine by altering the activity of the cytochrome P450 isoform 1_{A2} which is the enzyme that regulates caffeine metabolism (Van Soeren and Graham, 1998) there is no evidence to show any effect of high fat meal on the absorption, distribution, metabolism, or exertion of caffeine. Differences therefore in exercise performance, perceptual, metabolic and cardiovascular responses could be accounted by the ingestion of caffeine. Thus, caffeine could differentiate between peripheral and central aspects affecting exercise fatigue development enabling central components of fatigue examination.

Assuming therefore, that perception of effort reflects, in part at least, CNS responses (Hampson *et al.*, 2001; Utter *et al.*, 2004), the present results indicate a differentiation between the putative metabolic effects and the CNS actions of caffeine during constant-load exercise, as effort perception was reduced after caffeine ingestion (despite an elevation in cardiopulmonary and metabolic responses), but there were no differences in substrate utilisation and no improvements in exercise performance. Confirming the perception of effort results obtained from the present EXPs (n = 18), 12 out of 18 subjects ranked the FC trial as the easiest trial. This ranking response to the order of difficulty of the trials was irrespective of performance time since some subjects who ranked the FC trial as the easiest one did not have a better exercise performance in the same trial. These results demonstrate a dissociation between effort perception, metabolic responses and exercise performance.

It is difficult however, to explain why the subjects in the present experiments perceived it easier to exercise with caffeine than without, particularly when one considers the accompanying elevation in blood [lactate], $\dot{V}O_2$, $\dot{V}E$ (EXP 2) and heart rate that typically would be expected to augment, rather than attenuate perception of effort (Noble *et al.*, 1983). It is possible that these effects on perception of effort are the result of caffeine directly stimulating the CNS; the exact mechanism, however, remains unclear. Caffeine may reduce perception of effort by inhibiting brain adenosine receptors (A_1 and A_2) (Davis *et al.*, 2003), which otherwise suppress synaptic transmission within the motor cortex (Phillis and Wu, 1981), and/or by reducing the excitation threshold of motor-neurones facilitating motor unit recruitment (Waldeck, 1975). Alternatively, caffeine may attenuate perception of effort by enhancing the secretion of endorphins (Spindel *et al.*, 1984; Laurent *et al.*, 2000), which is well known to reduce pain perception and promote euphoria (e.g. Harber and Sutton, 1984). Furthermore, the significant increased $\dot{V}E$, observed during exercise on the FC trial (EXP 2) might be expected to increase dyspnoea (breathlessness) (e.g. Cullen and Rodak, 2002). In contrast, a mismatch between $\dot{V}E$ and perception of breathlessness was found after caffeine ingestion with a higher $\dot{V}E$ and lower rating of breathlessness reported during constant-load exercise. The exact mechanism, however, behind this dissociation during exercise is also unclear. Caffeine has previously been shown to reduce fatigue and effort sensation associated with inspiratory muscle contraction (Supinski *et al.*, 1986). Caffeine may also enhance respiration by blocking central adenosine receptors (Kawai *et al.*, 1995), which act to depress ventilation by inhibiting respiratory motor centres (Runold *et al.*, 1989).

Several studies utilising non-glycogen depletion exercise report an enhancement in high-intensity/incremental exercise performance after caffeine ingestion (McNaughton, 1986; Flinn *et al.*, 1990; Jackman *et al.*, 1996) and suggest CNS involvement in the fatigue process. In EXP 1 therefore, a non-glycogen depletion protocol (i.e. 30 min of constant-load exercise followed by incremental exercise to fatigue) was employed in an attempt to examine a possible metabolic effect of caffeine during the early stages of exercise and to further differentiate between a putative metabolic and CNS effect of caffeine during high-intensity exercise. The failure of caffeine to improve incremental exercise performance in EXP 1 is not

consistent with several previous reports (McNaughton, 1986; Flinn *et al.*, 1990). This may be due to the effect of caffeine in elevating $\dot{V}O_2$ and energy expenditure and therefore metabolic rate. Thus, the increased metabolic rate and therefore higher ATP demand (Engels *et al.*, 1999) during the 30 min constant-load exercise phase after caffeine ingestion may have negated any ergogenic effect of caffeine on incremental exercise performance.

To our knowledge, the present study (EXP 2) was the first which has examined the effects of caffeine on endurance performance during prolonged exercise at 10°C. Since caffeine ingestion did not appear to influence substrate utilisation, no improvement in exercise performance could be reasonably expected, as it is well established that fatigue during prolonged exercise to exhaustion at 10°C is due to glycogen depletion (Galloway and Maughan, 1997). Therefore, the improvements in endurance exercise performance observed in previous caffeine studies are unlikely to be associated with glycogen depletion, unless caffeine ingestion altered substrate utilisation.

Alternatively, it is possible that the pre-exercise high fat meal employed in the present experiments negated any increase in fat oxidation previously attributed to the effect of caffeine. Typically, the limitation in fat oxidation during the early stages of exercise is the inadequate FFA delivery to the active skeletal muscles, rather than the inability of the muscle to oxidise FFA (Frayn *et al.*, 1996). In the present experiments, however, the plasma [FFA] was elevated prior to exercise by acute fat ingestion. Although increased lipolysis was evident after caffeine ingestion (based on the higher plasma [glycerol]), the saturation threshold for FFA uptake, and possibly, oxidation by skeletal muscle was probably achieved on both fat trials due to the high fat meal. Consequently, any caffeine-induced lipolysis would not further enhance FFA utilisation. The performance results also confirm this. For example, if there was a significant contribution of the plasma [FFA] and [glycerol] on fat oxidation, endurance performance would be increased following caffeine ingestion.

Based on the present substrate oxidation findings, it is unlikely that the significant increase in $\dot{V}O_2$ observed during exercise after caffeine ingestion reflects a marked shift towards fat oxidation (Sherman and Leenders, 1995). It is possible that caffeine

increased $\dot{V}O_2$ due to its concomitant effect in increasing whole body metabolic rate without predominantly elevating the relative rates of CHO or fat utilisation (Engels *et al.*, 1999). In agreement with this is the higher energy expenditure observed at rest and during exercise on the FC relative to F trial. This elevation in energy expenditure observed in previous studies was ascribed to the thermogenic effect of caffeine via increased epinephrine secretion (Astrup *et al.*, 1990; Greer *et al.*, 2000). Increased epinephrine secretion is known to enhance lipolysis (Graham and Spriet, 1991) and muscle (e.g. Watt *et al.*, 2001) and liver (e.g. Graham *et al.*, 2000) glycogenolysis.

The higher blood [glucose] and [lactate] observed following caffeine ingestion in the present experiments is consistent with many previous studies (Gaesser and Rich, 1985; Spriet *et al.*, 1992; Jackman *et al.*, 1996; Laurent *et al.*, 2000; Graham *et al.*, 2000). The higher blood [glucose] has been suggested to be due to an increased liver glycogenolysis, although a reduction in blood-glucose uptake by skeletal muscle and/or adipose tissue cannot be excluded (e.g. Crist *et al.*, 1998; Thong *et al.*, 2002). Similarly, the increase in blood [lactate] may result from the inability of the mitochondria to handle the high pyruvate load, consequent to an increase in skeletal muscle glycogenolysis, therefore providing more substrate for lactate production (Holloszy, 1973). The higher [pyruvate] and L:P ratio observed in EXP 2 after caffeine ingestion supports this assertion. The elevation however, in blood [lactate] cannot solely be attributed to an increase in muscle lactate production through anaerobic metabolism. For example, Graham *et al.*, (2000) found an increase in blood [lactate] after caffeine ingestion, without a concomitant elevation in muscle lactate production at rest or during exercise, therefore implying inhibition of lactate uptake by non-exercising muscles and other tissues (e.g. liver).

Conclusions

In conclusion, while a number of metabolic responses were increased during exercise after caffeine ingestion, perception of effort was reduced; the exact mechanism however is still unclear. The reduction in effort perception after caffeine ingestion with a simultaneous elevation in a number of metabolic/cardiovascular responses and no differences in substrate oxidation would suggest a differentiation between putative altered substrate utilisation effects of caffeine and CNS actions.

Despite a possible CNS action, caffeine did not improve exercise performance, thus demonstrating a dissociation between effort perception and time to fatigue during incremental and endurance exercise performance. However, although central neural components may contribute to reduce effort perception after caffeine ingestion, whether this reduction is associated with brain serotonergic and dopaminergic modulators during exercise remains to be determined.

Chapter four

(Experiments 1 and 2)

Brain 5-HT and DA modulators, perceptual responses and exercise performance following caffeine co-ingested with a high fat meal in trained humans

4.1 Introduction

The results presented in Chapter 3 obtained from the EXPs 1 and 2 associated with metabolic, cardiovascular and perceptual responses have shown a strong association between effort (fatigue) perception and CNS after caffeine co-ingested with a high fat meal. However, whether the CNS implication in the RPE development during exercise (especially in relatively thermoneutral and mild-cold temperature that precludes thermo-physiological stressors involvement in effort perception development) in well-trained humans, is associated with brain 5-HT and DA function or any other central neural mechanism is not known yet.

As stated in Chapter 1 (section 1.2.5), an enhancement in circulating plasma [FFA] may displace Trp from albumin (Struder *et al.*, 1996) increasing plasma free [Trp]:[LNAA] ratio and possibly brain 5-HT synthesis (Bloxam *et al.*, 1980; Curzon *et al.*, 1973). The first human study for example, to show an improvement in exercise capacity following an acute increase in the circulating [FFA] by a high fat meal and heparin infusion, also reported the fat trial to be the more difficult trial and was accompanied by an early rise in effort perception (Pitsiladis *et al.*, 1999). On the other hand, caffeine was found to enhance exercise performance not via its classic CHO-sparing effect but by increasing central motivation to exercise (i.e. reduction in effort perception) (Cole *et al.*, 1996; Bridge *et al.*, 2000). Furthermore, although an increase plasma [Trp] relative to [LNAA] may enhance brain 5-HT synthesis (Bloxam *et al.*, 1980; Curzon *et al.*, 1973), the elevation in brain 5-HT biosynthesis is also depended on the expression of tryptophan hydroxylase (TPH), which catalyses the rate-limiting step of 5-HT biosynthesis in serotonergic neurons of the raphe nuclei (Lim *et al.*, 2001). A previous study for example, has shown that an increased TPH mRNA (gene) elevated TPH activity and contributed to the enhancement in 5-HT metabolism (Chamas *et al.*, 1999). On the other hand, an animal study has shown that prolonged exercise performance was diminished when the number of TRH-positive cells in the dorsal and median raphe regions had increased due to prolonged exercise but caffeine had the potential to reduce brain 5-HT biosynthesis during exercise by inhibiting the expression of tryptophan hydroxylase- (TPH) (TPH mRNA) positive cells in the dorsal and median raphe nucleus (Lim *et al.*, 2001). In addition, a recent study has been shown that caffeine reduced brain 5-HT:DA ratio by blocking adenosine receptors within the CNS,

which otherwise inhibit brain DA release (Davis *et al.*, 2003). Consequently, in the present EXPs the co-ingestion of caffeine with a high fat meal was used as a physiological strategy ('vehicle') in which the mechanism associated with a possible brain 5-HT and DA systems involvement in the perceptual and exercise fatigue development could be evaluated.

Assuming the increased effort perception following the high fat meal in the study by Pitsiladis *et al.* (1999) reflected a 5-HT mediated central neural adaptation to the high fat meal, this effect might therefore be reversed if caffeine were to be ingested simultaneously since caffeine is known to enhance brain DA metabolism (Davis *et al.*, 2003; Fredholm *et al.*, 1999; Garrett and Griffiths, 1997) and reduce effort perception (e.g. Cole *et al.*, 1996). Consequently, the aim of these EXPs was to examine the relationship between putative modulators of brain 5-HT and DA function, perceptual responses and exercise performance following caffeine co-ingested with a high fat meal in well-trained humans.

4.2 Methods

For information concerning the subjects' characteristics, experimental design and procedures see Chapter 3. Regarding subjects' medical screening, for cannulation, blood sampling, blood treatment, plasma amino acids and Prl analysis and the experimental protocol for the removal and statistical analysis are described in Chapter 2.

4.3 Results

Some of the results obtained from the present two EXPs relating to perceptual, metabolic and cardiovascular responses and exercise performance have been reported in the previous Chapter 3. In summary, in both EXPs ratings of perceived leg exertion were significantly lower during constant-load exercise following FC compared to the F trial (Fig. 3.4). Ratings of perceived breathlessness were lower during constant-load exercise following FC in both EXPs, significantly so at 10 min and 30 min time-points in EXP 1 (Fig. 3.4). In addition, $\dot{V}O_2$, $\dot{V}E$ (EXP 2), energy expenditure, (Tables 3.2, 3.3) blood [glucose] and [lactate] (Fig. 3.7) and plasma [glycerol] (Fig. 3.6) were significantly higher on the FC compared with the F trial in

both EXPs. Blood [pyruvate], the L:P ratio (Fig. 3.8), plasma [FFA] (Fig. 3.6) and heart rate (Fig. 3.6) were significantly higher on the FC relative to the F trial in EXP 2. Time to fatigue (Fig. 3.3), substrate oxidation (Tables 3.2 and 3.3) and sweat rate were not significantly different between the trials.

Plasma amino acids and prolactin concentrations

There were no significant differences between F and FC trials for total [Trp], [Tyr], [LNAA], total [Trp]:[LNAA] ratio total [Trp]:[Tyr] ratio in both EXP 1 (Table 4.1) and EXP 2 (Table 4.2). However, there was a tendency for plasma free-[Trp] ($F_{(1,9)}$ = 4.438; P = 0.064) and free-[Trp]:[Tyr] ratio ($F_{(1,9)}$ = 4.366; P = 0.066) to be higher on the FC compared to F trial (Fig. 4.1). Plasma free-[Trp]:[LNAA] ratio was significantly higher, at 90 min and at exhaustion, on the FC relative to F trial ($F_{(1,9)}$ = 6.700; P = 0.029) (Fig. 4.1). There was no main treatment effect on plasma [Prl] in both EXP 1 and 2 (Fig 4.2). In both EXPs plasma [Prl] was progressively increased over time in all trials relative to the resting levels. In EXP 1, plasma [Prl] has reached peak values at 5 min during recovery after the ramp phase; in EXP 2 the peak plasma [Prl] value was detected at exhaustion.

Table 4.1: Plasma concentrations of amino acids, serotonergic and dopaminergic modulators, in Control, F and FC trials in EXP 1. Values are given as mean ± s.d..

Variables	Trials	Blood collection time (min) Rest	30min	End	10min-post
Total [Trp] (μmol·l^{-1})	Control	34±4	33±6	34±7	34±6
	F	38±8	37±8	37±7	34±5
	FC	36±8	37±7	36±8	34±8
[Tyrosine] (μmol·l^{-1})	Control	58±7	62±11	73±13	74±16
	F	61±10	64±12	75±9§	69±11
	FC	60±9	66±12§	69±11	65±11
[LNAA] (μmol·l^{-1})	Control	536±81	573±130	665±171	661±172
	F	630±99	628±119	706±97	664±96
	FC	615±118	636±92	662±110	626±110
Total [Trp]:[LNAA] ratio	Control	.064±.009	.058±.009	.053±.008	.053±.010
	F	.062±.019	.059±.010	.053±.010	.052±.010
	FC	.059±.013	.059±.014	.054±.011	.059±.019
Total [Trp]:[Tyrosine] ratio	Control	0.59±.10	0.54±.10	0.48±.08	0.47±.09
	F	0.64±.16	0.58±.09	0.50±.11§	0.50±.10§
	FC	0.60±.13	0.58±.13§	0.52±.11§	0.58±.18

§: Indicates a significant difference over time relative to the resting values
s.d.: Standard Deviation

Table 4.2: Plasma concentrations of amino acids, serotonergic and dopaminergic modulators, in Control, F and FC trials in EXP 2. Values are given as mean ± s.d..

