FACTORS INFLUENCING MUSCLE PURINE NUCLEOTIDE METABOLISM.

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Abstract

The experiments in this thesis were designed to investigate the factors influencing the metabolism of purine nucleotides in human skeletal muscle, plasma and urine. Study one investigated the influence of the number of intermittent sprint bouts and the subsequent accumulation of plasma purines and urinary purine excretion. Study two investigated the influence of sprint training on urinary purine loss following intense exercise. Study three examined the influence of allopurinol on urinary purine loss after repeated sprint exercise in humans. The final study examined the combined effects of allopurinol and sprint training on purine nucleotide metabolism in humans at rest.

The loss of ATP observed in resting recovered muscle following sprint training is resultant of the intense metabolic stress imposed upon the working muscles and the slow rate of ATP restoration by recovery processes. The duration of repeated sprints during training sessions and the recovery time between sprint bouts on the plasma purine concentration have been manipulated and investigated. This study investigated the influence of the number of sprint bouts on purine loss in nine untrained male volunteers who performed one (B1), four (B4) or eight (B8) 10s sprint bouts separated by 50s of passive rest on a cycle ergometer, one week apart, in a randomised order. Plasma inosine, hypoxanthine and uric acid concentration were measured at rest and for 2 hr following exercise. Urinary excretion of these metabolites was also measured before, and for 24 hours of recovery following exercise.

Plasma inosine and Hx concentrations were progressively higher during recovery with an increasing number of sprint bouts. Plasma uric acid was higher following B8 compared with B1 and B4. The excretion of total purines (inosine + Hx +Uric acid) measured after 2 hours recovery following exercise was higher after B8 compared

with the other trials. Put together these results indicate that the loss of purine from the body was enhanced by increasing the number of successive intermittent 10s sprint bouts performed.

The second study re-examined the influence of sprint training with a focus on investigating purine excretion following an intense sprint bout. The study followed the fate of the endogenous purine metabolites produced following exercise, and the influence of sprint training, downstream of their accumulation in the plasma, via their loss in the urine. The loss of purines via urinary excretion following exercise has not been examined extensively. Thus, the influence of sprint training on endogenous urinary purine loss was examined in seven active, non sprint-trained, male subjects to investigate the role of an increased metabolic stress on purine excretion. Each subject performed a 30s sprint performance test (PT), before and after 7 days of sprint training. Training consisted of 15 x 10s sprints on an air-braked cycle ergometer, twice daily with at least 6 hours between sessions. Although there was no change in measured performance variables after sprint training there were some metabolic adaptations. Sprint training resulted in a 20% higher post-exercise muscle ATP content, a lower IMP (57% and 89%, post-exercise and 10 min recovery, respectively), and inosine accumulation (53% and 56%, post-exercise and 10 min recovery, respectively). Sprint training also attenuated the exercise-induced increases in plasma inosine, Hx and uric acid during the first 120 min of recovery and reduced the total urinary excretion of purines (inosine + Hx + uric acid) in the 24 hours recovery following intense exercise. These results show that intermittent sprint training reduces the total urinary purine excretion after a 30s sprint bout.

The third study introduced the inhibitory effect of allopurinol on xanthine oxidase on the dynamics of purine metabolite concentrations in the plasma and subsequent renal

handling and excretion of constituent endogenous purine degradation products. By utilising an intense exercise bout to vastly increased purine load for renal handling we were able to investigate the fate of endogenous purines during recovery. The influence of allopurinol on exercise-induced urinary purine loss was examined in seven active male subjects. These subjects performed, in random order, a trial with 5 days of prior ingestion of a placebo or allopurinol. Each trial consisted of 8 x 10 s sprints on an air-braked cycle ergometer, with a rest period of 50s separated between each repeated sprint. The two trials were separated by at least one week. Plasma Hx was elevated during recovery in the 120 min following exercise in the allopurinol, compared with placebo trial. Urinary Hx and xanthine excretion rates, were also higher ($P<0.05$) with allopurinol compared with the placebo trial following 24 hours recovery. In contrast, plasma uric acid concentration and urinary uric acid excretion rates during recovery were lower ($P<0.05$) with allopurinol. The urinary excretion of purines (inosine + Hx + xanthine + uric acid), taking into account the basal excretion rates, was higher in the allopurinol trial compared with placebo. These results indicate that the total urinary purine excretion after intermittent sprint exercise was enhanced with allopurinol treatment. Furthermore, the composition of urinary purines were markedly affected by this drug.

The fourth and final study was designed to further investigate the possibility of improved purine salvage with allopurinol. Intense intermittent sprint training can decrease resting ATP. Indirect evidence exists that the anti-gout drug allopurinol, increases cellular purine salvage. The potential, therefore exists which suggests that allopurinol may lead to a better maintenance of resting muscle ATP content with sprint training. To examine this possibility, twelve subjects trained using the aforementioned 7-day sprint training protocol. Resting muscle, blood and urine

samples were collected before, and 24-36 hours after, sprint training. Following the pre-training data collection, subjects were separated in double blind fashion into allopurinol or placebo groups (300 mg allopurinol/day (n=5) or calcium carbonate (n=7), respectively). The treatments were administered at least three days prior to, during, and until sampling following training. A training-induced decrease in resting muscle ATP was observed (main effect for training, $p<0.05$), although no difference in resting muscle ATP or AdN content was observed between the treatment groups before or after training. Consistent with the pharmacological effect of allopurinol, resting plasma uric acid concentration was lower relative to placebo with sprint training. There was no difference in resting plasma concentrations of inosine and hypoxanthine between the treatment groups. Furthermore, no difference was observed in basal urinary excretion of inosine, hypoxanthine and uric acid with allopurinol and sprint training. Sprint training reduced the resting ATP content of skeletal muscle, however, no differences were observed with allopurinol administration.

Declaration

I Christos George Stathis, declare that the thesis entitled Factors Influencing Muscle Purine Nucleotide Metabolism is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes.. This dissertation summarises original, previously unpublished work conducted at Victoria University in the School of Biomedical Sciences. With the exception of data collection, which required collaboration, and invasive procedures that were conducted by qualified medical personnel, this dissertation is the result of work performed solely by the author.

Christos G. Stathis

Preface

Part of the results in this thesis have been published or submitted for publication.

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Stathis C.G., M.F. Carey and R.J. Snow. The influence of allopurinol on urinary purinr loss after repeated sprint exercise in man. Metabolism 54: 1269-75, 2005.

Stathis C.G., M.F. Carey, A. Hayes and R.J. Snow. Sprint training reduces urinary purines following intense exercise in humans. Appl. Phys. Nutr. Metabol. In Press.

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Abbreviations

ADP	Adenosine 5'-diphosphate
fADP	free ADP
AdN	Total adenine nucleotides (ΣATP+ADP+AMP)
AdSL	Adenylosuccinate lyase
AdSS	Adenylosuccinate synthetase
AICAR	aminoimidazolecarboxamide ribotide
AMP	Adenosine 5'-monophosphate
AMPd	AMP deaminase
AMPD1	AMPd isozyme 1
AMPD2	AMPd isozyme 2
AMPD3	AMPd isozyme 3
fAMP	Free AMP
ATP	Adenosine 5'-triphosphate
Ca^{2+}	Calcium ions
C-N-I	Soluble cytosolic 5-NT isozyme I
C-N-II	Soluble cytosolic 5-NT isozyme II
CNT	Concentrative nucleoside transporter
Cr	Creatine
EC	Energy Charge
ENT	Equilibrative nucleoside transporter
FI	Fatigue index
FT	Fast Twitch
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate

GTP	Guanosine 5’-triphosphate
H^+	Hydrogen ions
hENT	human equilibrative transporters
HGPRT	Hypoxanthine guanosine phosphoribosyltransferase
HPLC	High performance liquid chromatography
Hx	Hypoxanthine
IMP	Inosine 5’-monophosphate
$KHCO_3$	Potassium bicarbonate
Mg2+	Magnesium ions
Mn2+	Manganese ions
NAD	Nicotinamide adenine dinucleotide
5-NT	5’-nucleotidase
PCr	Phosphocreatine
Pi	Inorganic phosphate
PNC	Purine nucleotide cycle
PNP	Purine nucleotide phosphorylase
PrN	Purine nucleotides
PRPP	5-phosphoribosyl-1-pyrophosphate
PT	Performance Test
SAICAR	succinylaminoimidazolecarboxamide ribotide
ST	Slow Twitch
TCA	Tricarboxylic acid cycle
VO_2max	Maximal oxygen consumption
VO_2 peak	Peak oxygen consumption
Vt	Ventilatory threshold

Chapter 1

Introduction

The primary function of skeletal muscle is to contract and the biochemical processes within the muscle are tailored to support this function and as a central part to this are the ATP hydrolysis and resynthesis pathways. The purine nucleotide pool (PrN) is defined as the sum of adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP) and inosine. At rest, ATP constitutes most (>90%) of the PrN pool and the maintenance of the ATP levels is an important link for the effective transfer of energy from nutrients to fuel cellular processes that supply the functions of cells.