Variables	Trials	Blood collection time (min) Rest	30min	90min	End
Total [Trp] ($\mu mol \cdot l^{-1}$)	Control	38±8	36±7	39±3	46±9
	F	38±7	39±7§	43±6§	42±9
	FC	38±7	39±7	43±9§	43±7§
[Tyrosine] ($\mu mol \cdot l^{-1}$)	Control	54±8	53±7	61±7	71±8
	F	52±3	58±6§	65±7§	68±5§
	FC	51±4	55±6§	64±8§	66±7§
[LNAA] ($\mu mol \cdot l^{-1}$)	Control	500±50	487±35	486±51	531±60
	F	522±46	532±50	518±45	518±54
	FC	505±40	499±48	504±48	506±44
Total [Trp]:[LNAA] ratio	Control	.076±.013	.077±.012	.081±.009	.088±.016
	F	.072±.012	.074±.013	.083±.015§	.083±.021
	FC	.075±.012	.080±.013	.085±.013§	.085±.015§
Total [Trp]:[Tyrosine] ratio	Control	0.72±.15	0.69±.13	.064±.08	0.66±.11
	F	0.72±.14	0.68±.13§	0.67±.11	0.63±.15§
	FC	0.74±.17	0.72±.14	0.67±.14	0.65±.10§

§: Indicates a significant difference over time relative to the resting values
s.d.: Standard Deviation

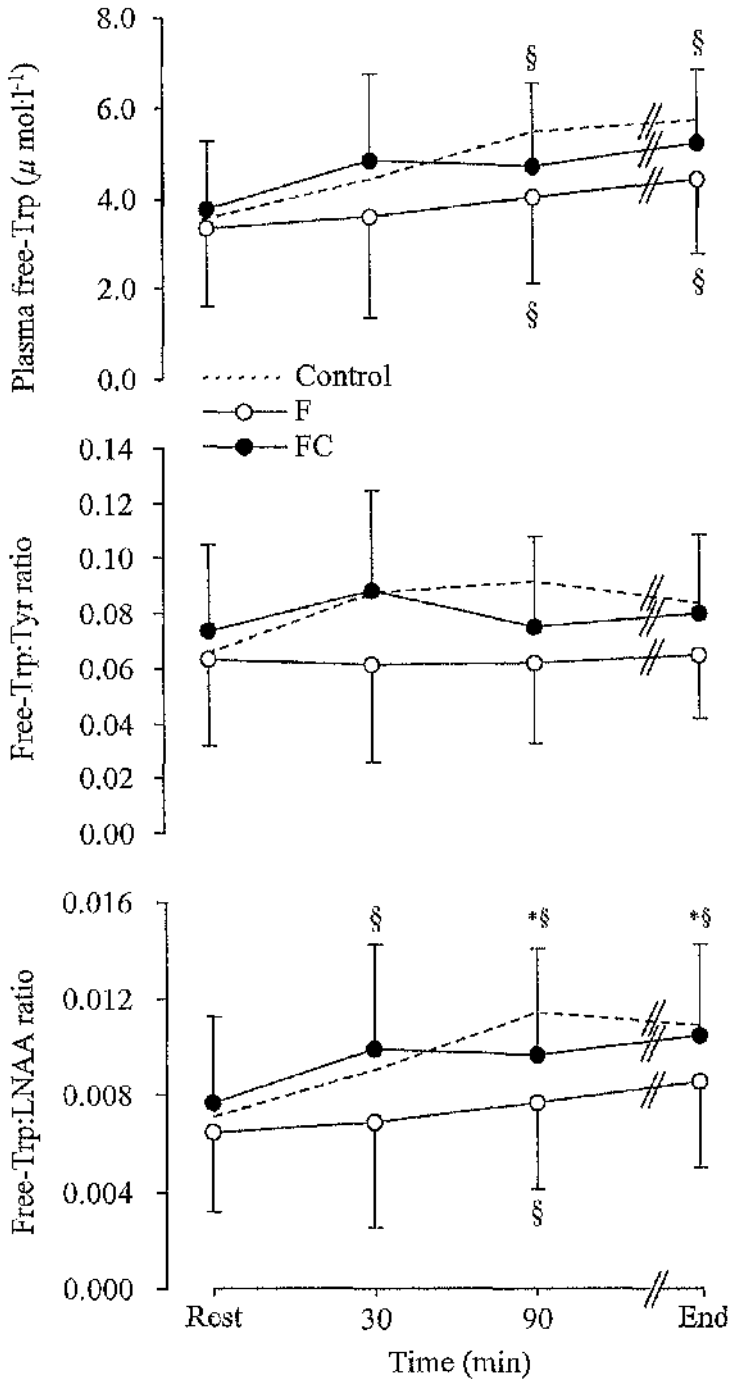


Fig. 4.1. Plasma free-[Trp]:[LNAA] ratio (bottom panel), free-[Trp]:[Tyr] ratio (middle panel) and plasma free-[Trp] (top panel) responses (mean ± s.d.) in EXP 2. *: indicates a significant difference between F (○) and FC (●) trials. §: indicates significant differences over time relative to resting value in both trials. The dash line indicates the Control trial.

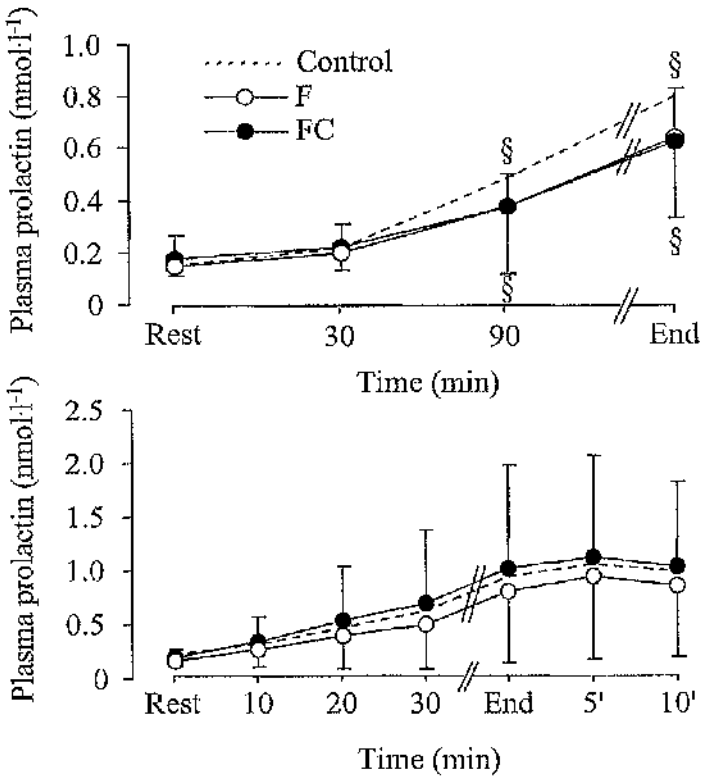


Fig. 4.2. Plasma prolactin responses (mean ± s.d.) between F (○) and FC (●) trials in EXP 1 (bottom panel) and in EXP 2 (top panel). §: indicates significant differences over time relative to resting value in both trials. The dash line indicates the Control trial.

Correlation analysis

There were no significant correlations between plasma free-[Trp] and RPE, between free-[Trp] and plasma [FFA], and between free-[Trp] and [Prl], when examined for each time-point separately.

4.4 Discussion

The present study examined the relationship between the putative modulators and indices of brain serotonergic and dopaminergic function, effort perception and performance during exercise in relatively thermoneutral (20 °C) and cold (10 °C) environments following caffeine co-ingested with a high fat meal in well-trained humans. In contrast to several previous studies that have examined putative modulators and indices of brain 5-HT and DA functions during exercise (e.g. Blomstrand *et al.*, 1988; 1989; 1997; Davis *et al.*, 1992; Bailey *et al.*, 1993b; van Hall *et al.*, 1995; Struder *et al.*, 1996) the 10 °C temperature, in association with the water replacement provided throughout exercise in EXP 2 has been chosen to

produce fatigue minimising thermo-physiological factors (dehydration, early increased core or brain temperatures, effort perception or reduction in cardiovascular capacity, systematic circulation and muscle blood flow) (see Febbraio *et al.*, 1996; Galloway and Maughan, 1997; Gonzalez-Alonso *et al.*, 1998; 1999; 2003; McConell *et al.*, 1997; Nybo *et al.*, 2001; 2002; Nielsen *et al.*, 1993; 1997).

The results presented here do not support a significant involvement of the putative modulators and indices of brain serotonergic and dopaminergic function in the fatigue process during submaximal constant-load exercise and maximal incremental exercise at moderate to low ambient temperatures. This lack of involvement of the putative modulators and indices of 'central fatigue' was observed despite a significant reduction in effort perception following caffeine ingestion that is most likely attributed to central neural mechanism(s).

Caffeine at the micromolar levels utilised in both studies has been shown to cross the blood brain barrier (BBB) with the potential to serve as a competitive antagonist of adenosine (Fredholm *et al.*, 1999). The net effect would be to increase central DA release by antagonising the inhibition of adenosine on DA activity, thus reversing the increased effort perception induced by the high fat meal in conjunction with the exercise stress (Pitsiladis *et al.*, 1999). This is consistent with the hypothesis that a high 5-HT:DA ratio may favour central fatigue, while a low 5-HT:DA ratio may favour increased arousal and motivation (Davis *et al.*, 1993; Davis and Bailey, 1997). Studies using rats for example, found a reduction in brain 5-HT synthesis and in the 5-HT:DA ratio, and an improvement in exercise performance after direct intracerebroventicular caffeine injection (Davis *et al.*, 2003). Similar results were found during exercise after a suppression of the expression of TPH-positive cells (which catalyses the rate-limiting step of serotonin biosynthesis in serotonergic neurons) in the dorsal and median raphe regions through caffeine administration (Lim *et al.*, 2001). In both studies, caffeine was effective in reducing effort perception, but did not enhance incremental or endurance exercise performance in relatively thermoneutral and low ambient temperatures respectively.

The failure of caffeine to significantly affect brain serotonergic function during exercise at thermoneutral and low temperatures in the present experiments is further reflected in the lack of difference in plasma [Prl] between trials (Fig 3). Previous

studies have shown that Ketanserin, a 5-HT antagonist drug, reduced Prl release during graded exercise to exhaustion (De Meirleir *et al.*, 1985a; b). A further study reported that Trp infusion reduced exercise performance and caused an earlier elevation in plasma [Prl] relative to placebo or glucose infusion (Farris *et al.*, 1998). If caffeine at the dosages used in the present experiments, can directly attenuate brain 5-HT biosynthesis (Lim *et al.*, 2001), Prl levels would be expected to be lower during the two exercise trials involving caffeine. The finding of no difference in [Prl] between caffeine trials in both experiments may imply that caffeine does not directly affect brain 5-HT function in well-trained humans when exercise is carried out in relatively thermoneutral and low ambient temperatures. Alternatively, circulating Prl levels may not be as sensitive a marker of 5-HT as previously proposed (De Meirleir *et al.*, 1985a; b).

Previous studies have demonstrated that elevation in plasma [FFA] displaces Trp from binding to albumin and consequently increases the free-Trp:LNAA ratio into the plasma (Bloxam *et al.*, 1980; Chaouloff *et al.*, 1985; Curzon *et al.*, 1973; Struder *et al.*, 1996). Since Trp and the other LNAAs share the L-system carrier for crossing the BBB, the elevation in plasma free-Trp:LNAA ratio may favour brain Trp uptake and potentially increase brain 5-HT synthesis (Pardridge, 1986), and hence central fatigue (Newsholme *et al.*, 1987; Blomstrand *et al.*, 1988). A recent study using analbuminaemic rats has shown an improvement in exercise performance after reducing brain Trp uptake by blocking the L-system carrier using 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid, a specific inhibitor of the L-system transporter (Yamamoto and Newsholme, 2000). Conversely, intracerebroventricular Trp injection in the same species was found to increase $\dot{V}O_2$ and reduce mechanical efficiency and exercise performance in rats (Soares *et al.*, 2003).

In EXP 2, the free-[Trp]:[LNAA] ratio was significantly higher although the increased was modest, and there was a tendency for free-[Trp] and the free-[Trp]:[Tyr] ratio to be higher following caffeine ingestion. This effect may have attributed to the action of caffeine in elevating adipose tissue lipolysis and thus plasma [FFA], results that are consistent with several previous reports (e.g. Costill *et al.*, 1978; Spriet *et al.*, 1992). This effect, in conjunction with a reduced effort perception following caffeine ingestion could reflect the two opposing actions of the high fat meal and caffeine interventions. The former potentially increasing 5-HT

function and consequently effort perception, and the latter increasing DA function, hence reducing effort perception. In addition, this may have caused the modest difference on plasma free-[Trp]:[LNAA] between F and FC trials because the high fat meal *per se* has increased plasma [FFA] to a similar magnitude in both trials. Consequently the modest elevation in plasma [FFA] observed in the FC trial was insignificant to produce massive differences in plasma precursors of brain 5-HT function (e.g. free-[Trp] and free-[Trp]:[LNAA] ratio.

Several previous studies found that caffeine attenuated effort perception during exercise in humans (Bridge *et al.*, 2000; Cole *et al.*, 1996; Jacobson *et al.* 2000; Laurent *et al.*, 2000; Motl *et al.*, 2003). It is not unreasonable therefore, to imply a direct elevation in brain DA function with caffeine which may have contributed to reduce effort perception. However, in the present EXPs, although caffeine may have effectively reduced effort perception by elevating brain DA function (Davis *et al.*, 2003), exercise performance was not enhanced. It should be noted, that in both studies, the effectiveness of caffeine to reduce effort perception via a central dopaminergic-mediated effect can only be speculated as there is currently no suitable available marker of brain dopaminergic function. However, plasma [Prl] is an index of hypothalamic 5-HT and DA metabolic interaction only (Ben-Jonathan *et al.*, 1989; Freeman *et al.*, 2000). It is possible therefore that caffeine reduced subjective fatigue by increasing brain DA metabolism in other brain regions than the hypothalamus without being able to significantly inhibit hypothalamic Prl release. Studies using animal models for example, have demonstrated an up-regulation of brain 5-HT synthesis or down-regulation of brain DA metabolism during exercise not only into the hypothalamus (Bailey *et al.*, 1993a) but also in the midbrain, hippocampus, striatum and frontal cortex (Bailey *et al.*, 1993b; Chaouloff *et al.*, 1989; Gomez-Marino, 2001; Meeusen and De Meirleir, 1995; Meeusen *et al.*, 1996). Consequently, plasma [Prl] in the present EXPs should not be considered to reflect changes of the whole brain 5-HT and DA synthesis.

Total CHO and fat oxidation was not different between both F and FC trials, confirming the lack of any ergogenic effect of pre-exercise caffeine ingestion when muscle metabolic adjustment is manipulated by a high fat meal prior to exercise and supporting the role of glycogen depletion in fatigue development during prolonged exercise in relatively thermoneutral environments.

Conclusion

In conclusion, both experiments presented demonstrate a dissociation between putative brain serotonergic modulators and indices and effort perception during exercise and a mismatch between effort perception and exercise performance after caffeine co-ingested with a high fat meal. The exact mechanism(s) for the attenuation of effort perception with caffeine is unlikely therefore to be brain 5-HT-mediated but most likely dopamine-mediated. Nevertheless, neither brain 5-HT nor DA systems would appear to be implicated in the fatigue process when exercise is performed without significant thermoregulatory stress, thus enabling fatigue development during exercise to occur due to cardiovascular and/or peripheral limiting factors (i.e. glycogen depletion).

Chapter five

Experiment 3

Measures of putative brain 5-HT and DA modulators during exercise in the heat following creatine supplementation in trained-humans

5.1 Introduction

It is well established that exercise performance is markedly impaired in hot environments but the precise mechanism(s) of fatigue have yet to be determined (see chapter 1, section 1.2.8.1). Although strategies like heat-acclimatization (Armstrong and Maresh, 1991), whole body or face pre-cooling (Olschewski and Bruck, 1988; Lee and Haymes, 1995; Booth *et al.*, 2001), fluid replacement (Galloway and Maughan, 2000), and plasma volume expansion (Watt *et al.*, 2000) may all extend time to fatigue by enhancing cardiovascular and thermoregulatory capacity, circulatory failure is not considered as the primary factor limiting exercise performance in the heat (e.g. Nielsen and Nybo, 2003). These observations, in conjunction with the maintenance of CHO reserves at exhaustion (Galloway and Maughan, 1997) and no impairment in the capacity of skeletal muscle to generate force during exercise with hyperthermia (Nybo and Nielsen, 2001b), may preclude peripheral factors as the main cause of fatigue during exercise in the heat.

Previously, Nielsen and colleagues (1993; 1997) proposed a core temperature increase to approximately 39.6 °C as a critical factor in the reduction of central neural motivation and exercise performance. In another study, Nielsen *et al.*, (2001) obtained electroencephalographic recordings from the frontal cortex during exercise in the heat and in the cold, in an attempt to examine alertness and central motivation. These authors found that the elevation in core temperature significantly reduced electrical activity of the frontal cortex and suggested that this reflected a reduction in central drive, a rise in effort perception, and consequently, a reduction in exercise performance. In subsequent studies, it was proposed that fatigue during exercise in the heat may occur at a critical brain temperature, through a reduction in cerebral blood flow (Nybo and Nielsen, 2001a; Nybo *et al.*, 2002) and brain glucose levels (Nielsen and Nybo, 2003; Nybo *et al.*, 2003). However, the exact mechanism(s) for these hyperthermia-induced effects and how thermal stress may affect brain neurotransmission during exercise in the heat are presently unknown.

Creatine (Cr) has extensively been shown to improve performance during short duration, high-intensity exercise by increasing intramuscular Cr and PCr levels (Harris *et al.*, 1992; Kreider *et al.*, 1998) and by accelerating the resynthesis rate of PCr following intense exercise (Greenhaff *et al.*, 1994). Cr has also been shown to increase total body water (TBW) (Hultman *et al.*, 1996), enhance body thermal

tolerance during exercise in the heat (Kern *et al.*, 2001; Ziegenfuss *et al.*, 1998) and improve performance during 35 min of submaximal exercise followed by 3 x 10 sec repeated sprints at an ambient temperature of 37 °C (Volek *et al.*, 2001). However, whether these effects of Cr supplementation on thermoregulation and exercise performance were due to an increase in intramuscular Cr and/or PCr levels, better maintenance of TBW or central neural effects, is presently unknown.

Several studies have attributed the changes in body temperature (Hasegawa *et al.*, 2000; Lin *et al.*, 1998), the higher effort perception and impaired exercise tolerance during exercise in the heat to events localized within the central nervous system, and in particular, the serotonergic and dopaminergic systems (Bridge *et al.*, 2003; Pitsiladis *et al.*, 2002). For example, when hypothalamic [5-HT] was increased in the rat by administration of Fluoxetine (i.e. 5-HT reuptake inhibitor) and 5-hydoxytryptophan (i.e. a 5-HT precursor), there was an increase in metabolic heat production with a concomitant reduction in heat loss (Lin *et al.* 1998). However, in the same species, moderate exercise induced an increase in DA release in the preoptic area of the anterior hypothalamus and this was associated with a reduced body heat storage and greater resistance to heat-stress (Hasegawa *et al.* 2000). In addition, Pitsiladis *et al.*, (2002) found a significant positive correlation between elevated serum [Prl] and rectal temperature and effort perception with a concomitant reduction in exercise capacity during exercise in the heat. On the other hand, Bridge *et al.*, (2003), using a combined Buspirone (i.e. 5-HT_{1A} agonists/dopamine D_2 antagonist) and Pindolol (5-HT_{1A} antagonist) neuroendocrine challenge, found the DA-induced Prl response to be significantly correlated both with exercise duration (73 % of $\dot{V}O_{2\,max}$ at 35 °C), rectal temperature and the rate of temperature rise. These authors concluded that high activity of the dopaminergic pathways in the hypothalamus was a predictor of exercise tolerance in the heat.

Interestingly, clinical studies have shown that oral Cr supplementation increased brain DA synthesis in the substantia nigra of mice by enhancing tyrosine hydroxylase (TH)-positive neurons and TH activation (the rate-limiting enzyme of brain DA biosynthesis) (Klivenyi *et al.*, 1999; 2004; Matthews *et al.*, 1999). Consequently, since the elevation in hypothalamic 5-HT and DA metabolism was found to increase (Lin *et al.* 1998) and reduce (Hasegawa *et al.* 2000) thermal-stress and to be a predictor of exercise tolerance in the heat (Bridge *et al.*, 2003) and Cr

supplementation was found to enhance TBW (Hultman *et al.*, 1996) and thermal tolerance (Kern *et al.*, 2001; Ziegenfuss *et al.*, 1998) it was not unreasonable to imply a possible role of Cr in thermoregulation via brain monoamine system. The purpose therefore of the present EXP was to examine the effects of oral Cr supplementation on thermal-stress and on putative modulators of brain 5-HT and DA function during exercise in the heat.

5.2 Methods

Subjects

Twenty-one endurance-trained male volunteers (n=18 cyclists; n=3 endurance runners) provided written informed consent (for subjects physical characteristics see Table 5.1) for the study. For more information regarding the subjects medical screening and study approval see Chapter 2. Subjects were not engaged in a structured weight-training program during the study and all were Cr free for at least 8 weeks prior to the study. The investigators did not reveal prior to interview that subjects would be excluded if they had supplemented with Cr in the 8 weeks preceding the study. One subject from the placebo group had previously supplemented with Cr. No Cr was detected in the baseline urine samples of any subject.

Experimental design

The determination of $\dot{V}O_{2\,max}$, LT and test workloads are described in Chapter 2. The schematic illustration of the experimental design is presented in Figure 5.1.

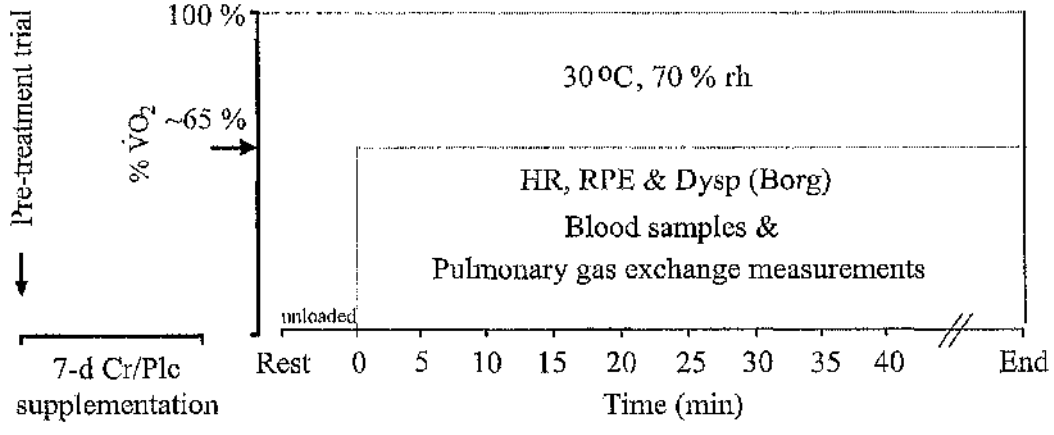


Figure 5.1 Schematic illustration of the experimental design in EXP 3

Briefly, following the $\dot{V}O_{2\,max}$ test, subjects visited the laboratory on at least two occasions (familiarisation to exercise in the heat), in addition, to establishing a suitable work rate that would elicit fatigue in the heat no less than 40-60 minutes. This was achieved by setting the work rate at 20 % Δ (i.e., 20 % of the difference between the $\dot{V}O_2$ at the LT and $\dot{V}O_{2\,max}$) during the initial familiarisation session and, where necessary, adjusting the work rate for subsequent trials to achieve the desired duration. This intensity of exercise was chosen to avoid fatigue occurring as a result of muscle glycogen depletion (Galloway and Maughan 1997). Following the familiarisation period, subjects were matched for body mass and were randomised, in a double-blind fashion, to receive either Cr or placebo. Subjects performed two constant-load exercise tests to volitional exhaustion pre- and post-supplementation. The first test was conducted at least 48 hrs after the subject's final familiarisation trial. The supplementation period for both groups started on the day after the first test and finished the day before the second test.

Cr supplementation consisted of 22.8 $g \cdot d^{-1}$ $Cr \cdot H_2O$ (equivalent to 5 g Cr × 4 daily) and 35 g of glucose polymer made up in 500 mls of warm to hot water for 7 days taken at equal intervals throughout the day. This protocol has been shown to increase resting muscle PCr levels within 5 days (Harris et al. 1992). The placebo group consumed 160 $g \cdot d^{-1}$ of glucose polymer (40 g × 4 daily) for 7 days, prepared and administered in an identical fashion to the Cr supplement. Both supplements had similar taste, texture and appearance and were placed in generic packets to ensure double-blind administration. During the Cr supplementation period subjects were advised to follow their normal diet and weight all food and drink consumed during the supplementation period, using digital weighing scales, readable to 1 g. The diet was analysed for energy intake and macronutrient content using commercially available software (Holland *et al.*, 1991). Subjects were provided with a dietary record book and weight scale and they were advised to record and keep their normal daily energy intake throughout the experimental period but to try to consume items that involve mostly CHO nutritional contents. A particular nutritional list was given to each subject. The subjects were advised to consume the same items with a similar weighted amount for three days prior to each experimental trial. Subjects were advised to avoid consuming items that involve caffeine and alcohol for at least 48 hours prior to each experiment or to ingest any vitamins or commercial ergogenic-

aid supplements or any medication throughout the experimental period and to avoid any strenuous activity for at least 72 hours prior to each exercise trial. At the end of the study all subjects gave verbal assurance that they had complied with these instructions.

Urinary Analysis and muscle Cr uptake estimation

Urinary [Creatinine] ([Crea]) and [Cr] were determined for each subject following 24 hour urinary collection for eight consecutive days (during the supplementation period). All urine was collected over a 24 hours period in a 5 L container provided by the investigators; subjects began urinary collection the day before supplementation in order to obtain baseline results. The volume of urine collected for each 24 hour period was measured and mixed thoroughly, with two representative 20 ml samples being stored at -20° C for subsequent analysis of [Cr] and [Crea] using a spectrophotometric enzymatic Crea Kit (Boehringer Mannheim MPR1 - Kit no. 839434) on an ABX Mira Plus Spectrophotometer (ABX Diagnostics, UK). The Crea content of the urine was measured by a sequence of four enzymatic steps as shown below:

1st step: Creatinine + H_2O creatininase Cr

2nd step: Cr + H_2O creatinase Sarcosine + Urea

3rd step: Sarcosine + H_2O + O_2 sarcosine oxidase Glycine + HCHO + H_2O_2

4th step: H_2O_2 + Phenol derivative + 4-aminophenazone peroxidase red benzoquinoneimine dye

Following the measurement of [Crea], the [Cr] content of the urine was then measured which involved the removal of the first step of the above creatininase reaction, with the remaining three enzymatic reactions (2nd to 4th steps) being completed as described by Oversteegen *et al.*, (1987). Total Cr excretion was corrected for any observed increase in Crea following supplementation. Estimated Cr uptake was calculated by subtracting the total Cr excreted, corrected for [Crea] excretion, from the total amount supplemented per day. Estimated intramuscular [Cr] (mmol·kg^{-1}.dry weight muscle) was calculated based on an estimated muscle mass amounting to 40% of body mass and average muscle water content approximating 77% of wet weight (Bergstrom *et al.*, 1971) as previously described by Maganaris and Maughan (1998). The criteria for subdividing the subjects into

'responders' and 'non-responders' of Cr were based on previous muscle biopsies studies which proposed an ergogenic threshold for intramuscular Cr uptake of ~ 20 mmo·kg^{-1}·dry weight muscle (e.g. Casey *et al.*, 1996; Greenhaff *et al.*, 1994) and on non-invasive muscle Cr uptake-estimation studies (based of urinary Crea exertion) which proposed < 25 mmo·kg^{-1}·dry weight muscle as 'non-responders' and > 25 mmo·kg^{-1}·dry weight muscle as 'responders' to Cr (Kilduff *et al.*, 2002; 2003).

Procedures

All exercise tests were carried out between 18:00 and 20:00 hr. Subjects reported to the laboratory on the day of testing after a standardised meal. Nude body mass were measured and body water compartments were estimated using a Bodystat Multiscan 5000 Bioimpedance analyzer (Bodystat Ltd., Isle of Man) (Van Loan, 1990). This method allows TBW and extra-cellular water (ECW) to be estimated; from these measurements ICW can also be deduced. These measurements allowed euhydration to be indirectly assessed prior to each experiment. The bioimpedance measurements were taken while the subjects lay comfortably in a supine position on a non-conductive surface with their arms and legs slightly abducted. Thereafter, flexible rectal thermistor was inserted 10 cm past the anal sphincter to measure rectal temperature (T_{rec}), an index of core temperature and thermistors (C6600 10-channel microprocessor, Comark, Hertfordshire, UK) attached to the chest, upper arm, thigh and calf for the determination of weighted mean skin temperature (T_{skin}). T_{skin}, [T_{sk} = 0.3 (T_{chest} + T_{arm}) + 0.2 (T_{thigh} + T_{calf})] (Ramanthan 1964) was calculated for each time point. Then, the subject's left hand and forearm were immersed in warm water. For arterialisation of the venous at rest and during exercise, cannulation, blood sampling, HR and RPE recording see chapter 2.

The subject was transferred to the climatic chamber (ambient temperature of 30.3 ± 0.5 °C with a relative humidity of 70 ± 2 % and air velocity of approximately 3.6 m·sec^{-1}) and remained seated on the cycle ergometer for a further 5 min while resting HR, T_{rec}, T_{skin} and gas collections were obtained. Subjects were then instructed to begin 5 min of unloaded cycling before further measurements and another blood sample were obtained. After 5 min of unloaded cycling, the work rate was increased in a 'single step' to the predetermined WR and subjects maintained a pedal cadence of 60-90 rpm throughout the test. Subjects exercised at the same WR for both experiments (i.e., 16 ± 11 % Δ or 63 ± 5 % $\dot{V}O_{2\,max}$). Exhaustion was

defined as the point at which the subject could no longer maintain the pedal cadence above 60 rpm. Blood samples and measurements of HR, T_{rec} and T_{skin} were obtained at 5 min intervals throughout exercise and at exhaustion (Figure 5.1). After exercise, nude body mass was measured. The difference in body mass before and after exercise was calculated and subsequently used to estimate sweat rate and sweat loss, after correcting for respiratory water loss and substrate oxidation (Mitchell *et al.* 1972). Time to exhaustion was recorded but withheld from the subject until all exercise tests had been completed. The experimental protocol for the removal, treatments and analyses of blood and plasma as well as gas exchange measurements and statistical methods used are described in Chapter 2.

5.3 Results

Table 5.1: Physical characteristics of the two groups of subjects. Values are presented as the mean ± s.d.

	Placebo Group (n=10)		Creatine Group (n=11)	
	Pre	Post	Pre	Post
Age (yr)	27 ± 4	-	27 ± 5	-
Height (cm)	181 ± 4	-	178 ± 7	-
Weight (kg)	71.0 ± 6.0	71.2 ± 6.0	72.7 ± 6.6	73.4 ± 6.6*
Total body water (L)	40.4 ± 3.3	40.5 ± 3.2	41.0 ± 3.1	41.6 ± 3.2*
Intracellular Water (L)	21.9 ± 1.8	21.9 ± 1.8	22.2 ± 1.8	22.7 ± 1.9*
Extracellular Water (L)	18.5 ± 1.5	18.5 ± 1.4	18.8 ± 1.4	18.9 ± 1.5
$\dot{V}O_{2\,max}$ ($L \cdot min^{-1}$)	4.3 ± 0.4	-	4.5 ± 0.4	-
$\dot{V}O_{2\,max}$ ($ml \cdot kg \cdot min^{-1}$)	60.5 ± 4.7	-	61.4 ± 4.6	-
Max work rate (watts)	350 ± 34	-	373 ± 31	-

*: Indicates a significant difference from pre-supplementation values

Some of the results obtained from this EXP relating to exercise performance, cardiovascular, metabolic and thermal responses have been reported elsewhere (Kilduff *et al.*, 2004). In summary, relative to the placebo (Plc) group, Cr supplementation increased ICW, TBW and body mass (Table 5.1) and reduced T_{rec}, mean body temperature (T_b) (Fig. 5.3), HR and sweat rate. Cr did not affect T_{skin} (Fig. 5.3), total sweat loss, changes in plasma volume, blood [glucose] and [lactate], $\dot{V}O_2$, $\dot{V}CO_2$, RER, $\dot{V}E$ and metabolic rate (Kilduff *et al.*, 2004). Although endurance performance was not different between Cr (as whole, n = 11) and Plc groups, Cr effectively enhanced performance in the group of subjects (n = 8) considered as 'responders' to Cr, based on estimated intramuscular Cr uptake (Placebo: 50.4 ± 8.4 min vs. 51.2 ± 8.0 min, $P = 0.119$; Cr group: 47.0 ± 4.7 min vs.

49.7 ± 7.5 min, $P = 0.095$; 'responders': 47.3 ± 4.9 min vs. 51.7 ± 7.4 min, $P = 0.031$) (Fig. 5.2). No side effects were reported following Cr supplementation.

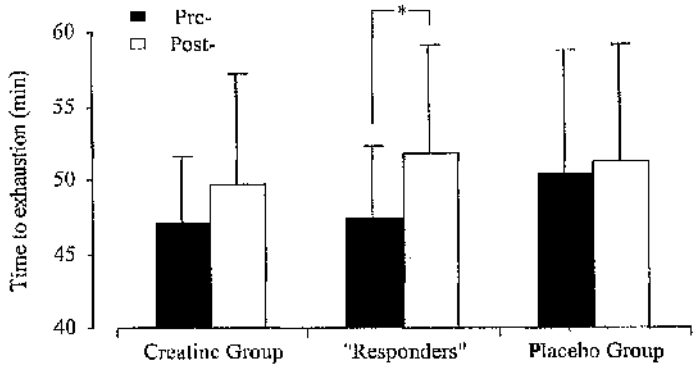


Fig. 5.2. Time to exhaustion (mean ± s.d.) in the Cr, 'responders' and placebo supplemented groups. *: indicates a significant difference between pre- and post-supplementation.

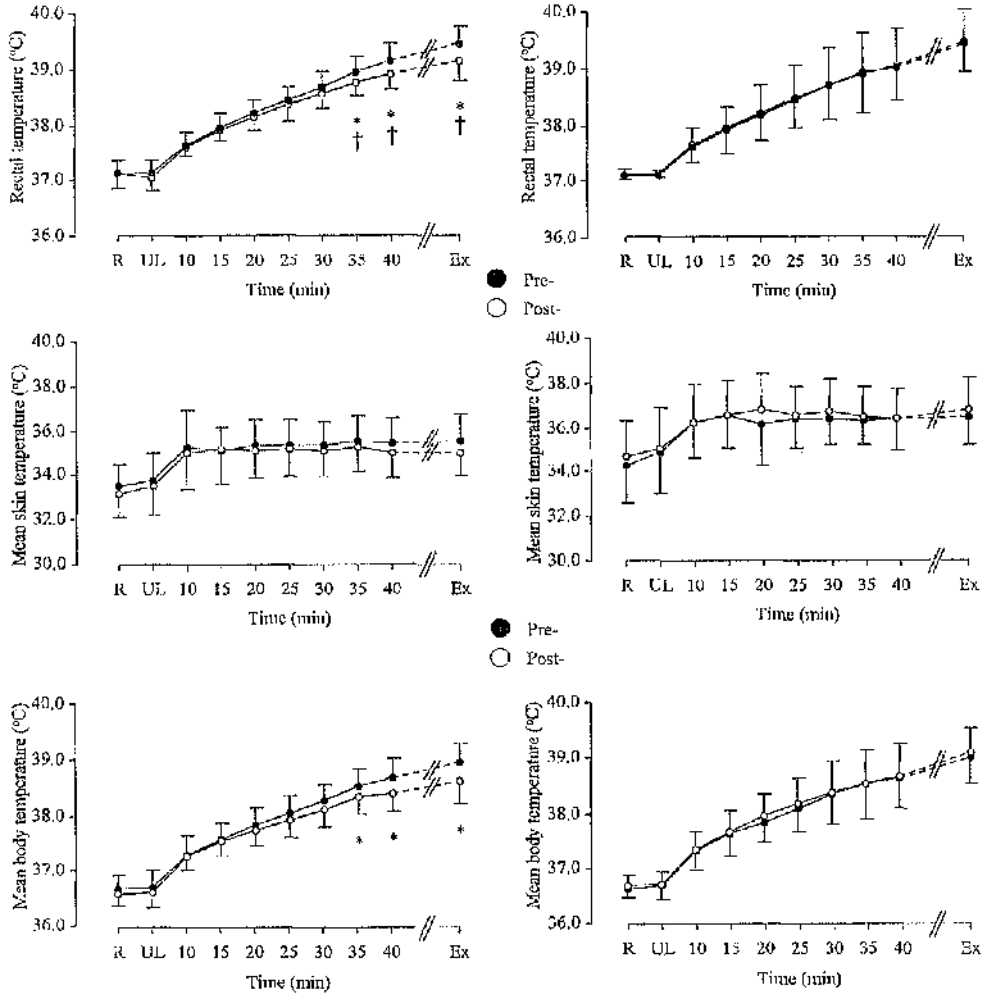


Fig. 5.3. Rectal temperature (top panel), mean skin temperature (middle panel) and mean body temperature (bottom panel) in the Cr (left side) and placebo (right side) supplemented groups. *: indicates a significant difference between pre (●) to post (○) supplementation. †: indicates a significant greater change in the Cr group compared with the placebo group. Values are given as mean (s.d.).

Estimated Cr uptake

Estimated Cr uptake was calculated by subtracting the total Cr excreted, corrected for the increase in Crn excretion, from the total amount supplemented per day. Estimated intramuscular [Cr] ($mmol \cdot kg^{-1}$·dry weight muscle) was calculated based on an estimated muscle mass amounting to 40 % of body mass and average muscle water approximating 77 % of wet weight (Bergstrom *et al.*, 1971). In the Cr group, Crn excretion increased from 1.4 ± 0.4 $g \cdot day^{-1}$ pre-supplementation to 2.4 ± 1.0 $g \cdot day^{-1}$ on the final day of supplementation. There was no increase in Crn excretion in the placebo group (1.5 ± 0.4 $g \cdot day^{-1}$ to 1.4 ± 0.4 $g \cdot day^{-1}$). Cr excretion increased from 8.7 ± 3.7 $g \cdot day^{-1}$ pre-supplementation to 17.4 ± 1.9 $g \cdot day^{-1}$; no Cr was detected in the urine of the placebo group. Estimated Cr uptake was maximal on the first day of Cr supplementation (12 (6-15) g, 61 (32-77) % being retained) and was lowest on the final day (3 (-2-4) g, 16 (-8-21) % being retained). The total amount of Cr retained over the supplementation period was 39 ± 14 g, with an estimated increase in intramuscular [Cr] of 51 (21-61) $mmol \cdot kg^{-1}$·dry weight muscle. Based on these estimates, 3 subjects were classified as 'non-responders' (21 (21-25) $mmol \cdot kg^{-1}$·dry weight muscle) and the remaining 8 subjects were classified as 'responders' (53 ± 5 $mmol \cdot kg^{-1}$·dry weight muscle).