During periods of muscular metabolic stress the central molecule of the metabolic energy system, ATP is rapidly de-phosphorylated to ADP and inorganic phosphate (Pi) with the release of energy fuelling the contraction. Re-phosphorylation of ADP occurs via aerobic (i.e. oxidative phosphorylation) and anaerobic (i.e. phosphocreatine (PCr) degradation, glycolysis) processes and restores ATP. Muscle PCr concentration can regenerate ATP for a few seconds at maximal ATP turnover rates (Gaitanos et al. 1993), whilst regeneration of ATP from glycolysis occurs at an inferior rate but plays an increasingly greater role in ATP resynthesis as maximal exercise continues (Spriet et al. 1995). Consequently, the rate of ATP dephosphorylation exceeds that of re-phosphorylation and result is an increase in free ADP (fADP), which is degraded via the myokinase and ADP deaminase pathways, to IMP. This has a short-term benefit by effectively maintaining a high intramuscular ATP/ADP ratio, however, IMP can be further degraded to purine bases inosine and hypoxanthine (Hx). During recovery these purines can, either be salvaged

intramuscularly and resynthesised to ATP, or efflux the muscle into the plasma and be oxidised to uric acid. Once in the plasma these endogenous purine metabolites (inosine, Hx and uric acid) are excreted and represent a loss of PrN from the muscle. Replacement of these purines must occur via the metabolically expensive process of *de novo* purine synthesis.

Sprint training involves repeated intermittent bouts of intense exercise and induces a large stress on skeletal muscle PrN metabolism and several studies have demonstrated a reduction in the resting PrN pool post sprint training. The nature of intermittent sprint training sessions ultimately enhances the degradation of ATP and compounds the net loss of purines from the muscle. The greater magnitude of purine loss relative to the aggregate purine salvage or de novo synthesis capacities is considered to be the cause of the lower ATP/PrN content of resting muscle after training.

Studies have demonstrated that an increased duration of intermittent sprint exercise and reduced recovery duration between intermittent sprints increases plasma purine concentration. No study has investigated the influence of the number of consecutive sprint bouts on plasma purine concentration and urinary excretion. Furthemore, sprint training reduces the plasma purine accumulation following a sprint trial. However, the extent of endogenous purine excretion (indicating the extent of loss from the muscle) during recovery after a trial in the post-trained state has not been investigated.

Additionally, allopurinol, a xanthine oxidase inhibitor, increased purine salvage in resting individuals (Edwards et al. 1981). An increase in purine salvage during sprint training could ameliorate the drop of ATP observed during a sprint training

regime. Few studies have investigated the influence of allopurinol ingestion on Muscle PrN content following intense exercise or during sprint training.

This dissertation will investigate human purine metabolism with a focus on skeletal muscle purine nucleotide metabolism and subsequent metabolism in the plasma following intense exercise. Specifically, this thesis will examine, 1) the effect of the number of consecutive sprint bouts, 2) the influence of allopurinol on the metabolism of purines with repeated sprint bouts and, 3) allopurinol combined with the repetitive stress of a week of intense sprint training on the accumulation of purine metabolites in plasma and excretion in urine.

Chapter 2

Review of Literature

2.1 Introduction

This review will begin with a brief overview of the intramuscular biochemical pathways of PrN degradation and synthesis and will be extended to the changes in metabolites that occur during, and in recovery from, intense exercise. Furthermore, the accumulation of purine metabolites in the plasma and their subsequent excretion during recovery will be discussed. Finally a summary of current knowledge on the influence of allopurinol and sprint training on purine metabolism will be presented. Unless specifically mentioned this review will describe human studies.

2.2 Brief Overview of the Adenine Nucleotide Metabolic Pathways

Intense exercise can produce a transient decrease in muscle ATP and a concomitant increase in IMP. This is due to a high turnover of ATP where the rate of muscle ATP hydrolysis is greater than the rate of ATP resynthesis. As a result there is an increase in fADP that drives the myokinase and AMP deaminase (AMPd) reactions and reduces the AdN (ΣATP + ADP +AMP) pool (Fig. 2.1; Hellsten et al. 1999; Stathis et al. 1994; Tullson and Terjung, 1991). The myokinase reaction transfers a phosphate moiety from one ADP molecule to another, producing a molecule of ATP and AMP (Fig 2.1). The AMP is subsequently deaminated by AMPd to produce IMP and ammonia (Fig 2.1; Lowenstein 1972). During intense exercise the increase in IMP is equimolar to the decrease in ATP in the muscle (Graham et al. 1990; Hellsten et al. 1999; Jansson et al. 1987; Katz et al. 1986a; Sahlin et al. 1978; Stathis et al. 1994). This highlights that AMP deamination is the dominant pathway in AMP catabolism in

skeletal muscle. The alternative pathway of AMP degradation, AMP dephosphorylation by 5'-nucleotidase (5'-NT), which produces adenosine and orthophosphate (Fig. 2.1; Meghji et al. 1988; Norman et al. 2001; Rubio et al. 1973; Truong et al. 1988), is relatively inactive in skeletal muscle (Norman et al. 2001; Sabina et al. 1984; Schopf et al. 1986; Tullson and Terjung, 1990; Zoref-Shani. et al. 1987). This is further highlighted by individuals deficient in AMPd who exhibit a 20 fold increase in adenosine accumulation (Norman et al. 2001). However, the extent of adenosine accumulation is still well below the magnitude of IMP production in individuals not AMPd deficient.

The high level of activity of the myokinase and AMPd enzymes ensure that changes in ADP and AMP concentrations are small and transient, effectively maintaining a high ATP/ADP ratio in the muscle under stress (Hellsten et al. 1999; Lowenstein, 1990; Tullson and Terjung, 1990). Initial studies proposed that maintenance of low ADP and AMP concentrations was important for muscle function in the maintenance of the energy charge (EC=(ATP+0.5ADP)/(ATP+ADP+AMP)) or a high energy potential (ATP/ADP) during intense exercise (Atkinson et al. 1968; Cooke and Pate, 1985; Dawson et al. 1980; Lowenstein, 1972; Sahlin and Katz, 1989). However, in several studies with asymptomatic homozygous AMPd deficient individuals, performance or muscle functional impairment was not evident with intense exercise (Norman et al. 2001; De Ruiter et al. 2000; Tarnopolsky et al. 2001) suggesting that AMPd in not important in maintaining contractile function. Interestingly, unlike the healthy controls, no reduction in muscle ATP or increase in IMP content was evident following a 30s maximal sprint exercise in AMP deficient subjects (Norman et al. 2001). Furthermore, AMPd deficient individuals exhibited no difference in EC, PCr hydrolysis, or tri carboxylic acid (TCA) cycle anaplerosis following intense