Ratings of perceived exertion (RPE)

A progressive increase in RPE both for breathlessness and perceived leg fatigue was found during exercise reaching near maximum ratings at exhaustion (Fig. 1). Significantly (3-way interaction, $F_{(2,8)} = 2.548$; $P = 0.013$) lower ratings of perceived leg fatigue were found in the Cr group after 10 min ($P = 0.043$) and 25 min ($P = 0.01$) of exercise, with tendencies at 15 min ($P = 0.076$); no such effect was found in the Plc group. There was also a tendency (3-way interaction, $F_{(2,8)} = 1.727$; $P = 0.097$) for subjects in the Cr group to report a feeling of less breathlessness following Cr supplementation; no such effect was observed in the Plc group. The Δ RPE for breathlessness and perceived leg fatigue was not different between Plc and Cr groups. Five out of the eleven subjects in the Cr group reported that they found the post-supplementation trial easier, while two out of the ten subjects in the Plc group rated the post-supplementation trial to be easier. All other subjects rated both trials similarly.

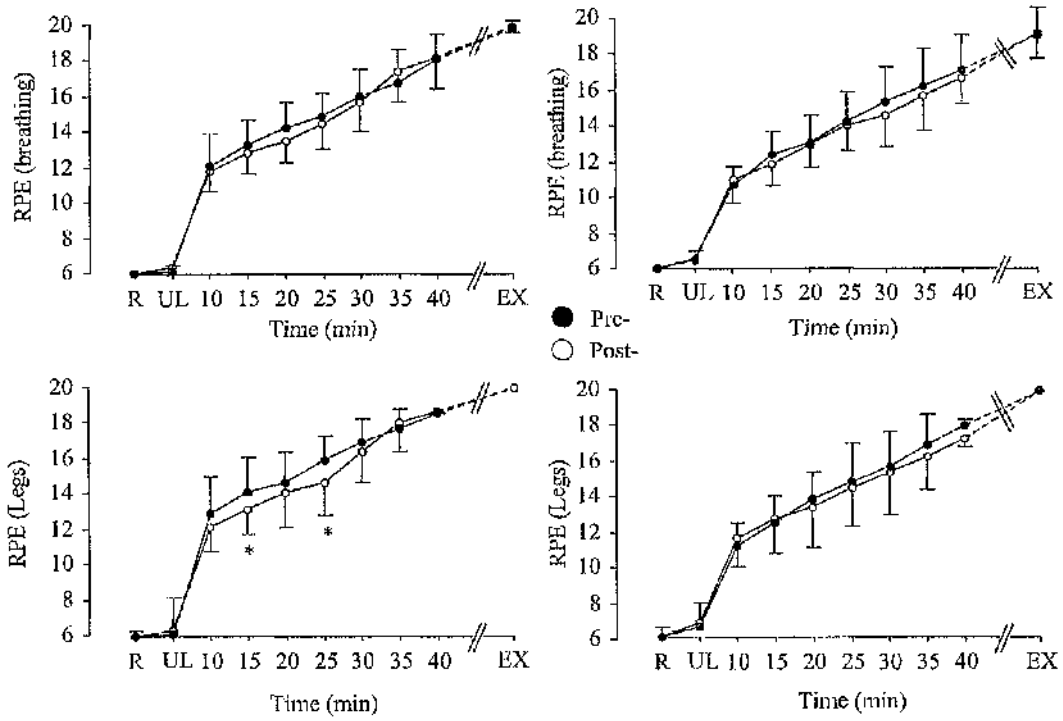


Fig 5.4. RPE (breathing) (top panel) and RPE (legs) (bottom panel) in the Cr (left side) and placebo (right side) supplemented groups during exercise. *: indicates a significant difference between pre (●) to post (○) supplementation. Values are given as mean (s.d).

Plasma amino acids, prolactin and free fatty acids concentrations

There were no significant differences between Plc and Cr groups in plasma total [Trp], [Tyr], [large neutral amino acids] (LNAA) (Table 5.2), total [Trp]:[LNAA] ratio, total [Trp]:[Tyr] ratio, free-[Trp]:LNAA ratio, and free-[Trp]:[Tyr] ratio (Table 5.3). The Δ plasma total [Trp], [Tyr] and [LNAA] and the ratios of total [Trp]:[LNAA], total [Trp]:[Tyr] and free-[Trp]:[LNAA] were also not different between the groups. Plasma free-[Trp] was significantly lower in the post-Cr supplementation trial in the Cr group ($\chi^2 = 33.909$, df = 5, $P < 0.0005$) and in the 'responders' ($\chi^2 = 25.786$, df = 5, $P < 0.0005$) when compared to the pre-supplementation trial (Table 5.2). The Δ free-[Trp] was also significantly lower during exercise in the Cr group ($\chi^2 = 15.881$, df = 1, $P = 0.017$) and in the 'responders' ($\chi^2 = 14.582$, df = 1, $P = 0.019$) when compared with Δ Plc group. In 'responders', the plasma free-[Trp]:[Tyr] ratio was significantly lower at rest and during exercise post-Cr, relative to the pre-supplementation trial, ($\chi^2 = 13.071$, df = 5, $P = 0.023$) (Table 5.3). Plasma total [Trp], [Tyr], [LNAA], free-[Trp]:[LNAA] ratio, total [Trp]:[LNAA] ratio, and total [Trp]:[Tyr] ratio were not different

between pre- and post-supplementation trials in both Plc and Cr groups as well as in the 'responders'. Plasma [Prl] was not different between the Plc group and the Cr group as a whole and between pre- and post-supplementation trials in both Plc and Cr groups (Table 5.4). In addition plasma [Prl] was not different at the end of exercise between 'responders' and 'non-responders' and between 'responders' and placebo groups ($P > 0.05$). In all trials, [Prl] increased significantly over time at all time-points when compared to the resting levels. Δ plasma [Prl] was also not different between the groups and between pre- and post-Cr supplementation trials. Plasma [FFA] was not different between Plc and Cr groups or between pre- and post-Cr supplementation trials (Table 5.4).

Table 5. 2: Concentrations of total Trp, Tyr, LNAA and free-Trp before and after Supplementation. Values are given as median (IQR).

			Blood collection time (min)		
	Group	Trial	Rest	40	End
Total [Trp] ($\mu mol \cdot l^{-1}$)	Plc	Pre	58.3(12)	56.7(22)	63.9(11)
		Post	55.2(14)	67.7(22)	75.5(16)§
	Cr	Pre	47.8(14)	71.1(16)§	69.9(17)§
		Post	53.4(5)	62.3(7)§	71.7(21)§
	Resp	Pre	47.5(6)	65.8(15)§	68.7(18)§
		Post	53.6(7)	62.4(8)§	71.6(20)§
[Tyr] ($\mu mol \cdot l^{-1}$)	Plc	Pre	85.3(18)	94.8(12)	116(24)§
		Post	76.9(41)	86.5(33)	104.1(36)
	Cr	Pre	71.9(37)	111.5(41)§	121.7(46)§
		Post	81.3(34)	104.9(31)§	112.4(52)§
	Resp	Pre	70.3(31)	112.1(36)§	118.3(45)§
		Post	74.6(33)	109.8(31)§	114.5(51)
[LNAA] ($\mu mol \cdot l^{-1}$)	Plc	Pre	831(29)	902(211)	986(254)
		Post	873(233)	897(171)	994(375)
	Cr	Pre	744(334)	1076(340)	945(326)
		Post	812(189)	806(372)	933(361)
	Resp	Pre	732(279)	1051(273)	962(267)
		Post	764(187)	864(473)	871(322)
Free-[Trp] ($\mu mol \cdot l^{-1}$)	Plc	Pre	2.5(0.6)	3.1(0.7)§	3.3(0.8)§
		Post	2.6(1.2)	3.2(0.7)§	3.6(0.9)§
	Cr	Pre	2.4(0.7)	2.9(0.6)§	3.5(0.5)§
		Post	2.4(0.5)	2.6(0.5)*§	3.0(0.7)*§
	Resp	Pre	2.6(0.9)	3.1(0.6)	3.4(0.6)§
		Post	2.3(0.7)	2.7(0.6)*	3.0(0.5)*§

*: Indicates a significant difference from pre-supplementation values.
§: Indicates a significant difference over time compared with the resting values
IQR: Interquartile range.

Table 5. 3: Total Trp:LNAA, total Trp:Tyr, free-Trp:LNAA and free-Trp:Tyr ratios before and after supplementation. Values are given as median (IQR).

			Blood collection time (min)		
	Group	Trial	Rest	40	End
Total [Trp]:[LNAA] ratio	Plc	Pre	.069(.016)	.071(.015)	.076(.016)
		Post	.070(.012)	.072(.017)	.084(.022)
	Cr	Pre	.062(.013)	.069(.010)	.075(.012)
		Post	.073(.016)	.080(.016)	.082(.007)§
	Resp	Pre	.062(.012)	.069(.010)	.076(.014)
		Post	.076(.016)	.077(.019)	.082(.006)§
Total [Trp]:[Tyr] ratio	Plc	Pre	0.65(.24)	0.61(.09)	0.58(.17)
		Post	0.71(.07)	0.68(.18)	0.74(.19)
	Cr	Pre	0.64(.14)	0.63(.07)	0.61(.15)
		Post	0.65(.16)	0.62(.14)	0.65(.09)
	Resp	Pre	0.66(.20)	0.62(.09)	0.61(.17)
		Post	0.71(.16)	0.62(.20)	0.62(.09)
Free-[Trp]:[LNAA] ratio	Plc	Pre	.0030(.0007)	.0040(.0010)	.0036(.0020)
		Post	.0031(.0024)	.0038(.0014)	.0048(.0012)
	Cr	Pre	.0034(.0021)	.0032(.0015)	.0037(.0016)
		Post	.0028(.0015)	.0038(.0017)	.0028(.0027)
	Resp	Pre	.0042(.0020)	.0034(.0010)	.0045(.0012)
		Post	.0027(.0015)	.0039(.0017)	.0043(.0025)
Free-[Trp]:[Tyr] ratio	Plc	Pre	.032(.017)	.035(.008)	.027(.014)
		Post	.035(.018)	.039(.012)	.043(.013)
	Cr	Pre	.034(.027)	.032(.013)	.027(.012)
		Post	.028(.016)	.028(.013)	.024(.019)
	Resp	Pre	.042(.025)	.033(.011)	.032(.013)
		Post	.032(.017)*	.026(.015)*§	.029(.018)

*: Indicates a significant difference from pre-supplementation values.
§: Indicates a significant difference over time compared with the resting values.
IQR: Interquartile range

Table 5.4: Concentrations of plasma prolactin and FFA before and after supplementation. Values are given as median (IQR).

			Blood collection time (min)		
	Group	Trial	Rest	40	End
Plasma [Prolactin] ($nmol \cdot l^{-1}$)	Plc	Pre	0.13(.04)	0.51(.38)§	0.94(.83)§
		Post	0.14(.06)	0.53(.24)§	0.93(.14)§
	Cr	Pre	0.15(.07)	0.84(.58)§	1.07(.63)§
		Post	0.15(.05)	0.79(.51)§	1.14(.56)§
	Resp	Pre	0.15(.04)	1.14(.39)§	1.27(.44)§
		Post	0.14(.04)	0.98(.46)§	1.39(.36)§
Plasma [FFA] ($mmol \cdot l^{-1}$)	Plc	Pre	0.53(0.47)	0.37(0.18)	0.40(0.34)
		Post	0.38(0.10)	0.28(0.14)	0.23(0.19)
	Cr	Pre	0.42(0.26)	0.29(0.11)	0.31(0.18)
		Post	0.48(0.27)	0.32(0.14)	0.34(0.18)
	Resp	Pre	0.48(0.24)	0.28(0.10)	0.30(0.15)
		Post	0.42(0.20)	0.29(0.10)	0.32(0.16)

§: Indicates a significant difference over time compared with the resting values.
IQR: Interquartile range.

Correlation analysis

There were no significant correlations between plasma free-[Trp] and T_{rec}, between free-[Trp] and RPE, between free-[Trp] and plasma [FFA], between T_{rec} and [Prl] and between free-[Trp] and [Prl], when examined for each time-point separately.

5.4 Discussion

The present study utilized prolonged exercise in the heat to examine modulators of brain 5-HT and DA function in association with perceptual, metabolic and thermoregulatory responses and endurance exercise performance after 7-days of Cr supplementation. Cr was used as a 'vehicle' to increase hydration status and reduce thermal stress. The most significant observations of this study were the effectiveness of Cr to directly or indirectly attenuate thermal stress (e.g. T_{rec}, sweat rate, HR), increase hydration (ICW, TBW), reduce effort perception and influence selected modulators of brain serotonergic and dopaminergic function (i.e. lower plasma free-[Trp] and free-[Trp]:[Tyr] ratio). These responses may therefore have contributed to enhancing endurance exercise performance in the heat in the 'responders' to Cr supplementation.

It is well established that there are 'responders' and 'non-responders' to Cr supplementation, with some researchers proposing an ergogenic threshold for intramuscular Cr uptake of 20 mmol·kg^{-1}·dry muscle weight following Cr supplementation (Casey *et al.*, 1996; Greenhaff *et al.*, 1994). In previous studies (Kilduff *et al.*, 2002; 2003), subdividing the Cr subjects into 'responders' and 'non-responders' on the basis of physiological measurement (e.g., estimated muscle Cr uptake) confirmed the ergogenic potential of Cr supplementation. However assigning a specific threshold value was not possible as Cr uptake was only estimated in these studies (Kilduff *et al.*, 2002; 2003). The present results provide support for this contention, again with results showing two very distinct groups (21 (21-25) mmol·kg^{-1}·dry weight muscle ("non-responders" n=3) and 53 ± 5 mmol·kg^{-1}·dry weight muscle ("responders" n=8) as previously reported (Kilduff *et al.*, 2002; 2003). Failure to discriminate between those who respond to Cr supplementation and those who do not could therefore mask any effect due to Cr supplementation. This may help account for some of the confounding reports in the literature with

regard to the ergogenic potential of Cr supplementation. However, it is not established yet why some people respond positively and some others negatively to Cr (Kilduff *et al.*, 2004; Lemon, 2002). Consequently, more studies are needed to establish why some subjects respond positively and some others negatively to Cr.

In the present study, subjects who had supplemented with Cr reported significantly lower ratings of perceived leg fatigue after 10 min of exercise, suggesting they were able to discern the benefit of this putative hyperhydration strategy. Five out of the eight 'responders' to Cr reported that they found the post-supplementation trial to be easier. These same five subjects also showed the largest estimated Cr uptake and performance gains (intramuscular Cr uptake: 'non-responders', n = 3: 21 (21-25) mmol·kg^{-1}·dry weight muscle; 'responders', n = 8: 53 ± 5 mmol·kg^{-1}·dry weight muscle). Time to exhaustion was significantly improved post-Cr supplementation and, although Δ performance failed to reach statistical significance in the 'responders' (P = 0.066), the significant positive correlation between estimated muscle Cr uptake and Δ performance (r = 0.75, n = 11; P = 0.008), is further indication that Cr can enhance prolonged exercise performance in the heat. Power tests calculations (Bolton, 1997) utilizing the performance data obtained from the Cr group as a whole (i.e., both 'responders' and 'non-responders') revealed that n = 39 subjects (90 % power) would be required in order to observe a significant performance effect, distinguishing the Cr group from the Plc group (P < 0.05). However, the power calculation utilizing the performance data obtained from only the 'responders' suggested that n = 9 subjects (90 % power) would be required to achieve statistical significance (P < 0.05).

In contrast to the present results, a previous study did not report an improvement in endurance performance following Cr supplementation when exercise was carried out in a relatively thermoneutral environment (Engelhardt *et al.*, 1998). This failure of Cr to enhance endurance exercise performance in such an environment would seem to preclude an increased intramuscular [Cr] or [PCr] being responsible for the ergogenic effect of Cr on endurance performance. In the absence of any differences between trials in blood metabolites, cardiovascular responses (with the exception of HR), changes in plasma volume and total sweat rate in the present study (see above: Results section; summarizing results from Kilduff *et al.*, 2004), the reduction in effort perception and the improvement in endurance performance in the heat in

'responders' to Cr supplementation may be due, in part at least, to the responses observed in the putative modulators of brain 5-HT and DA function. It has been suggested, for example, that a high brain [5-HT]:[DA] ratio increases effort perception (i.e. central fatigue) during prolonged exercise while a low [5-HT]:[DA] ratio may favor increased arousal and central neural motivation (Davis *et al.*, 1993; 2003; Davis and Bailey 1997). Furthermore, high hypothalamic dopaminergic activity has been shown to be a reasonable predictor of exercise tolerance during prolonged submaximal exercise in the heat (Bridge *et al.*, 2003). As oral Cr supplementation can increase brain DA synthesis (Klivenyi *et al.*, 1999; Mathews *et al.*, 1999), the finding of a lower plasma free-Trp:Tyr ratio (and by extrapolation a lower [5-HT]:[DA] ratio since both precursors share the same L-system transport across the blood brain barrier; Fernstrom and Wurtman 1972) may have contributed to attenuating effort perception and enhancing exercise performance in the 'responders' to Cr; no such response was observed in Plc trials. Previous studies have shown that DA microinjections into the hypothalamus and substantia nigra of the rat produced hypothermia through an increase in [DA] (Brown *et al.*, 1982; Cox and Lee, 1980). This notion is further supported by the recent work of Hasegawa *et al.*, (2000). These authors examined the role of monoamines in thermoregulation during prolonged exercise and observed that exercise in the heat did not influence brain [5-HT] and [5-HIAA], but elevated DA levels in the hypothalamus and substantia nigra and augmented heat loss mechanisms.

In a recent study, brain uptake of Trp and Tyr as well as brain DA release were not affected by hyperthermia, therefore failing to support the classic '5-HT-central fatigue hypothesis' and the involvement of brain DA function in thermoregulation during exercise in the heat (Nybo *et al.*, 2003). These authors suggested that brain glycogen depletion may have contributed to central fatigue during exercise in the heat. However, the results presented by these authors do not preclude the involvement of putative modulators of brain 5-HT function in central fatigue during exercise in the heat as a correlation was found between arterial free-[Trp] and brain Trp uptake (Nybo *et al.*, 2003). Brain Trp uptake has been shown on numerous occasions to be the rate-limiting step of 5-HT synthesis (Bloxam *et al.*, 1980; Fernstrom, 1990), thus an involvement of 5-HT during exercise in the heat as classically proposed cannot be excluded; 5-HT was not measured in the study by Nybo *et al.* (2003).

Although plasma free-[Trp] was significantly lower following Cr supplementation, [LNAA] and the ratios of free and total Trp:LNAA were unchanged; hence, the similar plasma [Prl] found during exercise in the heat in all trials is not entirely unexpected. According to Wurtman (1988) in order to elicit a reduction in brain Trp uptake and, therefore, attenuation in brain 5-HT turnover, a 5- to 6-fold elevation in plasma [LNAA] is required. In addition, Leathwood and Fernstrom (1990) suggested that a 13- to 26- fold elevation in total Trp:LNAA ratio is required to induce a change in the brain stem [5-HT] in monkeys. The association however, between peripheral modulators of brain 5-HT function and circulating [Prl] during exercise has not been fully explained. Fischer *et al.*, (1991) for example, observed an increase in [Prl] during exercise in proportion to the rise in plasma free-[Trp]. Other investigators found a positive correlation between serum [Prl] and T_{rec} (e.g. Brisson *et al.*, 1986; 1991) during exercise in the heat and this relationship has been used to justify brain monoamine system involvement in regulating T_{rec} and Prl secretion (e.g. Pitsiladis *et al.*, 2002). However, in the present study, T_{rec} was lower in the post-Cr supplementation trial and T_{rec} and plasma [Prl] were not correlated. These results are in agreement with a number of previous studies (e.g. Struder *et al.*, 1996) that showed that Prl secretion is not always related to plasma free-[Trp] and this may be more evident during exercise in the heat.

In the present study, Cr was effective in directly or indirectly attenuating thermal stress (e.g. T_{rec}, sweat rate, HR) and increasing hydration (ICW, TBW) but Prl levels were unaffected. The effects of dehydration on brain neurotransmission and hypothalamic Prl secretion has previously been examined and conflicting results have been reported. For example, Scacchi *et al.*, (1989) found that water deprivation elevated hypothalamic [5-HT] in the rat. On the other hand, rehydration (injection of 0.5 ml 10% saline solution) was found to decrease plasma [Prl] in rats (Nagy *et al.* 1992). Several other studies do not support the association between dehydration and changes in osmolality and plasma [Prl] but report a positive correlation between elevated body heat storage and plasma [Prl] (Brisson *et al.*, 1986; Falk *et al.*, 1991; Melin *et al.*, 1988; Laatikainen *et al.*, 1988; Pitsiladis *et al.* 2002). In particular, Brisson *et al.*, (1986; 1991) demonstrated that a body-temperature threshold value had to be reached for heat stress to induce a significant blood Prl response during active or passive heat exposure. These authors estimated this mean T_{rec} threshold to be approximately 1.3°C - 1.7°C above normal body temperature values. In the

present study, the rise in T_{rec} induced by exercise in the heat, far exceeded this threshold in both pre- and post-supplementation trials, thus resulting in a similar elevation in serum [Prl] (Table 4). Consequently, although Cr was effective in significantly reducing thermal stress, this reduction was insufficient to significantly alter plasma [Prl]. However, this finding does not necessarily preclude a difference in hypothalamic Prl secretion between Plc and Cr trials.

Another possible explanation for the reduction in effort perception and for the enhancement in endurance performance observed in 'responders' to Cr group is the attenuation in muscle contraction-stress with Cr, which may prevent an early ATP depletion (Guerrero and Willimann, 1998). Recently, for example, Terblanche and Nel (1998), using animal models, examined the effect of heat, submaximal exercise (10 min running x 3 in 30°C with 2 min recovery between exercise) and acclimation programme on the activity of creatine kinase (CK) in several tissues including kidneys, skeletal and heart muscles. They found that prolonged exercise in the heat has significantly reduced the activity of CK in all these tissues concluding that exercise in the heat may produce tissue damage. In the present EXP, it is possible that Cr has enhanced brain DA function (see Klivenyi *et al.*, 1999; Matthews *et al.*, 1999) that contributed to reducing thermal-stress (e.g. Hasagewa *et al.*, 2000), and therefore attenuating the negative effect of heat-stress on CK activation on skeletal muscle. However, more controlled human studies are needed in order to examine this particular speculation. In addition, it is possible that the increased in exercise performance observed in the 'responders' group to be due to the increased aerobic capacity that might have been elevated as a results of Cr supplementation. Unfortunately, the oxygen uptake capacity was not measured when subjects were on Cr supplementation regime.

A number of previous studies have reported a significant correlation between plasma [FFA] and plasma free-[Trp] primarily due to FFA displacing Trp from its binding to albumin (e.g. Curzon *et al.*, 1973; Spector *et al.*, 1975). In the present study, the rise in plasma [FFA] was modest and not different between trials but plasma free-[Trp] was lower during exercise and at exhaustion on the post-Cr supplementation trial. This somewhat surprising result, in conjunction with the lack of a significant correlation between plasma [FFA] and plasma free-[Trp] would suggest a more complex control of plasma free-[Trp] during exercise in the heat than previously

described. The exact mechanism(s) responsible for the observed reduction in plasma free-[Trp] and free-Trp:Tyr ratio after Cr loading (despite no difference in plasma [FFA]) and the significance of these observations remain to be determined.

It is possible that Cr to enhance tryptophan-2,3-dioxygenase (TDO) and/or indoleamine-2,3-dioxygenase (IDO) activation during exercise in the heat, via a protection against thermal-stress which otherwise induces liver metabolic dysfunction. This possible effect of Cr against thermal stress may enhance the conversion of Trp to kynurenine leading to a reduction in plasma free-[Trp]. It has been previously found that hyperthermia impaired liver activation by augmenting a non-specific proton leaking across the inner mitochondrial membrane, and the resultant degraded energy state offsets temperature stimulation of pyruvate carboxylase (Willis *et al.*, 2000). For this reason the pyruvate carboxylation rate of intact liver mitochondria may fail to exhibit a Q_{10} effect (Willis *et al.*, 2000). However, Cr supplementation was found to enhance liver function by elevating plasma transaminases, lactate dehydrogenase (LDH) and CK activation (Kreider *et al.*, 1998). Similarly, Cr was found to enhance others organs function, such as kidneys and heart by elevating CK activation during exercise in the heat (Terblanche and Nel, 1998). On the other hand, it has been shown that exercise activates the kynurenine pathway of Trp metabolism and made nicotinamide adenine dinucleotide (NAD) which will be concerned with energy metabolism in mitochondria (Ito *et al.*, 2003). Trp-NAD pathway was initiated by cleavage of indole-ring of Trp by TDO in the liver and IDO in other organs (Ito *et al.*, 2003). However, the hypothesis of Cr-induced an acceleration of kynurenic metabolic pathway during exercise in the heat by elevating CK-liver activation is only speculative on since no studies have examined yet any effect of Cr supplementation on TDO or IDO.

Conclusion

In conclusion, the possible effectiveness of Cr to alter key modulators of brain 5-HT and DA function and/or to increase TBW and hyperhydration may have contributed to the reduced thermal stress and effort perception during exercise in the heat and to the enhancement of endurance performance in the 'responders' to Cr supplementation. Although further research is warranted, these results provide some evidence of a centrally-mediated process of fatigue during strenuous exercise in the heat.

Chapter six

(Experiment 4)

Putative brain serotonergic and dopaminergic modulators in chronic fatigue syndrome: the effect of graded exercise stress

6.1 Introduction

As stated in Chapter 1 (section 1.2.9), chronic fatigue syndrome (CFS) is a debilitating condition, the precise pathology of which remains largely undefined, with both peripheral and central neural mechanisms hypothesised. CFS patients are reported to have lower physical fitness and quadriceps muscle strength and higher body mass ratio relative to sedentary and depressed controls (Fulcher and White, 2000). However, although these results may suggest peripheral incapability, they do not necessarily imply peripheral factors in the pathogenesis and maintenance of CFS. Alternatively, these peripheral responses may be due to the chronic physical deconditioning, which is common in this population (e.g. Montague *et al.*, 1989; Fulcher and White, 2000; Riley *et al.*, 1990). Other studies for example suggested no differences in $\dot{V}O_2$ capacity, HR and lactate metabolic responses between CFS and sedentary control groups during graded exercise, precluding the significant role of peripheral mediated-factors being responsible in the pathogenesis of CFS (e.g. Lloyd *et al.*, 1988; 1991; Sargent *et al.*, 2002).

In contrast also to the heterogeneity in studies on muscle metabolism, an exacerbated effort perception has been consistently associated with CFS, with patients reporting higher effort perception compared to healthy controls, during both submaximal and graded exercise (Cook *et al.*, 2003; Riley *et al.*, 1990; Gibson *et al.*, 1993). In addition, neuroendrocrinological studies support centrally-mediated factors in the pathogenesis of CFS implicating specifically, a metabolic up-regulation of brain 5-HT synthesis (e.g. Cleare *et al.*, 1995; Sharpe *et al.*, 1997), which may play a role in the development of central fatigue (e.g. Acworth *et al.*, 1986) and hypothalamic function (Komaroff and Buchwald, 1998). This evidence, however, is based predominantly on neuroendocrine challenge tests, with known methodological limitations. The extensive use for example, of brain 5-HT_{1A}-receptors agonist drug, Buspirone, has been suggested to be of doubtful pharmacological specificity (Sharpe *et al.*, 1996). This probably occurs because Buspirone has been suggested to be not only a 5-HT_{1A}-receptors agonist drug but also a D_2-receptors antagonist drug (Bridge et al., 2003). In addition, a double-blind trial suggested that Fluoxetine may be less tolerated in patients with CFS than those with major depression (Vercoulen *et al.*, 1996).

It is also interesting to note that most of the treatments in CFS, mainly antidepressants, have been suggested to be generally ineffective (e.g. Whiting *et al.*, 2001). The only evidence available to support relative effective treatments in CFS includes cognitive behavioural therapy (Prins *et al.*, 2001) and graded exercise (Fulcher and White, 2000). However, cognitive behavioural therapy has been extensively criticised because it does not prevent physical deconditioning, which contributes in maintaining and exacerbating CFS symptoms (e.g. Chaudhuri and Behan, 2001; Vercoulen *et al.*, 1997).

Although during resting conditions, clinical studies suggest a brain serotonergic system involvement in CFS, no studies to date have been conducted to examine, elucidate and understand the role of putative modulators of brain serotonergic and dopaminergic function during exercise in CFS. The aim therefore of the fourth and final experiment was to elucidate the role of putative modulators of central fatigue in CFS patients by examining circulating amino acids, which have been previously suggested to modify central serotonergic and dopaminergic functions, to evaluate the association between these modulators with perceptual and metabolic responses in CFS patients and to compare their results to those of matched sedentary controls.

6.1 Methods

Subjects

Twelve patients (ten female and two male) with a diagnosis of CFS [females: age, 39 ± 11 years (mean ± s.d.); height, 165 ± 8 cm; body mass, 71 ± 11 kg; male 1: age, 45 years; height, 177 cm; mass, 85 kg; male 2: age, 33 years; height, 167 cm; mass, 66 kg) and 11 sedentary Controls (nine females: age, 38 ± 12 years; height, 163 ± 8 cm; mass, 62 ± 9 kg; male 1: age, 48 years; height, 168 cm; mass, 76 kg; male 2: age, 33 years; height, 175 cm; mass, 65 kg) provided written informed consent prior to their participation in the study. For more information regarding the subjects medical screening, CFS patients' and controls' selection criteria, pre-exercise medical examination for CFS patients, and study approval see Chapter 2.

Experimental design and Procedures

The experimental equipments used are described in Chapter 2. The schematic illustration of the experimental design is presented in Figure 6.1.

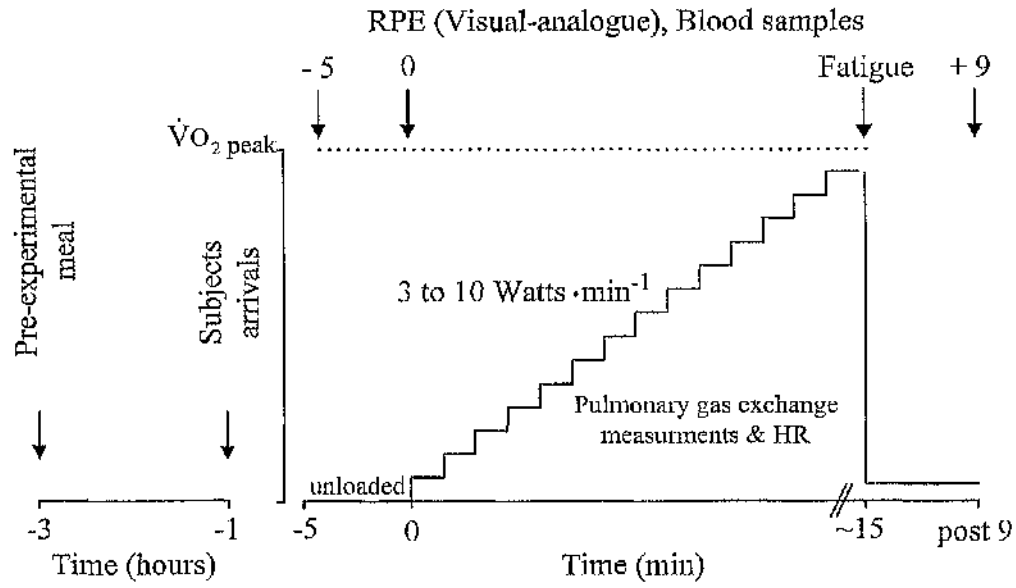


Figure 6.1. Schematic illustration of the experimental protocol in EXP 4

Prior to the experiments subjects were asked to keep their normal diet throughout the experimental period, were advised to consume the same weighted amount and same items prior to each trial and to consume their last meal the same time, (at least 3 hours) before each experimental trial. Subjects were advised to avoid consuming items that involve caffeine and alcohol for at least 48 hours prior to each experiment.

Subjects underwent a ramp-incremental exercise test on an electronically-braked computer-controlled cycle ergometer to limit of tolerance. Gas exchange variables and $\dot{V}O_{2\,peak}$ were determined using breath-by-breath mass-spectrometer analyser (QP9000, Morgan Medical, Gillingham, Kent, UK) based on previously derived algorithms (Beaver *et al.*, 1973) (see Chapter 2, section 2.3 for more details). The following criteria were used to define volitional fatigue in the present EXP: 1) near or maximal values of perceived exertion, 2) near or maximal HR, 3) inability to maintain a cadence of ~ 50 rpm. The incrementation rate for individual subjects varied between 3-10 W·min^{-1}, so as to elicit exhaustion within 10-15 min (Figure 6.1). The work-incremental rate has been determined for each subject separately based on medical history and physical activity questionnaires completed a week before the exercise testing. Subjects reported to the laboratory 1.5 hrs prior to the start of the tests. Following a brief familiarisation session with equipment and

procedures, subjects' body mass was measured and electrodes were introduced on five positions on the chest for on-line HR recording and cardiovascular medical examination inspection. Subsequently, subjects were seated comfortably with their right hand and forearm immersed for 15 min in warm water. Arterialised-venous blood samples were subsequently drawn at rest, unloaded exercise (Unl), exhaustion (Exh) and at 6 min of recovery (Rec). For arterialisation of the venous blood at rest and during exercise, cannulation, blood sampling, HR and RPE recording, the experimental protocol for the removal, treatments and analyses of blood and plasma as well as gas exchange measurements and statistical methods used see Chapter 2.

6.3 Results

Plasma amino acids concentrations

Table 6.1: Plasma concentrations of amino acid serotonergic and dopaminergic modulators in CFS and control groups; values: mean ± s.d.

	Rest	Unloaded Exercise	Exhaustion	Recovery
Total Trp ($\mu mol \cdot l^{-1}$)				
CFS	41.5±6.6	38.3±6.5	38.8±5.6	37.9±7.6
Controls	42.2±4.3	41.3±5.2	41.2±4.8	37.4±5.1
Free Trp ($\mu mol \cdot l^{-1}$)				
CFS	4.2±1.4	4.7±1.1	5.1±1.4	5.7±1.2
Controls	3.9±0.9	3.8±1.1	3.8±0.8*	4.6±1.0*
BCAA ($\mu mol \cdot l^{-1}$)				
CFS	351.9±74.2	338.9±71.6	348.7±50.7	343.5±73.5
Controls	391.9±68.6	391.6±78.9	394.4±51.1*	397.8±74.7
LNAA ($\mu mol \cdot l^{-1}$)				
CFS	531.6±97.5	506.8±95.1	526.5±61.3	516.9±95.8
Controls	587.4±87.9	584.4±103.7	596.1±66.4*	602.8±100.5*
Tyr ($\mu mol \cdot l^{-1}$)				
CFS	49.4±14.3	45.8±12.9	49.5±11.0	47.1±11.0
Controls	62.8±9.1*	61.9±10.0*	66.1±8.8*	65.0±13.1*

* indicates a significant difference between CFS and controls; $p < 0.05$

Plasma free-[Trp] was raised in the CFS group throughout the protocol, being highly significant at both Exh and Rec, with a tendency at Unl ($P < 0.1$); total [Trp], however, was not significantly different between groups at any of the time points (Table 6.1). [BCAA] and [LNAA], both of which compete with free Trp for entry into the brain, were lower in the CFS patients throughout, with highly significant differences at Exh, and for [LNAA] also in Rec (Table 6.1). Consequently, the central serotonergic modulators free-[Trp]:[BCAA] and free-[Trp]:[LNAA] were

higher in the CFS group, significantly at all time points except at rest (Fig. 6.2). On the other hand, significantly lower plasma [Tyr] was found in the CFS patients at all time points (Table 6.1). As a result, free-[Trp]:[Tyr] was significantly higher in the CFS group at all time points (Fig. 6.2).