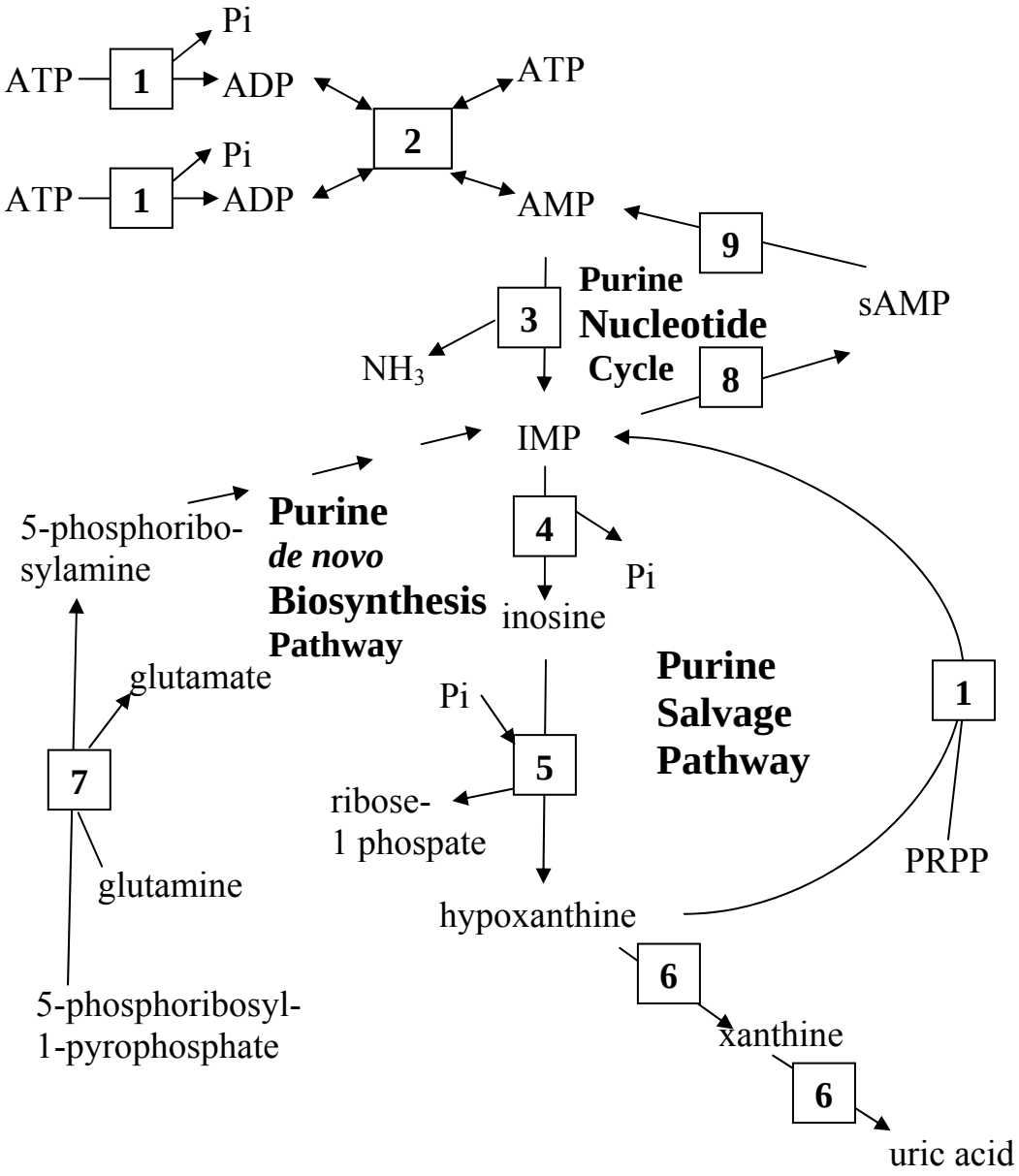


Figure 2.1; Major pathways of purine nucleotide synthesis and degradation in human skeletal muscle. 1. ATPase, 2. Adenylate kinase, 3. AMP deaminase, 4. 5'-nucleotidase, 6. Purine nucleoside phosphorylase, 7. 5-phosphoribosyl-1-pyrophosphate (PRPP) aminotransferase, 8.Adenylosuccinate synthetase, 9. Adenylosuccinate lyase, 10. Hypoxanthine/guanine 5-phosphoribosyl-1-pyrophosphate transferase.

exercise to fatigue relative to healthy controls (Tarnopolsky et al. 2001).

The IMP that accumulates in skeletal muscle can either be reaminated to AMP via the purine nucleotide cycle (PNC) to resynthesize the AdN pool (Lowenstein, 1972) or dephosphorylated to inosine by 5'-NT (Fig 2.1; Meghji et al. 1988). During intense exercise there is little, if any, reamination of IMP (Meyer and Terjung, 1980). The inosine which is produced can be further oxidised to hypoxanthine by purine nucleoside phosphorylase (PNP) (Schopf et al. 1986; Sjödin and Hellsten-Westing, 1990). Both inosine (Harkness et al. 1983) and hypoxanthine (Bangsbo et al. 1992; Harkness et al. 1983; Hellsten et al. 1998; Hellsten et al. 1999) can diffuse from the muscle. Bangsbo et al. (1992) estimated that at least 5 % of the AdN pool is lost from the muscle due to purine base efflux during the first 10 min of recovery following an intense exercise bout. More recently, it has been shown that the efflux of Hx and inosine from the muscle amounted to 9% of the ATP pool following intense exercise to fatigue (Hellsten et al. 1999). Hx can be further oxidised to uric acid by capillary endothelial cells (Hellsten-Westing, 1993a; Newsholme and Leech, 1983). Inosine, Hx and uric acid can be excreted by the kidney (Nasrallah and Al- Khalidi, 1964; Newsholme and Leech, 1983; Sutton et al. 1980), and represents a loss of purine base from the body. An additional fate of plasma uric acid is excretion via the gut (Sorensen and Levinson, 1975).

2.3 Detailed Discussion of Pathways of Adenine Nucleotide Metabolism.

2.3.1 Adenine Nucleotide Catabolism.

The AMP produced from ATP degradation is a common substrate to the branch point reactions catalysed by AMPd and 5'-NT (Fig 2.1). The different fates of AMP are

tissue specific and are highlighted by the difference between cardiac and skeletal muscle. The dominant pathway in skeletal muscle is AMPd, as indicated by the decrease in ATP and accumulation in IMP being equimolar following intense exercise (Jansson et al. 1987; Norman et al. 1987; Norman et al. 2001; Stathis et al. 1994; Zhao et al. 2000). The activity of 5-NT in contracting skeletal muscle is low (Hellsten et al, 1998; Norman et al, 2001; Rubio et al. 1973).

2.3.1.1 AMP Deaminase (EC 3.5.4.6)

AMPd is expressed widely in human tissues and the activity of AMPd in skeletal muscle is higher than that of any other tissue (Lowenstein, 1972). Regulation of AMPd is influenced by many factors including isozyme variation, substrate availability, allosteric control and myosin binding.

Three genes of AMPd have been identified (Mahnke-Zizelman and Sabina, 1992; Morisaki et al. 1990; Sabina and Mahnke-Zizelman, 2000) and they produce four distinct isozymes (Moriwaki et al. 1999; Ogasawara et al. 1982). Originally assigned A and B isoforms in rabbits and rats, the isozymes are now named after the tissues in which they were first purified and identified, i.e. M (muscle), L (liver) and E1 and E2 (erythrocyte) (Ogasawara et al. 1982; Ogasawara et al. 1984), and are encoded by the AMPD1, AMPD2 and AMPD3 genes, respectively (Mahnke-Zizelman et al. 1997). Skeletal muscle contains two isozymes, M and E, (Bausch-Jurken et al. 1992; Fishbein et al. 1993; Kaletha et al. 1987; Kaletha and Nowak, 1988; Mahnke-Zizleman and Sabina, 1992; Mahnke-Zizelman et al. 1996; Ogasawara et al. 1982; Ogasawara et al. 1983; Raggi et al. 1975) of which the M isoform is the most abundant of the two and is primarily found in the Type 2 fibres, whilst the E isoform

appears to be confined to Type 1 fibres (Mahnke-Zizleman and Sabina, 1992; Van Kuppevelt et al. 1994).

Many *in vitro* studies that have investigated the potential modulators of AMPd activity (Ashby and Frieden, 1978; Raggi and Ranieri-Raggi, 1987; Wheeler and Lowenstein, 1979) support the notion that a decreased energy charge results in the activation of this enzyme (Coffee and Solano, 1977). Physiological levels of guanosine triphosphate (GTP), Pi and low physiological concentrations of ATP have an inhibitory effect (Raggi and Ranieri-Raggi, 1987). However, the abovementioned inhibition that occurs at low levels of ATP is diminished at higher concentrations of ATP approaching the physiological range (Ashby and Frieden, 1978; Raggi and Ranieri-Raggi, 1987; Ronca-Testoni et al. 1970; Wheeler and Lowenstein, 1979). Positive modulation of AMPd by free AMP (fAMP), fADP and H^+ has been reported (Ronca-Testoni et al. 1970; Wheeler and Lowenstein, 1979). The most effective metabolite activating AMPd is likely to be ADP, which acts by reducing the inhibition caused by ATP, GTP and Pi (Ronca-Testoni et al. 1970; Rundell et al. 1992b; Wheeler and Lowenstein, 1979). The optimum pH for the enzyme is around 6.3 (Raggi and Ranieri-Raggi, 1987), thus during intense contraction, a simultaneous decrease in the energy charge and pH act to increase AMPd activity (Hellsten et al. 1999). A decrease in pH is not necessary for the activation of AMPd (Dudley and Terjung, 1985; Sahlin and Broberg 1990) but is often coincident with IMP formation.

Substrate availability is also an important factor in the AMDd reaction. The Km for AMPd is around 1.0 mM AMP (Raggi-Ranieri-Raggi, 1987; Wheeler and Lowenstein, 1979) which is well above resting fAMP concentrations, calculated from the myokinase equilibrium at less than 1 μM (Tullson and Terjung, 1990). Hence any

increase in the fAMP (ie AMP transients in exercise) concentration will increase the rate of deamination (Sahlin et al. 1990).

The binding of AMPd to myosin increases the activity of AMPd in rat (Rundell et al. 1992a) and chicken (Ashby et al. 1979) skeletal muscle and provides another potential regulatory mechanism of AMPd activity. However, AMPd-myosin binding does not occur in humans after exhaustive exercise (Tullson et al. 1995), and suggests that the human variants of AMPd found in skeletal muscle are incapable of binding to myosin. This appears suprising given the high degree of homology that occurs between rat and human isoforms when comparing amino acid sequences (92%) and nucleotide sequence (87%) (Sabina et al. 1992). However, most of the variability occurs in the N-terminal third of the gene, which is also the section carrying the AMPd binding domain, and the region influenced by truncation (Mahnke-Zizelman et al. 1998, Sims et al. 1999). The 90-95 amino N-terminal acid residues encoded by AMPD1 and AMPD3 recombinant polypeptides enzyme is where the differences are found between rats and humans (Mahnke-Zizleman et al. 1998) and thus explain the above mentioned presence of binding in rats and lack of binding in humans.