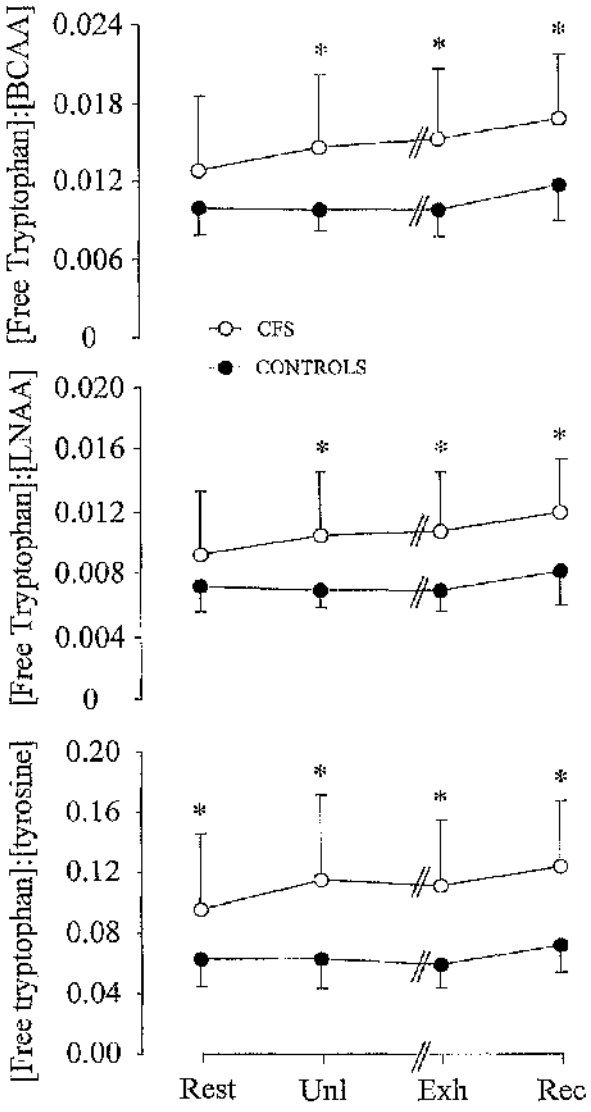


Fig. 6.2 The ratios of free-[Trp]:[BCAA] (top panel), free-[Trp]:[LNAA] (middle panel) and free-[Trp]:[Tyr] (bottom panel) (mean ± s.d.) in CFS and control groups for Rest, Unl, Exh, and Rec. * indicates a significant difference between groups.

Plasma FFA and blood metabolites concentrations

There were no differences between CFS and control groups for plasma [FFA], blood [glucose] or blood [lactate] (with the exception of a significantly lower Rec value in the CFS group) (Fig. 6.3).

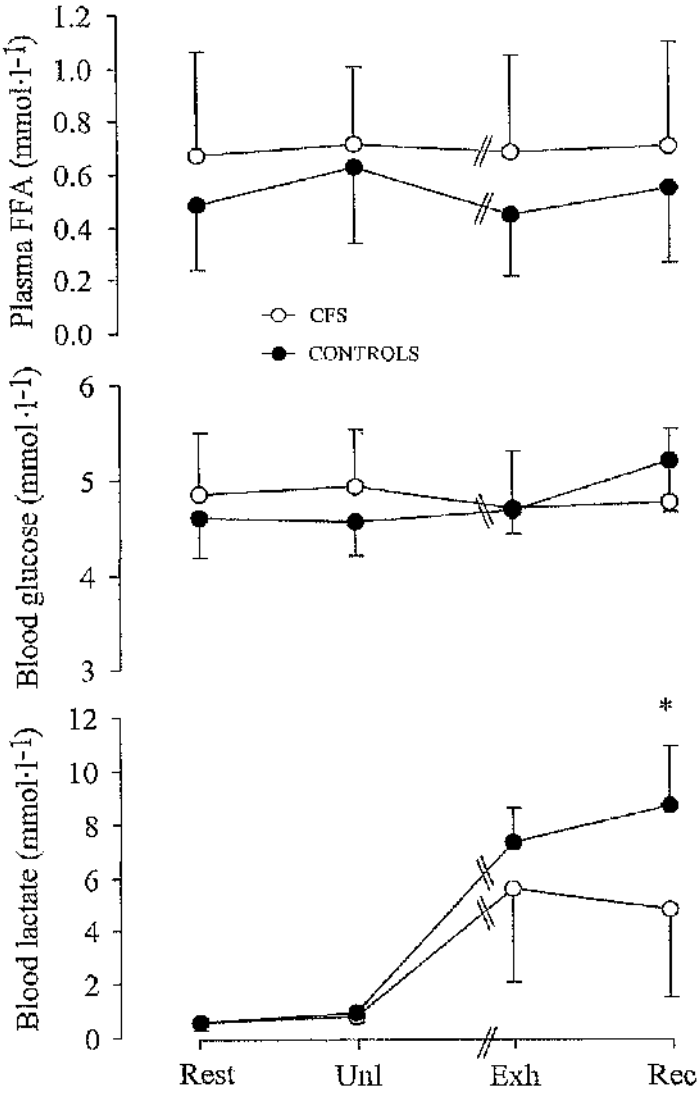


Fig. 6.3. Plasma [free fatty acids] (top panel), blood [glucose] (middle panel) and [lactate] (bottom panel) (mean ± s.d.) in CFS and control groups for Rest, Unl, Exh, and Rec. * indicates a significant difference between groups.

Cardiopulmonary and perceptual responses

$\dot{V}O_{2\,peak}$ in the CFS group (21.2 ± 6.3 ml · min^{-1} · kg^{-1}) was significantly lower than in the control group (28.3 ± 6.4 ml · min^{-1}·kg^{-1}). Peak heart rate was lower in the CFS group than in the control group, although this difference was not statistically significant (CFS, 160 ± 29 beats · min^{-1}; control, 176 ± 13 beats · min^{-1}; $P = 0.12$); this corresponded to 88% of the maximal age-predicted value for the CFS patients, compared with 97% for the controls. The peak RER was significantly lower in the patients (0.98 ± 0.06) than in the control group (1.15 ± 0.40). RPE was significantly higher in the CFS group at all time points, compared with controls: rest: CFS, 14 (2–63) [median (range)]; control, 2 (0–6); unloaded exercise: CFS, 24 (2–99), control, 2 (0–8); at exhaustion (near-maximal values): CFS, 98 (91–100), control, 90 (51–100) (Table 6.2). The total work-load completed was significantly lower in CFS relative to Control group; CFS: 62 ± 8.5, Control: 106 ± 12; $P < 0.1$).

Table 6.2: Perception of effort in CFS and Control groups. Values are given as median (range)

Effort perception	Rest	Unloaded Exercise	Exhaustion
CFS	14(2-63)	24(2-99)	98(91-100)
Controls	2.0(0-6) *	2.0(0-8) *	90(51-100) *

* indicates a significant difference between CFS and controls; $p < 0.05$

Correlations

Significant positive correlations were found between free-[Trp] and [FFA] for all time points collectively, both for the CFS group ($r = 0.44$) and the control group ($r = 0.47$). Significant positive correlations were found between $\dot{V}O_{2\,peak}$ and the putative central 5-HT modulators, i.e. free-[Trp] (r=0.73, n=12, P=0.011), free-[Trp]:[BCAA] (r=0.80, n=12, P=0.003) and free-[Trp]:[LNAA] (r=0.78, n=12, P=0.005), at exhaustion in the CFS group, but not in the control group. Significant negative correlation was found between RPE and [tyrosine] at rest (r=-0.60, n=12, P=0.05) and during unloaded exercise (r=-0.65, n=12, P=0.029) in the CFS group only. $\dot{V}O_{2\,peak}$ and free-[Trp]:[tyrosine] ratio were also significantly correlated at exhaustion in the CFS group (r=0.69, n=12, P=0.018), but not in the control group.

6.4 Discussion

In the present experiment, significant differences between CFS patients and healthy sedentary controls were observed in a number of key central serotonergic and dopaminergic modulators both at rest and during exercise. This point towards a possible role for central neural mechanisms of physical and perceptual fatigue and probably contributes, in part at least, to the pathogenesis of CFS.

CFS patients showed marked exercise intolerance, associated with an exacerbated RPE result that is consistent with previous studies (e.g. Riley *et al.*, 1990). The demonstration of lower values for HR and RER at peak exercise in the CFS patients relative to controls, in combination with an almost maximal value for RPE, is consistent with the involvement of a centrally mediated increase in RPE (rather than O_2 transport) being the limitation to exercise performance (e.g. Gibson *et al.*, 1993). That is, it appears likely that the abnormally high RPE may translate into an inability or unwillingness to effect the power outputs necessary to achieve maximal performance (Inbar *et al.*, 2001), thus precluding the 'classic' criteria for attainment of maximal effort from being fulfilled. Thus the limit of tolerance provides a symptom-limited peak $\dot{V}O_{2\,peak}$ value, rather than a true maximum.

The raised plasma free-[Trp] in CFS patients during exercise and subsequent recovery, in combination with the high baseline levels of free-[Trp] reported by Castell *et al.* (1998) imply increased concentrations of Trp in the brain (Davis and Bailey, 1997) and, potentially, an increase in 5-HT turnover. As we saw in Chapter 3 and 4, the association between increased 5-HT turnover and the subjective feeling of fatigue is one that is widely documented. Increases in brain [5-HT] have been associated with lethargy, sleepiness and mood changes (Young, 1986), all of which have also been linked to increase RPE (Davis and Bailey 1997). It was also demonstrated that elevated levels of plasma free-[Trp] and, consequently, of brain 5-HT are associated with decrements in physical performance (Blomstrand *et al.*, 1989). Free Trp, however, shares the same mechanism for transport into the brain as other LNAAs with consequent competition for entry into the brain. In the present experiment, LNAA levels were lower at Exh, and also in Rec in CFS patients. The resulting increases observed in the ratios of free-[Trp]:[BCAA] and free-

[Trp]:[LNAA] in CFS group strengthens the proposed hypothesis linking fatigue in CFS with an abnormally high brain 5-HT activity (Bakheit *et al.*, 1992).

The dopaminergic system has also been implicated in central fatigue during exercise (Heyes *et al.*, 1985; Bailey *et al.*, 1993b). It is of interest, therefore, that we found significantly lower levels of tyrosine, the rate-limiting dopaminergic precursor, in the CFS group at all time-points, including rest. To our knowledge, this is the first study to demonstrate low DA precursor levels in patients with CFS. Since fatigue during prolonged physical activity has been shown to be associated with increased brain 5-HT and reduced brain DA levels (Bailey *et al.*, 1993a) a high 5-HT:DA ratio should favour reduced exercise performance and indeed, the ratio of the precursors of 5-HT and DA, free-[Trp]:[Tyr], was consistently higher in CFS patients at all time-points when compared to the control group. The increased baseline [free Trp]:[Tyr] ratio may also explain the evident fatigue and associated high RPE, even prior to physical exertion, in this patient population.

It should be noted that although the patient group has shown different plasma amino acid ratios (free-[Trp]:[BCAA], free-[Trp]:[LNAA] and free-[Trp]:[Tyr]) relative to control group none of these ratios seem to change massively with exercise. Consequently, based on these responses someone could argue that this makes it difficult to relate these differences in amino acid ratios to any problem with exercise its self. However, the correlation analysis revealed a significant positive correlation between $\dot{V}O_{2\,peak}$ and the putative central 5-HT modulators, i.e. free-[Trp], free-[Trp]:[BCAA] and free-[Trp]:[LNAA], at exhaustion in the CFS group, but not in the control group. In addition, a significant negative correlation between RPE and [Tyr] was observed at rest and during unloaded exercise and between and $\dot{V}O_{2\,peak}$ and free-[Trp]:[Tyr] ratio at exhaustion in the CFS group only.

At present, the physiological mechanisms underlying the apparent 5-HT over-activity and the concurrent depression of the dopaminergic system in CFS (the latter possibly being a direct result of the inhibitory effect of increased serotonergic activity; Bailey *et al.*, 1993a) cannot be readily explained. The altered levels of circulating amino acids demonstrated in the present study may indicate a dysfunction in amino acid metabolism, either within the CNS or peripherally (e.g., uptake by liver or skeletal muscle). One cannot, therefore, entirely exclude involvement of peripheral mechanisms, which could ultimately affect levels of the

amino acids measured in the present study, in the pathogenesis of CFS. Although plasma concentrations of serotonergic and dopaminergic modulators have been shown to be closely associated with brain 5-HT and DA levels, respectively (Meeusen and De Meirleir, 1995) we did not measure the latter and, thus, can only speculate on a 'true' central component in the disorder. Plasma levels of free-[Trp] have also been shown to be highly dependent on plasma [FFA], FFA having been shown to displace Trp from binding to albumin (Curzon *et al.*, 1973). However, there were no differences in the correlations between free-[Trp] and [FFA], between CFS and control groups. This close association between free-[Trp] and [FFA] in both groups would imply normal Trp-albumin binding properties.

Conclusion

In conclusion, the significant differences between patients with CFS and healthy controls that have been observed with several key CNS serotonergic and dopaminergic modulators, especially during resting conditions, suggest that central neural mechanisms may contribute to the increased RPE and impaired exercise tolerance in CFS. However the precise role of the serotonergic and dopaminergic systems in the pathogenesis of CFS remains to be elucidated.

Chapter seven

General discussion

7. General discussion

The primary objectives of experiments (EXPs) 1-4 were to i) clarify and improve the current understanding of the role of putative brain 5-HT and DA modulators and therefore brain serotonergic and dopaminergic function in exercise fatigue development; ii) elucidate the relationship among these brain 5-HT and DA modulators and function, perceptual, metabolic and cardiovascular responses; and, iii) differentiate between the mechanism(s) responsible for peripheral and central fatigue development in health and exercise-intolerance patients. The general observations of these EXPs are discussed in the present chapter, and where possible, conclusions are drawn regarding the factors that may affect brain 5-HT and DA modulators and indices, perceptual and metabolic responses and exercise capacity.

7.1 Putative central components affecting fatigue in health and disease

No relationship between brain 5-HT modulators, indices, RPE or exercise performance was observed in EXPs 1 and 2 after co-ingestion of caffeine with a high fat meal. Only in well-trained cyclists in the heat, following Cr supplementation (EXP 3), and in CFS patients (EXP 4) was there a relationship between modulators of brain 5-HT and DA function (being lower and higher respectively in trained humans and reverse in CFS), RPE or exercise tolerance.

In EXPs 1 and 2, these results may suggest that elevating circulating plasma [FFA] by a pre-exercise high fat meal have effectively contributed to a similar displacement of Trp from plasma albumin and a parallel adjustment of plasma [amino acid] and substrate oxidation in both F and FC trials. This is supported by the observation that there were no differences between the trials in key brain 5-HT and DA modulators (plasma free and total Trp, LNAA, Tyr) as well as in the rate and total CHO and fat oxidation. However, although a differentiation between putative metabolic and CNS effects of caffeine was achieved following caffeine co-ingestion with a high fat meal, (as RPE was reduced with caffeine despite the elevation in cardiopulmonary and metabolic responses, but there were no differences in substrate utilisation and no improvement in exercise performance), caffeine had no effect on peripheral brain 5-HT and DA modulators or any positive action on incremental and endurance exercise performance. Consequently, brain 5-HT and DA systems are unlikely to play an important role in the exercise fatigue process of

well-trained humans and this may be more evident when exercise is performed without significant thermal stress, thus enabling fatigue development to occur due to cardiovascular and/or peripheral limiting factors (i.e. glycogen depletion). The limit therefore in endurance exercise capacity in low temperatures is most likely accounted for by glycogen depletion since the rate and total substrate oxidation were not different between the trials (EXP 2).

In EXPs 1, 3, and 4, it is unlikely that substrate oxidation limited exercise capacity as muscle glycogen depletion does not limit performance during incremental exercise (EXPs 1, 4) (Coyle *et al.*, 1986) or during prolonged exercise in the heat (EXP 3) (Galloway and Maughan, 1997). It has been shown that the reduction in exercise capacity in the heat is highly associated with elevated core/brain temperature (Nielsen *et al.*, 1993; 1997; Nybo and Nielsen, 2001b; Nybo *et al.*, 2002). In addition, evidence suggests that the reduction in central neural-drive motivation to exercise, during exercise in the heat, is highly associated with progressive increase in hyperthermia-induced alterations in brain activation (Nybo and Nielsen, 2001c), and in particular, with an up-regulation of brain serotonergic function (Bridge *et al.*, 2003; Marvin *et al.*, 1997; Pitsiladis *et al.*, 2002).

In the present EXP 3, plasma free-[Trp] and free-[Trp]:[Tyr] ratio, were significantly lower (even at rest) following Cr supplementation indicating that the subjects in the Cr trial may have had the advantage of feeling more motivated to exercise than the subjects in the Plc trial. These results may suggest that a down-regulation of brain Trp uptake and/or an up-regulation of brain DA function may have contributed to increase central motivation to exercise and enhanced endurance performance in the 'responders' to Cr supplementation during exercise in the heat. It is possible that this brain DA metabolic up-regulation has contributed in reducing thermal-stress (T_{rec}) during exercise in the heat. Clinical studies for example, have demonstrated that two weeks of oral Cr administration was able to increase brain DA synthesis and function in rats (Klivenyi *et al.*, 1999; Mathews *et al.*, 1999). Subsequently, in the same species an increased brain DA synthesis was found to reduce core temperature during passive or active heat exposure (Feldberg and Myers, 1964; Hasegawa *et al.*, 2000; Lin *et al.*, 1998; Sugimoto *et al.*, 2000). However, the exact mechanism(s) of the reduction in thermal-stress during exercise

in the heat following Cr supplementation (through a possible elevation in brain DA function) remains to be determined.

It should be noted that the enhancement in exercise performance observed in the Cr-'responders' group relative to Plc should be treated with caution. Although a positive correlation was found between estimated Cr uptake (Cr group including 'responders' and 'non-responders') and Δ exercise performance (r=0.75, n=11; P=0.008) and power calculation revealed n=9 'responders' for securing significant differences in performance; the weak statistical power of the present EXP 3 should not be ignored (for review see Lemon, 2002).

In EXP 4, plasma baseline [Tyr] was lower and the ratios of free-[Trp]:[Tyr] and free-[Trp]:[LNAA] were higher in the CFS relative to the control group. These results coupled with the higher RPE, even at rest, and with exercise intolerance in CFS group. This indicates that CFS patients had a poor central neural motivation to start exercising and it may explain, in part at least, the lack of engagement in any kind of physical activity characterising CFS patients. Consequently, in line with several other studies (e.g. Cleare *et al.*, 1995; Dimitrack *et al.*, 1992), the putative brain 5-HT modulators results may reflect a metabolic up-regulation of brain 5-HT synthesis in CFS. In addition, the novel observation of the concurrent lower plasma baseline [Tyr] could suggest a down-regulation of brain DA function, which may contribute to exercise intolerance, pathogenesis and maintenance of CFS symptoms.

In the EXPs 1-3, a dissociation between brain 5-HT and DA modulators and plasma Prl was observed. In EXP 2 for example, plasma free-[Trp]:[LNAA] ratio was significantly higher with caffeine and there was a tendency for plasma free-[Trp] (P = 0.064) and free-[Trp]:[Tyr] ratio (P = 0.066) to be higher with caffeine but plasma [Prl] results was close to P = 0.9 between the trials. Similarly, in EXP 3, plasma free-[Trp] and free-[Trp]:[Tyr] ratio were significantly lower with Cr but plasma [Prl] was not different between Cr and Plc trials. In addition, in all EXPs 1-3 there was no correlation between plasma free-[Trp] and plasma [Prl]. The lack of positive correlation however, between modulators and indices of brain 5-HT and DA function is consistent with some previous studies. For example, Biggio *et al.*, (1977) found an increased intestinal [Trp] but a reduced brain 5-HT synthesis. Teff and Young (1988) found a decline in plasma [Trp] (21%) but 5-HT was unaffected.

Similarly, Struder *et al.*, (1995) observed an elevation in plasma free-[Trp] and free-[Trp]:[BCAA] ratio after endurance activity but plasma [Prl] was not affected.

In the present study, it is possible that the dissociation between modulators and indices of brain serotonergic and dopaminergic functions is due to the high fitness levels of the subjects participating in these experiments. It has been suggested, for example, that the HPA axis is both stimulated by muscle exercise and subjected to adaptations with endurance training (Luger *et al.*, 1987). Well-trained endurance athletes may therefore have a metabolic down-regulation of Trp uptake and brain 5-HT synthesis (Jakeman *et al.*, 1994). This probably occurs because they have developed, through the chronic exercise training, the appropriate physiological adaptations to cope from the negative consequences of exercise-induced elevation of 5-HT turnover. Trained rats for example, have delayed to develop onset of central fatigue, associated with elevation in brain 5-HT, during prolonged exercise when compared with untrained rats (Acworth *et al.*, 1986). Consequently, although it is possible that exercise-stress, caffeine and Cr administrations have influenced brain 5-HT and DA modulators and functions, this was not to the extent to significantly affect plasma [Prl] in well-trained humans.

The lack of changes in plasma [Tyr] observed in EXPs 1-3 after caffeine and Cr administration does not necessarily mean that both treatments could not directly affect brain DA metabolism irrespective of their influence on plasma DA precursor or brain 5-HT synthesis. For example, caffeine (e.g. Bridge *et al.*, 2000; Cole *et al.*, 1996; Jacobson *et al.* 2000; Laurent *et al.*, 2000; Motl *et al.*, 2003) and Cr administrations (e.g. Kern *et al.*, 2001; Volek *et al.*, 2001) were found to attenuate effort perception during exercise in thermoneutral or hot environments respectively. It is not unreasonable therefore, to imply a direct elevation in brain DA function with caffeine and Cr treatments. Davis *et al.* (2003), for example, found a reduction in rats brain 5-HT:DA ratio after caffeine administration and Mathews *et al.* (1999) found an enhancement in mice's brain DA metabolism resulting in a neuroprotective effect under specific stress following an oral Cr administration. However, in the present study, this claim is speculative since plasma [Prl] was the only index of hypothalamic 5-HT and DA metabolic interaction (Ben-Jonathan *et al.*, 1989; Freeman *et al.*, 2000).

It is possible that caffeine and Cr reduced subjective fatigue (i.e. in EXPs 1-3) by increasing brain DA metabolism in other brain areas than the hypothalamus without being able to significantly inhibit hypothalamic Prl release. Studies using animal models for example, have demonstrated an up-regulation of brain 5-HT synthesis during exercise not only into the hypothalamus (Bailey *et al.*, 1993a) but also in the midbrain, hippocampus, striatum and frontal cortex (Bailey *et al.*, 1993b; Chaouloff *et al.*, 1989; Gomez-Marino, 2001; Meeusen and De Meirleir, 1995; Meeusen *et al.*, 1996). Consequently, plasma [Prl] in the present EXPs should not be considered to reflect changes of the whole brain 5-HT and DA synthesis. For these reasons, more in vivo studies, using predominantly human volunteers, are warranted to examine a possible direct effect of caffeine and Cr administration on DA metabolism in various brain regions.

The reason however, why only in CFS patients (EXP 4) an association between brain 5-HT and DA precursors (being significantly higher and lower respectively in CFS relative to control group) and an early elevation in subjective and objective fatigue was observed is not easy to explain. CFS patients have been suggested to have a metabolic up-regulation of brain 5-HT synthesis (Behkeit *et al.*, 1992; Cleare *et al.*, 1999). This response may be due to the elevation in plasma Trp uptake by the brain *per se* (Cleare *et al.*, 1995) or in combination with the increased plasma and brain [5-HT] both of which were found to induce BBB permeability and neurotoxicity (Bested *et al.*, 2001). Supporting this contention, several studies observed a positive correlation between plasma/brain [5-HT] and BBB permeability under various stressors such as summer-heat and forced-swimming (Sharma and Dey, 1986; 1987; Sharma *et al.*, 1996).

Nevertheless, a metabolic down-regulation of plasma Tyr uptake by the brain should not be underestimated in the development of an early onset of fatigue and increased effort perception in CFS. Acute tyrosine depletion for example, was found to inhibit brain DA synthesis and function in humans (Montgomery *et al.*, 2003). Subsequently, the reduction in brain DA metabolism, through an acute tyrosine depletion, was found to reduce mood-state, motivation to physical activity, exercise tolerance (Avraham *et al.*, 2001) and motor co-ordination in humans (Meeusen and De Meirleir, 1995). It is possible therefore, that in CFS the pathogenesis of the disease is the concomitant result of both down-regulation of plasma Tyr (which may

induce Tyr deficiency) and up-regulation of brain 5-HT function. As a result, the altered levels of circulating amino acids demonstrated in the EXP 4 may indicate a dysfunction in amino acid metabolism, either within the CNS or peripherally (i.e. uptake by liver or skeletal muscle). Consequently, one cannot entirely exclude the involvement of peripheral mechanisms (which could ultimately affect levels of the amino acids measured in the present study) in the pathogenesis of CFS. More studies are needed to examine the association between circulating 5-HT levels, brain neurotoxicity rate and 'central fatigue' development in CFS.

One of the attempts in EXP 1 was to examine whether anaerobic exercise, reflected by elevation in muscle lactate production during incremental exercise, may stimulate 5-HT synthesis and therefore Prl secretion (De Meirleir *et al.*, 1985a, b; Newsholme and Blomstrand, 1996; Shangold *et al.*, 1981) and if this could be reversed by a positive action of caffeine on brain DA metabolism (Davis *et al.*, 2003). However, blood [lactate] was significantly higher on the FC relative to F trial during constant-load exercise, at exhaustion and recovery after incremental exercise but neither exercise performance nor plasma [Prl] was different between the trials. In addition, similar pattern between blood [lactate] and plasma [Prl] have observed in EXP 2 at exhaustion. In this EXP, at the end of exercise, especially on the FC trial, blood [lactate] indicates anaerobiosis (blood lactate > 4 mMol) but plasma [Prl] was not different between the trials. These results may therefore dispute the hypothesis of a linear relationship between blood [lactate] and brain 5-HT stimulation during exercise. However, these results do not preclude that muscle acidosis may stimulate hypothalamic 5-HT synthesis via an autonomic nervous system information supplied irrespectively from blood [lactate] *per se*. For example, in the studies by De Meirleir *et al.*, (1985a and b) where a positive correlation between blood [lactate] and 5-HT synthesis was observed, the blood [lactate] may represent an absolute muscle lactate production and not an attenuation in blood lactate clearance due to caffeine ingestion for example (see Graham *et al.*, 2000).

As stated in Chapter 3, blood [lactate] does not necessarily indicate muscle lactate production and/or muscle acidosis especially in well-trained subjects implying an attenuation effect of caffeine on blood lactate clearance (e.g. Graham *et al.*, 2000). Consequently, it is possible that anaerobiosis stimulates brain 5-HT synthesis only when an early muscle acidosis is developed irrespective of blood [lactate] and this is

probably more evident in untrained subjects. It has been well established that during high-intensity exercise untrained individuals can develop muscle acidosis earlier relative to well-trained individuals due to the lack of anaerobic enzymatic capability and glycolytic adaptations in type IIA and IIB muscle fibres (Jansson *et al.*, 1990). This, in association with the observation that untrained subjects do not have the training adaptations to down-regulate brain 5-HT synthesis (Acworth *et al.*, 1986; Jackeman, 1996) might have caused the inconsistency between the present study and those by investigators who tested untrained subjects (e.g. De Meirleir *et al.*, 1985a, b; Newsholme and Blomstrand, 1996; Shangold *et al.*, 1981).

7.2 Plasma FFA and displacement of tryptophan from albumin

Release of FFA from adipose tissue appears to be the primary factor underlining the prolonged exercise-induced elevation of plasma free-[Trp]. Trp is liberated from its binding to albumin when the plasma [FFA] rises and competes with Trp for the binding to albumin (Curzon *et al.*, 1973; Spector, 1975). The higher plasma free-[Trp] observed at rest, during exercise or recovery in CFS group relative to control in EXP 4 are unlikely to be associated with the displacement of Trp from albumin by plasma FFA. This is because there were no differences in plasma [FFA] between CFS and Control groups and there was a positive correlation between plasma free-[Trp] and [FFA] in both CFS and Control groups. Similarly, in EXP 2, although significantly higher plasma [FFA] was found, at rest and during exercise following caffeine ingestion, there was only a tendency for plasma free-[Trp] to be higher in the same trial. In EXP 3 also although plasma [FFA] was not different between the pre- and post-Cr supplementation trials, plasma free-[Trp] was lower with Cr.

The reason for this dissociation between plasma [FFA] and [Trp] observed in all EXPs is intriguing. In EXP 2, although a small increase in plasma free-[Trp] was observed with caffeine at the beginning and at the end (increased 11.3% and 15.5% respectively) of exercise relative to Plc counteract time points, the lack of significant differences in free-[Trp] between F and FC trials ($P = 0.064$) may be explained by the pre-exercise high fat meal employed in both trials. This concentration probably of the artificially elevated plasma FFA can occupy the biding site of plasma albumin to its higher threshold binding to FFA attenuating any further displacement of Trp from albumin (Struder *et al.*, 1996).

In the present EXPs 1 and 2, the elevation in plasma [FFA] following the high fat meals was at around 0.6mmol/l at rest, 0.35mmol·l^{-1} during exercise and 0.8mmol/l at the end of exercise. In contrast, Struder *et al.* (1996) observed that circulating plasma [FFA] was ~3.3mmol·l^{-1} following soy oil/heparin administration. Consequently, due to the different diet manipulation protocol between Struder *et al.* (1996) study (which caused this massive elevation in circulating plasma [FFA]) and the present EXPs 1 and 2, the study by Struder *et al.* (1996) cannot be in general comparable with present EXPs. It should be noted that this high amount of the artificially elevated plasma [FFA] (hyperlipemia) is characterised by negative muscle metabolic and cardiovascular physiological responses elevating the risk of sudden-death (Newsholme and Leach, 1991).

The reason however, why plasma free-[Trp] was significantly higher and lower in CFS group (EXP 4) and in Cr group (EXP 3) respectively but plasma [FFA] was not different between the groups may implicate the role of liver function in this dissociation. It is well established that Trp is taken up and metabolised by the liver (Newsholme and Leech, 1983). Consequently, in CFS patients the dissociation between plasma [FFA] and free-[Trp] may suggest an abnormal liver Trp metabolic regulation leading to an inhibition of plasma free-Trp uptake. This may elevate plasma free-[Trp] irrespectively from plasma [FFA]. This possible liver dysfunction concerning Trp metabolism may be the same reason for the lower plasma [Tyr] observed in CFS group. This may lead to a metabolic down-regulation of plasma [Tyr] inducing lower brain Tyr uptake and DA synthesis. Consequently, there is a possibility that one of the pathogenic factors in CFS is due to the liver dysfunction enabling an elevation in plasma free-[Trp] and brain 5-HT up-regulation. On the other hand, it is possible that Cr supplementation contributes to enhancing liver free-Trp uptake and re-conversion of free-Trp to total Trp resulting in lowering plasma free-[Trp] irrespectively from plasma [FFA]. However, this possible liver re-conversion of free-Trp to total Trp was not to the extent to elevate plasma total [Trp] because free-Trp generally approximates to about only 10% of the total Trp levels (Curzon *et al.*, 1973). More controlled studies are needed in order to examine the role of liver on Trp metabolic regulation in CFS and in health after Cr supplementation at rest and during exercise.

7.2.1 One step forward into the role of Trp metabolism in 'central fatigue'

As stated in Chapter 1 (section 1.2.1), after the Trp uptake by the liver, Trp can enter three main metabolic pathways. These include Trp metabolism i) to kynurenine, in periphery and brain (Lapin, 1980; Stone and Connick, 1985; Moroni, 1999), ii) to brain 5-HT (Newsholme *et al.*, 1987) and iii) to tryptamine in the brain, which is further converted to kynurenine (Stone and Darlinghton, 2002). It has been reported that only 1% - 5% of dietary Trp is metabolised to brain 5-HT and more than 95% is converted to kynurenine (Stone, 1993).

One of the significant reactions after the conversion of Trp to kynurenine in the liver is that, kynurenine can be easily transferred to cerebrospinal fluid or directly cross the BBB and distributed in various brain regions by the LNAA-carrier (Fukui *et al.*, 1991; Heyes and Quearry, 1990). For this reason, its levels may be elevated into the brain. It was previously found for example, that around 60% of the brain [kynurenine] has a peripheral origin (Gal and Sherman, 1980). In the brain, after the conversion of Trp to kynurenine, the latter can further be metabolised to kynurenic acid, to quinolinate acid (Stone and Darlinghton, 2002) or to antharnilic acid (Amirkhani *et al.*, 2002). The significance of this kynurenine sub-conversion is that kynurenic acid was found to be neuroprotective but quinolinate acid is highly neurotoxic and may cause mitochondrial dysfunction, increase free-radical generation and produce neuronal damage in vivo (for review see Stone and Darlinghton, 2002). However, what is not known to date is whether kynurenine metabolites, especially quinolinate acid, could affect central fatigue in health and CFS, especially during prolonged exercise. It is possible therefore that kynurenine metabolites play a role in the development of central fatigue during exercise in the heat for example, and in the pathogenesis, exercise intolerance and exacerbated effort perception in CFS patients. More controlled studies are needed to examine the Trp rate conversion to kynurenines in periphery and brain at rest and during exercise at various temperatures in humans and to elucidate the role of kynurenine metabolites in putative central fatigue in health and CFS patients.

7.3 Brain 5-HT and DA modulators and perceptual responses

Several observations linking effort perception with peripheral sensory cues include (apart from sensations arising from the exercising muscles and joints) blood lactate

levels (Ekblom and Goldbarg, 1971; Mihevic, 1981; Noble *et al.*, 1983). Other mediators in the effort perception development include body core and skin temperatures and sensation of thermal-stress (Noble and Robertson, 1996). However, the association between increased 5-HT turnover and the subjective feeling of fatigue is one that is widely documented but less well understood. Increased brain [5-HT] has been associated with lethargy, sleepiness, poor mood (Young, 1986) and increased core temperature (Feldberg and Myers, 1964; Lin *et al.*, 1998; Sugimoto *et al.*, 2000). All these responses have also been linked to reduction in central motivation to exercise and rise in effort perception during exercise in thermoneutral (Davis *et al.*, 1993; Davis and Bailey, 1997) or hot environments (Bridge *et al.*, 2003; Nybo and Nielsen, 2001c; Pitsiladis *et al.*, 2002).

In EXPs 1 and 2, leg muscular discomfort was significantly lower during constant-load exercise following caffeine ingestion but this reduction was not found to be associated with brain 5-HT modulators as previously proposed (e.g. Blomstrand *et al.*, 1997; Davis *et al.*, 1993; Davis and Bailey, 1997). During exercise in the heat however, the reduction in leg muscular discomfort could be attributed to the lower plasma free-[Trp] and free-[Trp]:[Tyr] ratio, observed following Cr supplementation, leading to a reduction in brain 5-HT synthesis and/or to an enhancement in brain DA metabolism. However, the lack of correlation between plasma free-[Trp] and [Prl] and between plasma free-[Trp] and effort perception, does not preclude an alternative mechanism in this effort perception reduction.