2.3.1.2 5'-Nucleotidase (EC 3.1.3.5)

Two reactions, the hydrolysis of ribose and deoxyribose nucleotides, to their respective nucleosides and phosphate are catalysed by 5'-nucleotidase.

$$AMP + H_20 \rightarrow adenosine + Pi$$

$$IMP + H_20 \rightarrow inosine + Pi$$

Multiple mammalian isoforms of 5'-NT exist and are characterised according to their cellular location; a membrane bound ecto and several soluble cytosolic isoforms (Camici et al. 1985; Frick and Lowenstein, 1976; Menghji et al. 1988; Newby et al. 1975; Truong et al. 1988; Zimmerman, 1992). Furthermore, the two soluble cytosolic isozymes, c-N-I and c-N-II, are distinguished by their different affinities for AMP and IMP, respectively (Skladanowski and Newby, 1990; Zimmerman, 1992). This highlights that both the availability and type of the substrate will play a role in 5'-NT activity.

Skeletal muscle inosine production is a consequence of the prolific production of IMP during an intense metabolic challenge in skeletal muscle (Norman et al. 2001; Stathis et al. 1994), thereby exposing 5'-NT to its substrate for inosine formation over an extended period. Early *in vitro* studies reported the total maximal activity of human skeletal muscle 5'-NT to be 4.0 ± 0.1 μmoles.g^{-1} protein.min^{-1} (Schopf et al. 1986). The *in vivo* activity can be estimated from studies measuring muscle inosine accumulation following intense exercise. From these studies the *in vivo* activity of 5'-NT was 160-320 μmol/kg dw/min {0.1-0.2 μmoles.g^{-1} protein.min^{-1}, assuming skeletal muscle protein content was 15% (Chesley et al. 1992) and dry weight to wet weight ratio was 23%} during 30 sec of "all out" exercise and 50-110 μmol/kg dw/min (0.03-0.07 μmoles.g^{-1} protein.min^{-1}) in the immediate 3-5 min of recovery following the exercise (Stathis et al. 1994; Zhao et al. 2000).

Substrate concentration plays a role in 5'-NT activity as the production of IMP from AMP is dependent on many factors (see section 2.3.1.1). It is presumed that only small amounts of IMP are required as the accumulation of IMP after intense exercise is substantial compared with the extent of inosine production. Other factors that influence 5'-NT activity include nucleoside di- and tri-phosphates, which are

powerful inhibitors of the 5'-NT ectozyme in guinea-pig skeletal muscle (Camici et al. 1985) and rat cardiac muscle (Itoh et al. 1986). This inhibition by ATP and ADP is partially reversed by Mg^{2+}, Mn^{2+} and Ca^{2+} (Camici et al 1985). In contrast, the cytosolic 5'-NT found in cardiac muscle is activated by ATP, ADP and decreasing energy charge and is inhibited by Pi (Itoh et al. 1986). Additionally, the cytosolic isozyme is activated by Mg^{2+} and is inhibited by H^{+} (Bak and Ingwall, 1998).

Differences in 5'-NT activities across fibre types have been reported in rats (Rubio et al. 1973; Tullson and Terjung, 1991) and cats (Bockman and McKenzie, 1983). The evidence suggests that the red ST muscle is more likely to form nucleosides and bases than the white FT muscle. The extent of purine base loss from each fibre type by these mechanisms is unknown but it could be related to the 5'-NT to AMPd activity ratio of the different fibres (Tullson and Terjung, 1990).

2.3.1.3 Purine Nucleoside Phosphorylase (EC 2.4.2.1)

The action of purine nucleotide phosphorylase (PNP) results in the degradation of inosine to Hx (Fig 2.1; Moriwaki et al. 1999; Tullson and Terjung 1990). This is widely expressed in tissues although the activity varies across different tissues. Histochemical localisation of PNP has demonstrated that the enzyme is found in the capillary endothelial cells of many tissues including the heart (Berne and Rubio, 1974; Borgers and Thone, 1992; Rubio et al. 1972), lung (Mentzer et al. 1975), brain (Rubio et al. 1978) and liver (Rubio and Berne, 1980). It has been isolated in skeletal muscle homogenates (Schopf et al. 1986), myocardial cells (Bowditch et al. 1985) or in the plasma (Harkness et al. 1983), but aside from its wide distribution in tissues and varied activities (Moriwaki et al. 1999) relatively little information exists on the regulation of PNP. Interestingly the PNP activity in the heart of various mammalian

species, including humans, has been isolated to the vascular epithelium with no activity reported in the cardiomyocytes (Borgers and Thone, 1992).

The maximal *in vitro* activity of PNP in human skeletal muscle has been reported to be 2.6 ± 0.3 μmol.g protein^{-1}.min^{-1} (Schopf et al. 1986). Intense metabolic stress results in inosine accumulation in skeletal muscle (Hargreaves et al. 1998; Stathis et al. 1994; Zhao et al. 2000) indicating that the *in vivo* activity of PNP is lower than that of 5-NT. This is only a qualitative observation because both the substrate and the product of PNP can efflux from the myocyte (Hellsten et al. 1999).

2.3.1.4 Xanthine Oxidase (EC 1.2.3.2)

Xanthine oxidase and xanthine dehydrogenase are interconverting forms of the one enzyme complex (Batelli et al. 1973; Hellsten-Westing, 1993). During normal resting conditions 80-90% of the enzyme is in the dehydrogenase form (Bindoli et al. 1988; McCord et al. 1985; Parks et al. 1988). However, during metabolic stress the dehydrogenase form is converted to the oxidase form (Corte and Stirpe, 1972).

The xanthine dehydrogenase/oxidase enzyme plays a role in the production of oxygen free radicals, which can cause traumatic processes at the tissue level by initiating damaging chemical reactions. Irrespective of the form, both catalyse the conversion of hypoxanthine to xanthine and the further oxidation of xanthine to uric acid (Fig 2.1). A conformational change converts the dehydrogenase form that uses nicotinamide adenine dinucleotide (NAD) as an electron acceptor compared with the oxidase form, which utilises molecular oxygen. Molecular oxygen is thereby reduced and the superoxide radical is formed (Kuppusamy & Zweier, 1989). For the purpose of this review xanthine oxidase will define both forms.

Immunohistochemical studies have located the presence of xanthine oxidase in the capillary endothelium of most human tissue including liver, intestine, heart, lung and skeletal muscle (Hellsten-Westing et al. 1993a; Jarasch et al. 1981). The activity of xanthine oxidase varies widely between animal species and across different tissue types (Hellsten, 1994). The activities reported by several studies and tabulated by Hellsten (1994) span a large range of values. Nevertheless, the enzyme activity in liver endothelial cells is relatively high when compared to skeletal muscle. This suggests that the liver endothelium is responsible for converting a majority of the Hx to uric acid (Hellsten et al 1994). The xanthine oxidase reaction is an irreversible reaction of purine nucleotide degradation in humans with most of the uric acid end product being excreted (Sorensen and Levinson, 1975). An additional fate of uric acid during exercise is further oxidation to allantoin (Hellsten, 1996).

2.3.2 Adenine Nucleotide Recovery.

2.3.2.1 Introduction

As previously discussed the skeletal muscle AdN pool can be transiently reduced during exercise when IMP is produced and accumulates within the muscle (Hellsten et al. 1999; Stathis et al. 1994). Most of the ATP is restored early in recovery via the reamination of IMP via the PNC. The subsequent production of inosine and Hx and efflux from the muscle (ultimately resulting in a loss of AdN), provides a challenge for the muscle to restore the AdN levels after exercise. Purine salvage recovers the purine bases within the muscle, whilst replacement of purines that have left the muscle via inosine and Hx efflux occurs via the purine *de novo* synthesis pathway. The resultant product of both of these pathways is IMP, although

purine *de novo* synthesis is more metabolically expensive than salvage (Newsholme and Leech, 1983).

2.3.2.2 IMP reamination

The restoration of AdN from IMP occurs via the two-step reamination pathway of the PNC. The reactions are catalysed by adenylosuccinate synthetase (AdSS) and adenylosuccinate lyase (AdSL). AdSS catalyses the conversion of IMP and aspartate to adenylosuccinate as described in the following reaction,

IMP + aspartate + GTP $\rightarrow$ adenylosuccinate + GDP + Pi

The maximal activity of this enzyme in rodent skeletal muscle is 0.74 $\mu mol.g^{-1}$ $ww.min^{-1}$ (Goodman and Lowenstein, 1977) and it is the rate limiting reaction of the PNC (Fig 2.1 ; Lowenstein, 1990). The regulation of AdSS is influenced by the availability of its substrates (IMP, GTP, aspartate) with each having a specific substrate binding site (Stayton et al. 1983). Of these three substrates, only IMP is considered to exert any significant regulatory influence during muscular activity (Stayton et al. 1983). Several authors (Goodman and Lowenstein, 1977; Muirhead and Bishop, 1974; Ogawa et al. 1977; Stayton et al. 1983) report that skeletal muscle AdSS has a Km for IMP in the range 0.2-0.7 mM. The IMP concentration in resting skeletal muscle is around 0.07 mM (Goodman and Lowenstein, 1977) and therefore small increases in IMP would be expected to elevate enzyme activity.

Product inhibition of AdSS by GDP, adenylosuccinate and AMP has been demonstrated using purified AdSS (Muirhead and Bishop, 1974; Ogawa et al. 1977). Nucleotide di- and monophosphates are also inhibitors of AdSS (Ogawa et al. 1977), of which GDP is the most potent (Stayton et al. 1983). Although small increases in

IMP activate AdSS, elevated concentrations of IMP inhibit the rate of the reaction *in vitro* (Goodman and Lowenstein, 1977; Stayton et al. 1983). Thus, with intense exercise where IMP concentrations are substantially elevated one would expect marked inhibition of AdSS. The extent of this inhibition *in vitro* does not appear to be confirmed by *in vivo* studies since the rate of IMP disappearance post exercise is not apparently dependent upon IMP concentration. It is acknowledged that this argument presupposes that IMP reamination rates account for most of its disappearance, as supported by the close relationship between ATP recovery and IMP removal (Katz et al 1986a). Additionally, PCr and Pi inhibit AdSS at physiological levels *in vitro* (Stayton et al. 1983). Hence, the inhibition by high resting PCr stores is probably relieved during intense exercise. Conversely, Pi accumulates in contracting muscle and is likely to inhibit the enzyme (Stayton et al. 1983). Clearly, the regulation of this enzyme is complex and it's activation appears to be determined by the balance of activators and inhibitors. During intense exercise it has been suggested that AdSS is inhibited such that the PNC is not operational and that reamination occurs only during recovery (Meyer and Terjung, 1980).

AdSL catalyses two reactions in the genesis of PrN as summarised below, succinylaminoimidazolecarboxamide ribotide (SAICAR) to aminoimidazolecarboxamide ribotide (AICAR) occurs early in the purine *de novo* synthesis pathway. The second reaction of AdSL, the second step in IMP reamination to AMP, is shared by the purine *de novo* synthesis and salvage pathways converting of adenylosuccinate to AMP and fumarate (Fig 2.1).

SAICAR $\rightarrow$ AICAR + Fumarate

Adenylosuccinate $\rightarrow$ AMP + Fumarate

This enzyme has been purified from rat skeletal muscle and its Km for adenylosuccinate was reported to be 1.5 μM and its activity in converting adenylosuccinate to AMP is 11 $\mu mol.min^{-1}.mg^{-1}$ of protein (Casey and Lowenstein, 1987). Adenylosuccinate concentrations range from undetectable levels at rest to approximately 20 $nmol.g^{-1}$dw after *in situ* stimulation of rat skeletal muscle (approximately 8 μM) (Lowenstein and Goodman, 1978). The Km values, therefore, suggest that AdSL is sensitive to small changes in adenylosuccinate concentrations but during high intensity exercise may be operating at its maximal rate.

Little, if any, IMP reamination occurs during intense exercise (Katz et al. 1986a; Katz et al. 1986b; Meyer and Terjung, 1980). Investigations into the operation of the PNC suggest, however, that there is some concurrent reamination during exercise of moderate intensity (Aragón and Lowenstein, 1980) indicated by an increase in adenylosuccinate concentration during exercise (Goodman and Lowenstein, 1977; Lowenstein and Goodman, 1978). The high IMP concentrations following sprint exercise may result in substrate inhibition of AdSS activity (Goodman and Lowenstein, 1977; Stayton et al. 1983) and may, in part, reduce the reamination rates. Meyer and Terjung, (1980) show that most of the IMP is converted to ATP during recovery from intense exercise in rats and this has also been confirmed in humans (Hellsten et al. 1999; Zhao et al. 2000).

The reamination rates of IMP after exercise in humans have been estimated indirectly from the ATP reappearance rates, i.e re-synthesis (Katz et al. 1986b) and the difference in IMP concentration at various time points during recovery (Graham et al. 1990; Sahlin and Ren, 1989). From these measurements it appears that the maximal *in vivo* rate of IMP reamination is between 0.4 and 0.6 $mmol.kg^{-1}$dry weight (dw).min^{-1}

(0.26-0.39 μmoles.g^{-1} protein.min^{-1}) which is not as rapid as the maximal rate of IMP production (calculated from AdN degradation) 14.4-31.0 mmol.kg^{-1}(dw).min^{-1} (9.4-20.2 μmoles.g^{-1} protein.min^{-1}) (Gaitanos et al. 1993; Nevill et al. 1989).

2.3.2.3 Purine Nucleotide Salvage

The purine salvage pathway acts to limit purine loss from skeletal muscle by resynthesizing IMP from hypoxanthine (Fig 2.1; Edwards et al. 1979). The purine salvage pathway is catalysed by hypoxanthine-guanosine phosphoribosyltransferase (HGPRT -EC 2.4.2.8), which combines hypoxanthine with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form IMP (Fig 2.1; Namm, 1973; Wiedmeier et al. 1972). Hence reducing the cost of ATP replacement by utilising preformed purine bases for nucleotide synthesis (Manfredi and Holmes, 1984) and avoiding the metabolically expensive *de novo* synthesis pathway.

At rest, HGPRT is estimated to be responsible for the recovery of approximately 75% of the intramuscular Hx production (Edwards et al. 1979). Purine base recovery by HGPRT following exercise is unknown. Control of the activity of this enzyme has not been thoroughly investigated, however, the critical role of HGPRT in AdN metabolism is evident in patients with Lesch-Nyhan syndrome, a complete deficiency in HGPRT, who exhibit debilitating physical and neurological symptoms (Edwards et al. 1979; Lesch and Nyhan, 1964). The maximal activity of HGPRT in human myocardium and quadriceps femoris muscles is 0.13±0.04 and 0.28±0.06 μmol.g^{-1} protein.min^{-1}, respectively (Schopf et al. 1986). The activity of the HGPRT in rats ranges between 19.2±1.6 and 31.8±2.2 nmol.h^{-1}.g^{-1}ww (0.05-0.08 nmoles.g^{-1} protein.min^{-1}, respectively; Brault et al. 2001). The *in vivo* activity is considerably lower than the maximal activity mentioned by Schopf et al. (1986). The

factors influencing the activity of HGPRT have not been extensively studied in skeletal muscle. To date, most studies of the salvage pathway have used rodent heart (Harmsen et al. 1984) and liver (Kim et al. 1992). These studies have demonstrated that the availability of PRPP limits the rate of IMP formation. Kim et al. (1992) suggest that the provision of ribose-5-phosphate may ultimately determine the availability of PRPP as they report that changes in PRPP are correlated with changes in ribose-5-phosphate. This is further supported by enhanced purine nucleotide synthesis from hypoxanthine when ribose was added to the fluid perfusing the myocardium (Harmsen et al. 1984). Ribose supply to rat hindlimb preparations increased hypoxanthine salvage rates by three to eightfold (Brault et al. 2001). Studies investigating the factors regulating HGPRT in human skeletal muscles have not been reported, however ribose supplementation increased the rate of recovery of muscle ATP content in the 72 hours after the cessation of 1 week of intense sprint training (Hellsten et al. 2004).

2.3.2.4 *De novo* Adenine Nucleotide Synthesis.

The *de novo* purine biosynthesis pathway has been established *in vitro* (Tully and Sheehan, 1979; Zoref-Shani et al. 1982) and *in vivo* (Brosh et al. 1982). The *de novo* synthesis rate *in vitro* is two-fold higher in the skeletal muscle of mice compared with that of liver (Brosh et al. 1982) and is critical to skeletal muscle AdN metabolism, continually supplying and replenishing the muscle AdN pool. The initial substrate for *de novo* synthesis is PRPP and in an elaborate series of reactions carbon and nitrogen atoms, provided by glutamine, aspartate, glycine, formate and bicarbonate ions, are added to PRPP to produce IMP, which is then incorporated into the AdN pool via the PNC (Newsholme and Leech, 1983). The production of one IMP

molecule via this mechanism is metabolically expensive and involves the consumption of six high-energy phosphate bonds (Newsholme and Leech, 1983). The resting *de novo* AdN synthesis rates of fast-twitch and slow-twitch muscle in rats are reportedly about 25 and 60 nmol.g^{-1} ww.h^{-1} (0.06 and 0.15 μmoles.g^{-1} protein.min^{-1}, respectively) which equates to a replacement of about 0.3-1.0% of the AdN pool per hour (Tullson et al. 1988).