The reduction therefore, in effort perception in EXP 3 may be due to the effect of Cr supplementation in reducing thermal-stress and dehydration rate (since both ΔT_{rec} and sweat rate were lower during exercise with Cr than without). This contention is consistent with some previous reports (e.g. Kern *et al.*, 2001; Noble and Robertson, 1996). Another possible explanation for this reduction is the attenuation in muscle contraction-stress with Cr, which may prevent an early ATP depletion (Guerrero and Willimann, 1998). Although this prevention was not to such an extent to enhance endurance performance in the heat in the whole Cr group, it was effective to increase performance in the estimated 'responders' to Cr supplementation. Recently, for example, Terblanche and Nel (1998), using animal models, examined the effect of heat, submaximal exercise (10 min running x 3 in 30°C with 2 min recovery between exercise) and acclimation programme on the activity of creatine kinase

(CK) in several tissues including kidneys, skeletal and heart muscles. They found that prolonged exercise in the heat has significantly reduced the activity of CK in all these tissues concluding that exercise in the heat may produce tissue damage. In the present EXP 3, it is possible that Cr has enhanced brain DA function (Klivenyi *et al.*, 1999; Mathews *et al.*, 1999) that contributed to reducing thermal-stress (e.g. Hasagewa *et al.*, 2000), and therefore attenuating the negative effect of heat-stress on CK activation on skeletal muscle. This may have contributed to the reduction in effort perception. However, more controlled human studies are needed in order to examine this particular speculation.

It is difficult however, to explain why the subjects in the EXPs 1 and 2 perceived it easier to exercise with caffeine than without. Particularly when one considers the accompanying elevation in key blood metabolites and cardiovascular responses (see Chapters 3 and 4) that typically would be expected to augment, rather than attenuate the response (Borg, 1982; Noble *et al.*, 1983). Although, as stated in chapter 3, the reduction in effort perception may be due to the effect of caffeine on enhancing the secretion of endorphins (e.g. Laurent *et al.*, 2000), which is well known to reduce pain perception and promote euphoria (e.g. Harber and Sutton, 1984) alternative mechanisms should not be precluded. Caffeine has the potential to easily cross the BBB and serves as a central competitive antagonist of adenosine (e.g. Fredholm et al., 1999). The net effect could then be to increase brain DA synthesis by antagonising the inhibition of adenosine on DA activity (Davis et al., 2003) (For more details see Chapter 1, section 1.2.6.3). However, the similar plasma modulators and indices of brain 5-HT and DA functions observed between the trials in EXPs 1 and 2 in association with the lack of performance improvement but lower effort perception found in both EXPs are in contrast to the hypothesis that physiological caffeine doses attenuates brain 5-HT synthesis and enhance exercise performance in well-trained humans. It is therefore possible that caffeine may significantly reduce brain 5-HT:DA ratio only when is directly administrated in small animals' brain (Lim *et al.*, 2001; Davis *et al.*, 2003); or circulating [Prl] may not be as sensitive a marker of 5-HT and DA metabolic interaction as previously proposed (e.g. Ben-Jonathan *et al.*, 1989; De Meirleir *et al.*, 1985a; b).

Alternatively, the inconsistency between the present EXPs 1 and 2 and some other animal studies on caffeine (e.g. Davis *et al.*, 2003; Lim *et al.*, 2001) may be due to

different mechanistic action of caffeine between animals and humans (see Chapter 1, section 1.2.6.2). In animals for example, caffeine administration leads to high CNS [theophylline] and in humans [paraxanthine] (Fredholm *et al.*, 1999). Theophylline is a more potent inhibitor factor of brain adenosine receptors than caffeine *itself* or paraxanthine (Benowitz *et al.*, 1995). Consequently, this could cause a discrepancy between studies that used animals or humans to examine the effect of caffeine on brain 5-HT:DA ratio and/or exercise performance. In addition, it was reported that caffeine half-life, for doses lower than $10mg \cdot kg^{-1}$, range from 0.7 to 1.2 hours in rat and mouse, 3 to 5 hours in monkey and 2.5 to 4.5 hours in humans (Fredholm *et al.*, 1999). For these reasons, more control studies are required, utilising the same exercise protocol and same amount of caffeine ingestion, to determine possible different actions of caffeine in humans and animals particularly during exercise.

Only in CFS patients was effort perception significantly higher at exhaustion relative to sedentary control subjects (EXP 4). In the EXPs 1-3, although effort perception was significantly lower during prolonged exercise there were no differences between the trials and groups in perceptual and cardiovascular responses at exhaustion. These results may imply a central neural component being responsible for the inability and/or unwillingness of CFS patients to achieve peak performance. Conversely, in EXPs 1-3 the effort perception results at exhaustion may suggest a similar central neural components being responsible for effort perception development in all trials irrespectively from caffeine and Cr administration. These may indicate that both caffeine and Cr administrations can effectively increase central motivation to exercise in thermoneutral, mild-cold and hot environments before the fatigue point is reached. However, when the metabolic and cardiovascular systems are stressed in the heat or in the cold neither caffeine nor Cr contributes in attenuating peak subjective fatigue.

7.4 General conclusions

From this series of EXPs a number of conclusions could be drawn. These conclusions apply specifically to protocols where constant-load or incremental exercise is performed to volitional fatigue in well-trained individuals and CFS patients. It is unknown to what extent the results obtained from these experiments

and conclusions drawn could successfully be applied to different exercise protocols, to untrained or less-fit subjects and to different kind of exercise-intolerance patients other than CFS.

For the present experiments it may be concluded:

i) a dissociation between effort perception, putative modulators of brain 5-HT function, and exercise fatigue development in well-trained humans during exercise (EXPs 1-2).

ii) a lack of implication of brain 5-HT and DA modulators and functions in exercise fatigue process (EXPs 1-2), especially in a low temperature where the primary cause of fatigue during prolonged exercise is due to glycogen depletion (EXP 2).

iii) a lack of caffeine effectiveness in enhancing incremental and endurance exercise performance following an acute pre-exercise muscle metabolic adjustment with a high fat meal (EXPs 1-2).

iv) a dissociation between metabolic responses and effort perception development during constant-load exercise in the heat, thermoneutral and low temperatures in well-trained humans (EXPs 1-3) and during grated exercise in CFS patients (EXP 4).

v) an association between increased effort perception and exercise intolerance and increased effort perception and metabolic up- and down-regulation of brain 5-HT and DA modulator respectively in well-trained humans during exercise in the heat (EXP 3) and in CFS patients at rest and during grated exercise to volitional fatigue (EXP 4).

Overall, the results obtained from all four EXPs may suggest that neither brain 5-HT nor DA systems are implicated in the fatigue process when exercise is performed without significant thermoregulatory stress thus enabling fatigue development during exercise to occur due to peripheral factors (i.e. cardiovascular factors and glycogen depletion). Consequently, the increased central drive motivation to exercise (reduction in effort perception) *per se* is not capable to contribute in enhancing exercise performance of well-trained humans in relatively thermoneutral temperatures. The exact mechanism therefore for the attenuation of effort perception with caffeine is unlikely to be brain 5-HT-mediated but a DA-mediated effect

should not be precluded. However, the effectiveness of Cr supplementation to reduce brain 5-HT modulators and/or directly elevate brain DA function may have contributed to ameliorate thermal-stress, reduce RPE and enhance endurance performance in the 'responders' to Cr supplementation during exercise in the heat. Similarly, a metabolic up- and down-regulation of brain 5-HT and DA functions respectively may be involved in the early elevation in RPE and in onset of exercise intolerance in CFS and these may be important aspects even for the pathogenesis and maintenance of the disease. The present study was the first to demonstrate significantly lower baseline DA precursor levels in patients with CFS when compared with sedentary control.

7.5 Direction for future research

A. The EXP 4, was the first that demonstrated abnormalities in precursors and peripheral modulators of brain 5-HT and DA functions, even in resting conditions. These results implicate the involvement of central neural mechanisms in the increased effort perception and impaired exercise tolerance in CFS, and warrant future intervention strategies aimed at correcting these abnormalities. Tyr depletion for example, was found to attenuate DA function in health (Harmer *et al.*, 2001). It is possible that Tyr supplementation contributes in enhancing central motivation to exercise and exercise tolerance and progressively attenuating the acute CFS symptoms. However, no studies to date have examined this particular hypothesis.

B. One of the metabolic pathways of Trp in the liver and brain leads to conversion of kynurenine, which is further converted mainly to kynurenic acid and quinolinate acid. What is not known to date is whether kynurenine metabolites, especially quinolinate acid, which is highly neurotoxic (causing mitochondrial dysfunction, increase free-radical generation and produce neuronal damage in vivo), could contribute in central fatigue process in health and CFS either at rest or during prolonged exercise in the heat. In addition, whether those kynurenine metabolites play a role in the pathogenesis, exercise intolerance and exacerbated RPE in CFS. More controlled studies are required to examine the rate of Trp conversion to kynurenines in periphery and brain at rest and during exercise in various

temperatures in humans and to elucidate a possible role of kynurenine metabolites in central fatigue in health and CFS patients.

C. Further research is required to examine the effect of Cr supplementation on putative modulators of brain 5-HT and DA systems at rest but especially during prolonged exercise to fatigue in the heat.

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APPENDIX A

Medical History Questionnaire

CENTRE FOR EXERCISE SCIENCE AND MEDICINE

MEDICAL HISTORY

(CONFIDENTIAL)

Please read.

It is important to take a record of your medical history. You may have, or may have once had a condition that would make this type of testing unsuitable for you. For this reason we ask you to be as truthful and detailed as possible. At no point will this information be made available to any one other than the principal investigators for this study. If you have any doubts or questions, please ask.

SUBJECT DETAILS:

NAME:

AGE: D.O.B:

SEX (M/F):

GP NAME & ADDRESS:

SMOKING:

Never Smoked .....
Not for >6 months .....
Smoke <10 per day .....
Smoke >10 per day .....

ILLNESSES:

ALLERGIES:

HOSPITALISATIONS:

MUSCULO-SKELETAL DISORDER:
(Arthritis, Joint Pain, Fractures, Sports injury, Others)

CARDIOVASCULAR DISORDER: (Fever, Heart Murmurs, Chest Pain, Palpitations, High Blood Pressure, Others)

RESPIRATORY DISORDER: (Asthma, SOB, Cough, URTI, Others)

GASTROINTESTINAL DISORDER: (Jaundice, Bleeding, Others)

DIABETES:

CNS DISORDER: (Fits, Blackouts, Tremor, Paralysis, Epilepsy, Other)

PSYCHIATRIC TREATMENT:

FAMILY HISTORY: (Sudden death in a first degree relative under the age of 35 years)

ARE YOU CURRENTLY TAKING ANY MEDICATION? No / Yes*

(*Please specify)_____

ARE YOU CURRENTLY TAKING ANY SUBSTANCES TO HELP IMPROVE YOUR TRAINING OR CONTROL YOUR WEIGHT i.e. CREATINE, PROTEIN SUPPLEMENT? No / Yes*

(*Please specify) _____

ARE YOU CURRENTLY TAKING ANY OTHER SUPPLEMENTS i.e. FOOD SUPPLEMENTS, VITAMINS? No / Yes*

(*Please specify) _____

CAN YOU THINK OF ANY OTHER REASON WHY YOU SHOULD NOT TAKE PART IN ANY OF OUR TESTS?

_____

SYMPTOMS:

Do you experience any of the following, particularly on exercise?

Breathlessness	No / Yes
Chest Pain	No / Yes
Dizzy Fits/Fainting	No / Yes
Palpitations	No / Yes

Please note that if you feel unwell on the day of the proposed test, or have been feeling poorly over the preceding day or two, please inform the investigators and DO NOT TAKE PART in the exercise test.

DECLARATION:

I have completed this questionnaire fully and truthfully. I have not kept any information from the investigators that may put myself at risk during high-intensity exercise, or affect the results that they obtain. I understand that I may withdraw from any one test or the study as a whole if I feel unwell, or feel uncomfortable with any part of the testing procedure.

(Signature).....

(Date) .....

PHYSICAL EXAM:

WEIGHT: _____ HEIGHT: _____

PULSE (Resting): _____ BP (Resting): _____

Screened by: .....

(Signature) ..... (Date) .....

APPENDIX B

Consent Form

UNIVERSITY of GLASGOW

CONSENT FORM

TITLE OF PROPOSED STUDY

Name of volunteer: .....

Principle Investigator: **Dr. Y. P. Pitsiladis**

I have read the volunteer information form on the above-mentioned study and I have had the opportunity to discuss the details and ask relevant questions. The nature and the purpose of the experimental tests to be undertaken have been explained to me. I understand fully what is proposed to be undertaken.

I have agreed to take part in the study as it has been outlined to me, but I understand that I am completely free to withdraw from the study at any time I wish.

I understand that these practical trials are part of a research project design to promote scientific and medical knowledge, which has been approved by the Joint Ethical Committee, and may be of no benefit to me personally.

I hereby fully and freely consent to take part in the study, which has been explained to me.

Signature of volunteer .....

Date .....

I confirm that I have fully explained to the volunteer named above, the nature and purpose of the experimental tests to be undertaken.

Signature of investigator .....

Date .....

APPENDIX C

HPLC buffers, mixed-reagent and calibration standard preparation

Buffer A- 0.04 NaAc + 0.25% THF (1L)

0.03M NaAc 2.46g 1L

pH ~7.5 – adjust to 7.2 with dilute Acetic Acid, add 2.5ml THF

Buffer B – 200ml 0.1 NaAc + 800ml Acetonitrile

0.1M 1.64g NaAc in 200ml H_2O_d

Calibration Standard 0.25mM:

5mM L-Tryptophan	50µl
2.5mM Sigma	100µl
2.5mM internal standard	100µl (0.0045g L-Methionine Sulfine in 10ml)
0.1mM HCl	750µl

Reagent C – 500mg OPA in 10ml methanol

Increase volume to 100ml with borate buffer

Add 500 µl of 2-mercaptoethanol (MCE).

Running samples composition:

80µl plasma + 20µl (1.375) / 0.25 ISl + 10µl PCA (3.3mM)

Calibration Std 0.005mM:

0.25 χ Dilution factor = 0.005

Dilution factor = 0.02 i.e., 50 times dilution

APPENDIX D1

Glucose Method

The following is an enzymatic method for the measurement of blood glucose on the Cobas Mira (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK).

Reagent:

Add contents of 1 bottle of Glucose PAP (unimate 7) to 100mls of distilled water

Standard:

5.56 mmol/L standard (Sigma diagnostics)

Use Standard neat

APPENDIX D2

Lactate Method

The following is an enzymatic method for the measurement of blood lactate on the Cobas Mira (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK).

Reagents:

Hydrozine buffer: 5g Hydrozine hydrate + 0.2g EDTA disodium (Diaminoethanetetra-acetic acid disodium salt) + 1.3g Hydrozine sulphate. Make up to 85ml using distilled water. Check pH is 9 (using HCL to ↓pH and KOH to ↑pH). Top up to 100ml with distilled water.

NB: Calibration of pH meter. Use pH 7 to set offset. Rinse with distilled water. Use pH 4 to set slope. Rinse with distilled water.

Lactate reagent: 10ml Hydrozine buffer + 0.02g NAD + 150µl Lactate dehydrogenase (mix well).

Standards:

Lactate standard 3mmol: 9,970µl distilled water + 30µl L-Lactat(e) standard 1mol (Boehringer Mannheim).

APPENDIX D3

Pyruvate Method

The following is an enzymatic method for the measurement of blood pyruvate on the Cobas Mira (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK).

Reagents:

Main Reagent solution: Trizma base solution (Sigma)

Start Reagent solution 1: 2mmol/L NADH (Roche). 14mg (0.014g) of NADH dissolved in 10ml of distilled water.

Start Reagent solution 2: Lactate dehydrogenase (Boehringer Mannheim)

Both main reagent and start reagent 2 are used neat from the bottle.

Standards: Pyruvate acid standard (450umol/L)

Use standard neat

APPENDIX D4

Glycerol Method

The following is an enzymatic method for the measurement of plasma glycerol on the Cobas Mira (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK).

Reagents:

Reagent 1 - 2-amino-2-methylpropan-1-ol Buffer (0.1mol/L, pH 9.9)

891.4mg (0.8914g) 2-amino-2-methylpropan-1-ol 37.2mg (0.0372g) EDTA Disodium salt

1mL hydrazine hydrate

Dissolve in approx 90mL distilled was, adjust pH to 9.9 with HCl and dilute to 100mL.

Reagent 2 – Phosphate Buffer 0.1mol/l pH 7.4

0.1778g potassium orthophosphate

0.7628g di-sodium hydrogen orthophosphate

Dissolve in 100ml distilled water and check pH is 7.4

Co-enzyme – Dissolve 25mg (0.025g) NAD in 1ml phosphate buffer (reagent 2)

Enzyme – Use Glycerol Dehydrogenase (GDH) from Boehringer (Cat No. 258555)

Working Enzyme Reagent:

Add 150µl of NAD solution (co-enzyme) and 30µl of GDH (enzyme) to 8.1 ml of amino methyl propanol buffer (reagent 1)

Standards:

2.29 mmol/L (1.14mmol/L) (Precimat Glycerol, Roche)

APPENDIX D5

Free Fatty Acid Method

The following is an enzymatic method (colorimetric method, Roche Diagnostics GmbH, Germany) for the measurement of FFA on the Cobas Mira (ABX Mira Plus Spectrophotometer; ABX Diagnostics, UK).

Reagent:

Using Free fatty acid, Half-micro test (Boehringer Mannheim). Prepare reaction mixture A (*Main reagent*) -dissolve one tablet of bottle 2 in bottle 1 (NB. Use forceps to take tablets out of bottle 2).

Start reagent:

Using Free fatty acid, Half-micro test (Boehringer Mannheim). Prepare reaction mixture B (*Sr1*)- dissolve one tablet of bottle 5 in bottle 4 (NB. Use forceps to take tablets out of bottle 5). Mix reaction mixture B with an equal volume from bottle 3.

Palmitic acid standard

1.5mmol: 0.0384g Palmitic Acid (Sigma) / 100ml = 1.5mmol. Transfer Palmitic acid into 100ml volumetric flask and dissolve in 25ml warm ethanol (exact volume not crucial, make sure stopper is in place on flask to prevent evaporation). Make volume up to 100ml with Triton X solution, stirring throughout. Continue to stir for a further 30mins.

Triton X solution: Warm 80ml distilled water to approx. 30°C - 40°C (microwave 30s). Pipette in 7.5ml Triton X (NB. Triton X is difficult to pipette i.e. very thick, pipette slowly accuracy is not crucial). Leave to cool to room temperature then top up to 100ml mark.

Ethanol: Warm 25ml of ethanol. Seal in a beaker (Parafilm) and leave for 5 minutes in a basin of warm water.