The availability of substrates, particularly that of PRPP, appear to be the rate limiting factor of *de novo* synthesis *in vivo* (Fox and Kelly, 1971; Harmsen et al. 1984; Tullson and Terjung, 1991a; Zimmer and Gerlach, 1978; Zoref-Shani et al. 1982). The synthesis of PRPP is catalysed by PRPP synthetase and this enzyme is inhibited by various nucleotides, particularly AMP, ADP and GDP (Mathews and van Holde, 1990). The rate of *de novo* synthesis may be limited by the flow through the hexose monophosphate shunt, which produces the ribose for PRPP production (Zimmer and Gerlach, 1978). An increase in the concentration of ribose in the perfusate of rat skeletal muscle increased the *de novo* AdN synthesis 3- to 4- fold (Tullson and Terjung, 1991a). Consistent with this faster rate is a quicker restoration of the ATP and purine nucleotide content in skeletal muscle following vigorous sprint training with ribose supplementation (Hellsten et al. 2004). None of the other substrates which are sequentially added in the purine *de novo* biosynthesis pathway, including glycine, glutamine, formate and bicarbonate (Newsholme and Leech, 1983), are likely to be limiting due to their relative abundance in skeletal muscle.

Tullson et al. (1988) and Tullson and Terjung, (1991a) have proposed that *de novo* AdN synthesis occurs in favourable energetic conditions, particularly where a high ATP:ADP ratio exists. The decrease in energy charge may reduce *de novo* AdN synthesis by inhibiting PRPP-aminotransferase (Fig. 2.1). This enzyme catalyses the

conversion of PRPP to 5'-phosphoribosylamine and is inhibited allosterically by AMP and GMP (Mathews and Van Holde, 1990).

A fibre type difference is also reported in the capacity of AdN *de novo* synthesis in rat (Tullson et al. 1988) with the highest rate exhibited in FT red, then the ST red muscle and the lowest rate in FT white muscle. This trend appears to be influenced by the relative oxidative capacity (Tullson et al. 1988) and by a greater capacity of oxidative muscle fibres to degrade purine nucleotides to purine bases more readily (Arabadjis et al. 1993). This would require an increased demand, via *de novo* synthesis, for the production of purines in the ST fibres to maintain constant AdN levels (Tullson et al. 1988). In rat muscle, endurance training does not appear to increase AdN *de novo* synthesis (Tullson and Terjung, 1991a). However, these results may be influenced by limitations in the substrate supply (Section 2.3.3.2). There is, as yet, no information on the *de novo* synthesis capacity of human skeletal muscle.

2.4 Muscle Purine Accumulation and Efflux

The accumulated IMP following exercise is exposed to subsequent degradation by 5'-NT, producing inosine in the muscle (Hargreaves et al. 1998; Hellsten et al. 1999; Stathis et al. 1994; Zhao et al. 2000). Following the cessation of exercise, IMP remains elevated compared with pre-exercise resting muscle and the muscle inosine content increases after 3-10 min recovery (Hellsten et al. 1999; Stathis et al. 1994; Zhao et al. 2000). The inosine and Hx release from the muscle at rest is small (almost zero in both cases) and following intense exercise rises to peak at 8 and 35 μmol.min^{-1}, respectively (Hellsten et al. 1999). Repeating the exercise after a short rest period also results in a higher accumulation of inosine in the muscle (Hargreaves et al. 1998). Interestingly, intramuscular Hx accumulation is not evident following an intense

exercise bout (Hargreaves et al. 1998; Hellsten et al. 1999; Stathis et al. 1994; Zhao et al. 2000). This indicates that the combination of the intramuscular purine salvage of Hx (Edwards et al. 1979) and Hx efflux from the muscle (Hellsten et al. 1999) matches the rate of Hx production.

Inosine, Hx and uric acid accumulate in the plasma (Harkness et al. 1983; Hellsten et al. 1994; Hellsten 1999; Stathis et al. 1994; Sutton et al. 1980). All of the aforementioned purine metabolites can be removed via the kidney (Harkness et al. 1983; Nasrallah and Al-Khalidi, 1964; Sorensen and Levison, 1975; Sutton et al. 1980). Additionally, uric acid can also be excreted via the gut (Sorensen and Levison, 1975). As discussed earlier the net efflux of purine base represents a loss of AdN precursor molecule from the muscle and complete recovery of the resting ATP levels is reliant upon the relatively slow and metabolically expensive AdN *de novo* biosynthesis pathway (Tullson and Terjung, 1991a).

There is growing evidence of nucleoside transporter involvement in the permeation of purine bases across biological membranes. In mammalian cells, two major types of transporter groups exist. Firstly, the equilibrative nucleoside transporters (ENTs) are facilitated transport carrier proteins that translocate nucleosides and nucleobases down their concentration gradients (Griffiths et al. 1997; Hyde et al. 2001; Osses et al. 1996, Yao et al. 2002). Secondly, the concentrative nucleoside transporters (CNTs; Cabrita et al. 2002), which are Na^+-dependent secondary active transporters that co-transport nucleosides and sodium uni-directionally against the nucleoside concentration gradients (Cabrita et al. 2002). In humans three ENTs have been reported, hENT1 (Griffiths et al. 1997), hENT2 (Osses et al. 1996, Yao et al. 2002) and hENT3 (Hyde et al. 2001). Of these, hENT2 transporters (Osses et al. 1996, Yao et al. 2002) and hENT2 mRNA has been reported in skeletal muscle (Pennycooke et

al. 2001). The possibility of restricted movement of purine bases across membranes provides some support for the unidirectional flux of Hx (Hellsten et al. 1998; Hellsten et al. 1999). However, Brault et al. (2001) demonstrated an influx of Hx into the muscle with high, non-physiological extracellular concentrations of Hx. An understanding of the human ENT function may provide further information about the movement of purines across the sarcolemmal membrane and excretion in the urine and gut.

2.4.1 Plasma Markers of Muscle Purine Catabolism

2.4.1.1 Inosine

Resting plasma inosine levels have been measured at around 1μM and this can increase with exercise peaking at around 4-6 μM (Hellsten et al. 1999). The extent of the accumulation depends on the intensity of the exercise as cycle exercise up to 40 W (Kono et al. 1986) does not increase plasma inosine concentrations in healthy individuals, but 2 min of intense cycle exercise (Harkness et al. 1983), 2 min of ischaemic forearm exercise (Mineo et al. 1985) or leg extension to fatigue (Hellsten et al. 1999) all lead to significant plasma inosine accumulation.

2.4.1.2 Hypoxanthine

The plasma concentration of hypoxanthine at rest is around 1-2 μM (Harkness et al.1983; Ketai et al. 1987; Mineo et al. 1985; Sahlin et al. 1991). The plasma concentration of Hx increases following intense exercise (Harkness et al. 1983; Hellsten-Westing et al. 1991; Ketai et al. 1987; Sjödin and Hellsten-Westing, 1990; Stathis et al. 1994). The intensity of the exercise is a major determinant of plasma hypoxanthine levels (Hellsten-Westing et al. 1991; Ketai et al. 1987; Sahlin et al.

1991; Sjödin and Hellsten-Westing 1990). Sjödin and Hellsten-Westing (1990), report that there is an inflexion point around 110% VO_2 max for the rise in plasma hypoxanthine during incremental exercise. Data by Ketai et al. (1987) shows that hypoxanthine increases in the plasma at intensities above ventilatory threshold (Vt). A maximal 30s sprint exercise bout does not increase plasma levels of hypoxanthine at the end of exercise but there is a subsequent increase to a peak level around 15-20 min into recovery (Hellsten-Westing et al. 1989). This reflects the time course of the balance between muscle Hx production and efflux and removal from the plasma and is supported by other studies investigating the levels of hypoxanthine during recovery that report peak concentrations 15-30 fold higher than resting levels at about 15-20 mins after intense exercise (Hellsten-Westing et al. 1989; Ketai et al. 1987; Sjödin and Hellsten-Westing, 1990). Plasma concentrations remain elevated above resting levels for at least 30 mins after 30 s of "all out" cycling (Stathis et al. 1994) and for 120 min after approximately 2 min of intense maximal running (Hellsten-Westing et al. 1989). Hypoxanthine could be removed from the plasma by inactive skeletal muscle when circulating concentrations are high (Bangsbo et al. 1992). Other tissues such as the heart (Harmsen et al. 1984) and liver (Kim et al 1992) also act to remove Hx from the circulation.

2.4.1.3 Xanthine

Xanthine is produced as an intermediary of the two reactions catalysed by xanthine oxidase (Hellsten, 1994). Few studies have measured plasma xanthine concentrations during or following exercise. In a study using horses, the xanthine concentration of blood following exercise did not increase significantly during 20 min of recovery (Mills et al. 1997). This is likely to be due to an insufficient intensity of

exercise to induce significant muscle ATP depletion and subsequent IMP degradation and downstream purine degradation products.

2.4.1.4 Uric Acid

The concentration of uric acid in the blood of healthy individuals is around 250-300 μM at rest (Hellsten-Westing et al. 1989; Sjödin and Hellsten-Westing, 1990). The production of uric acid at rest arises from the conversion of Hx to uric acid catalysed by xanthine oxidase, which is located in endothelial cells of blood vessels (Jarasch et al. 1981). At rest, two thirds of the circulating uric acid is removed by the kidney and one third via the gut (Sorensen and Levison, 1975).

Increases in plasma uric acid are observed 20-30 min after intense exercise (Helsten-Westing et al. 1989; Hellsten-Westing et al. 1991; Sjödin and Hellsten-Westing, 1990; Stathis et al. 1994). The intensity (Sjödin and Hellsten-Westing, 1990) and duration (Hellsten-Westing et al. 1991) of the exercise influences the rise in concentration of uric acid in the blood. Like Hx, plasma uric acid levels post-exercise are unchanged unless the exercise intensity is above a threshold intensity of 115% VO_2max (Sjödin and Hellsten-Westing, 1990).

The levels of plasma uric acid begin to rise 15 min into recovery from maximal, short-distance running efforts and peak after 45 min (Hellsten-Westing et al. 1989). This delay is probably due to the fact that the formation of uric acid is the final step in a cascade of reactions (Fig. 2.1), in which many of the enzymes have low maximal reaction rates. The fact that it takes somewhat longer for uric acid to peak suggests that much of the time taken for the accumulation of uric acid is probably due to the activity of xanthine oxidase. Xanthine oxidase, the enzyme responsible for the production of uric acid, is located in endothelial cells of the blood vessels of skeletal

muscle (Jarasch et al. 1981) and other tissues (Wajner and Harkness, 1989). Xanthine oxidase activity is relatively low in skeletal muscle compared with other tissues, particularly the liver (Wajner and Harkness, 1989). Hence, formation of uric acid occurs predominantly after Hx has diffused out of the muscle, with most conversion of Hx to uric acid occurring in the liver (Hellsten-Westing et al. 1994). The absolute change in plasma uric acid is three times greater than plasma Hx after intense exercise (Hellsten-Westing et al. 1989), most likely due to the fact that uric acid is the end point of AdN degradation.

2.5 Urinary Excretion of Purines

The increase in urinary excretion of uric acid and purine bases with exercise and intense labour activities have been reported early last century (Cathcart et al. 1907; Kennaway, 1909). In general, these reports presented data at rest or following exertion in a qualitative sense (i.e. daily activities). However, even then, it was recognised that "severe" exercise was required to produce a marked increase in the output of endogenous uric acid (Cathcart et al. 1907). The purine bases that increased with exercise were identified some time later as Hx and Xanthine (Harkness et al. 1983; Nasrallah and Al-Khalidi, 1964; Sutton et al. 1980). Studies have attempted to measure the increase in purines in the urine after exercise (Harkness et al. 1983; Sutton et al. 1980). Purine excretion patterns are influenced by the fractional clearance rates of the purines, of which, Hx and xanthine is many fold higher than uric acid (Moriwaki et al. 2002). Furthermore, a rise in plasma lactate reduces the excretion of purines (Nichols et al. 1951), via the inhibition of lactate on renal purine transport mechanisms (Yamamoto et al. 1993).

2.5.1 Inosine

Early studies reported the excretion of purines (Rundles et al. 1966; Klinenberg et al. 1965; Goldfinger et al. 1965). This included inosine, Hx and xanthine and were unable to distinguish between the three. The excretion of inosine has been reported at 4.7 μmole/mg creatinine at rest and rises to 36.9 μmole/mg creatinine following strenuous exercise (Sutton et al 1980). No other studies have investigated the influence of intense exercise on inosine excretion patterns.

2.5.2 Hypoxanthine

The basal excretion rates of Hx have been reported to be 53 $nmol.hr^{-1}.kg^{-1}$ (Harkness et al. 1983) or 65 $nmol.hr^{-1}.kg^{-1}$ (Nasrallah and Al-Khalidi, 1964; assuming 70 kg total body weight). Whilst only a small amount of Hx is excreted in the resting state, it is by far the major purine intermediary excreted in the urine early in recovery following intense exercise (Sutton et al. 1980). Nasrallah and Al Khalidi (1964) have also demonstrated that Hx excretion averages a five-fold increase and could be as high as 20-fold that of resting concentration following strenuous activity for long periods of time (hours). Furthermore, after a 2 min bout of severe exercise a 20-fold increase in Hx excretion in untrained individuals has also been observed (Harkness et al. 1983).

2.5.3 Xanthine

At rest the excretion of xanthine has been reported at 31 $nmol.hr^{-1}.kg^{-1}$ (Harkness et al. 1983) and 70 $nmol.hr^{-1}.kg^{-1}$ (Nasrallah and Al-Khalidi, 1964). The increased excretion of xanthine with exercise was reported to be substantially lower

(≈half) than the increase observed for Hx (Harkness et al. 1983; Nasrallah and Al-Kahlidi, 1964).

2.5.4 Uric acid

Uric acid excretion in healthy men on a purine free diet has been shown to be around 1.63 $\mu mol.hr^{-1}.kg^{-1}$ and does not change after almost an hour recovery following intense running for 9 mins (Nichols et al. 1951). Others have reported no change in urinary uric acid excretion during recovery following graded exercise (Sutton et al. 1980). When interpreting this result it should be noted that urine was collected in the 30 mins starting from the commencement of exercise. Thus the recovery period was very short and the opportunity to measure changes in uric acid excretion within this time is limited. A rise in uric acid does not occur in the plasma until at least 15 min after an intense exercise bout (Hellsten-Westing et al. 1989). Hypoxic breathing ($8\%O_2$) induced a 30% reduction in uric acid excretion early (15-25 min) in recovery from exercise due to an increase in lactate production (Nichols et al. 1951). Thus exercise intensity and the subsequent accumulation of lactate in the plasma will play a role in uric acid excretion. Maximal "all out" sprinting and intermittent bouts of "all out" sprinting are likely to produce the highest concentrations of plasma lactate and thus influence renal transport in the kidneys following exercise. Further research is required on the excretion pattern of uric acid during or following intense exercise.

2.5.5 Alternative excretion of plasma purines.

Intestinal and urinary excretion and sweating are possible avenues of plasma purine loss. At rest, removal of plasma uric acid via the gut amounts to one third of the total

with kidney accounting for the remaining two thirds (Sorensen and Levinson, 1975). Most of a bolus of infused isotopic label is accounted for via urinary or intestinal avenues of excretion (Sorensen and Levinson, 1975). This is confirmed by reports that the loss of uric acid in sweat is minimal (Huang et al. 2002). Very little information of extrarenal excretion pathways of the other purine intermediates is available. In mice less than 3% of the Hx injected into the plasma was lost in the gut (Bradford et al. 1968).

2.6 Factors Influencing Adenine Nucleotide Metabolism

The factors that regulate and determine resting skeletal muscle ATP concentrations are not fully understood. However, the rate of production of ATP must be equal to the rate of loss in skeletal muscle for ATP levels to remain constant. Resting ATP concentrations in human skeletal muscle are usually reported between 21-28 $mmol.kg^{-1}dw$ (Cheetham et al. 1986; Hargreaves et al. 1998; Hellsten-Westing et al. 1993a; Hellsten et al. 1999; Katz et al. 1986a; Katz et al. 1986b; Nevill et al. 1989; Stathis et al. 1994; Zhao et al. 2000). Several studies demonstrate no difference in resting ATP concentrations in Type I and Type II fibres of untrained muscle at rest (Ball-Burnett et al. 1991; Karatzaferi et al. 2001a; Soderlund and Hultman, 1990). There is evidence of a continual basal turnover of ATP from the incorporation of radiolabelled glycine into AdN in resting rat skeletal muscle (Tullson et al. 1988; Tullson and Terjung, 1991a). During intense exercise, the balance is shifted towards a net loss of muscular purines due to the increased metabolic load on the skeletal muscle. The extent of muscle purine loss or efflux into the plasma is influenced by the intensity and duration of the exercise performed. Furthermore training status and

pharmacological intervention may also be factors in determining muscle purine content.

2.6.1 Exercise Intensity and Duration

The intensity of exercise plays a role in purine nucleotide metabolism. There is no change in skeletal muscle ATP (Baldwin et al. 2003; McConell et al. 2000; Snow et al. 2000) or plasma purine concentration (Sjödin and Hellsten-Westing, 1990) following low or moderate exercise. Sjödin and Hellsten-Westing, (1990) demonstrate a break point in plasma Hx concentrations at about 110%VO_2 max indicating an increase in the production of purines with increased intensity.

Maximal sprint exercise generates large disturbances in muscle ATP content. Substantial decreases have been demonstrated for exercise bouts of 6-30s duration (Bogdanis et al. 1998; Cheetham et al. 1986; McCartney et al. 1986; Nevill et al. 1989; Stathis et al. 1994; Withers et al. 1991; Zhao et al. 2000) and no further decrease is evident beyond 30 s of maximal sprint exercise (Withers et al. 1991).

2.6.2 Recovery

During recovery from high intensity exercise there is a close relationship between the ATP recovery and IMP disappearance. This demonstrates that a majority of the restoration of AdN occurs via the reamination of accumulated IMP (Sahlin and Ren, 1989). The time course of recovery determines the extent of time the IMP is exposed to further degradation by 5-NT. The reamination rate of IMP is influenced by the metabolic stress placed on the muscle as many of the metabolites produced during intense exercise influence AdSS activity (refer section 2.3.2.2). Bogdanis et al. (1998) demonstrated that although the extent of ATP degradation during a 10s and 20s sprint

was similar, resynthesis rates of ATP after 2 min of recovery were different. This is probably due to lower PCr levels in the 20s sprint increasing inhibition of AdSS (Tullson et al. 1996). Thus, the recovery time for complete restoration following intense exercise depends on the extent of ATP degradation and the cellular conditions upon cessation of exercise.

The initial rate of AdN recovery in the muscle following intense sprint cycling is minimal. No significant recovery is reported 1.5-6 min following maximal sprint cycling (Bogdanis et al. 1995; Bogdanis et al. 1998; Stathis et al. 1994) or 2.9 min after cycling to fatigue at 120-130% VO_2 max (Tullson et al. 1995). However, ATP recovery and IMP reduction has been reported in the muscle after 4 min recovery of leg kicking exercise to fatigue (Graham et al. 1990; Sahlin and Ren, 1989).

With further recovery time the concentration of ATP returns to pre-exercise resting levels. This has been reported 10 min after the cessation of a 30 s maximal cycle sprint (Zhao et al. 2000), 10 min recovery from intense knee extensor exercise to fatigue (Hellsten et al. 1999) and 7 min after exercise greater than 80% VO_2max (Sahlin et al. 1978), respectively.

These studies highlight that exercise intensity, and consequently the metabolic stress, plays a role in the time-course of ATP resynthesis from IMP during recovery. Intense exercise produces the intramuscular milieu when the inhibitory metabolite concentrations are at their greatest. For example a high IMP content (Goodman and Lowenstein, 1977; Stathis et al. 1994), low pH (Davey, 1961; Ogawa et al. 1977; Zhao et al. 2000) and a reduced PCr content (Tullson et al. 1996) inhibit AdSS activity. Thus the extent to which this inhibition occurs is influenced by the recovery of the inhibitors. Bogdanis et al. (1998) also demonstrate that PCr and pH levels are lower after the 20 s compared with the 10 s sprint. The recovery of IMP to ATP is

also linked to the aerobic capacity of the fibre and its ability to restore PCr. PCr resynthesis rates may play a role in the restoration of AdN and the lack of PCr recovery and IMP reamination following the occlusion of blood flow to the muscle following exercise (Tullson et al. 1996) demonstrates it importance for the restoration of ATP in the muscle.

2.6.3 Influence of multiple sprint bouts on purine metabolism

Several sprint training studies have demonstrated a decrease (Harmer et al. 2000; Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994) in resting muscle ATP and/or AdN content. This is in contrast to other studies that found no difference (Neville et al. 1989), or an increase (Thorstensson et al. 1975) in resting ATP levels in the post-trained relative to the pre-trained status. A variation in the sprint training protocols may explain these conflicting findings. The efflux of purine bases from the muscle is affected by the different intensities, number of exercise bouts, and/or the duration of the exercise bouts. The training protocols employed in the studies that result in a decreased resting muscle ATP/ AdN (Harmer et al. 2000; Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994) share some common variables including short duration exercise and multiple intermittent repetitions. However, variations between the training regimes existed including a difference in the number of sprints employed per training session, the duration of sprint times, rest interval between bouts, recovery time between successive sessions and the length of the overall training program. This section of the review will consider the effect of such variables with intermittent protocols on purine metabolism.

2.6.3.1 Intensity

The influence of exercise intensity on purine metabolism was discussed in section 2.6.1. In sprint training studies that employed “all out” intermittent sprint protocols many have resulted in a significant reduction of AdN or ATP in the muscle due to a net loss of purine from the muscle (Harmer et al. 2000; Hellsten-Westing et al. 1993b; Hellsten et al. 2004; Stathis et al. 1994).

2.6.3.2 Number of Sprint Bouts

One study has provided some insight into the influence of a successive sprint bout on muscle purine metabolism (Hargreaves et al. 1998). They measured the muscle inosine content 4 minutes after two successive 30s sprint bouts separated by 4 min recovery. Muscle inosine was approximately two-fold higher than that reported between 3-5 min following a single sprint bout (Stathis et al. 1994; Zhao et al. 2000). Although the comparison is not across the same subject populations it provides an indication as to the influence of a second subsequent bout on muscle purine production. No studies have investigated the influence of a varying number of sprint bouts on muscle content or plasma purine concentration. It is likely that increasing the number of sprint bouts will result in an extended elevation of IMP and consequently an increased purine efflux and loss from the muscle.

2.6.3.3 Sprint Duration

Balsom et al. (1992a) demonstrated an increase in the duration of intermittent sprint bouts elevates plasma Hx accumulation. These results indicate that the duration of the sprints may influence the extent of the IMP accumulation following each successive sprint and may translate to the substrate availability for the 5’-NT pathway. This is

supported by a study using a protocol of 10 x 6s sprints separated by 30s rest intervals (Gaitanos et al. 1993). They demonstrated a 12.9% decrease in ATP during the first 6s sprint and subsequent degradation to 31.7% of the resting ATP after the tenth bout (presumably converted to IMP with high intensity exercise).

The aforementioned relationship between the duration of the intermittent bouts and the extent of plasma Hx accumulation has not been demonstrated for bouts higher than 6 seconds. It is likely, however, that the extent of the plasma Hx accumulation will plateau, since the maximal rate of its production via the 5'-NT pathway is small, as is the amount IMP required to supply it. Furthermore, the magnitude of ATP degradation does not increase significantly following sprint durations greater than 10 (Bogdanis et al. 1998) and 15s (Withers et al. 1991). Gaitanos et al. (1993) also reports no change in ATP during the last bout indicating that the maximal magnitude of degradation was reached somewhere earlier in the intermittent sprint protocol. In another experiment, there was no further decrease in ATP observed following the first sprint of a 4x30s intermittent sprint protocol (McCartney et al. 1986), hence ATP degradation following one 30s sprint appears to be maximal. Although the IMP content was not measured in these studies, it is assumed that a reduction in ATP content equates to elevated IMP content and degradation products (see section 2.1).

2.6.3.4 Recovery Between Intermittent Bouts

The influence of recovery following a single bout of intense exercise has been discussed previously (Section 2.6.2). There are no reports investigating the influence of recovery on purine degradation products in the muscle during intermittent sprint exercise. Balsom et al. (1992b) have manipulated the recovery times between bouts and demonstrated plasma concentrations of Hx at 15 min recovery are higher when

15x40m sprints are performed 30 s apart than when they are performed 60 or 120s apart.

2.6.4 Sprint Training

In many studies intense intermittent sprint training reduces the resting ATP content of skeletal muscle (Section 2.6.3). Hellsten-Westing et al. (1993a) report that there was no difference in plasma Hx 15 min into recovery after completing an intense intermittent sprint training session in the sprint trained, relative to control subjects. Stathis et al. (1994) reported that sprint training attenuated the plasma Hx concentration 20 and 30 min into recovery from a 30 s sprint bout. The discrepancy may simply be due to a more accurate picture obtained by multiple sampling for a longer duration during recovery (Stathis et al. 1994), compared with a single sampling time point (Hellsten-Westing et al. 1993a).

The sprint training induced attenuation of plasma Hx reported by Stathis et al. (1994) is due to a decreased purine efflux into the blood and/or an increased capacity for plasma Hx removal following sprint training. Hellsten-Westing et al. (1993b) demonstrated an increased maximal activity of HGPRT in skeletal muscle after training that may support a decreased efflux from the muscle into the blood. They also show that during recovery from 2 min exercise bout of various intensities the plasma concentrations of Hx and uric acid decreased in the post-trained state.

There are no studies available that directly examine the effects of training on the removal mechanisms of Hx from the blood. It may be an increased capacity for removal by the kidneys following training. No human study has investigated the effects of sprint training on plasma and urine uric acid concentration in recovery from intense exercise.

2.6.5 Influence of Fibre Type

The aforementioned influence of exercise intensity on PrN metabolism may be due to intrinsic differences within the different muscle fibres. As a result the fibre type composition of the muscles involved in the exercise may play a role in PrN metabolism Several studies have demonstrated high levels of ATP reduction and IMP accumulation in Type II compared with Type I fibres following intense exercise in humans (Jansson et al. 1987; Karatzaferi et al. 2001a; Karatzaferi et al. 2001b). In fact, to induce IMP accumulation in the Type I fibres requires extremely challenging metabolic conditions (25s vs 10s maximal sprints; Karatzaferi et al. 2001a vs Karatzaferi et al. 2001b). Interestingly, Jansson et al (1987) reported no change in IMP production in Type I fibres following intense knee extension and Karatzaferi et al. (2001a) clarify their results by noting in their results that 70% of type I fibres actually had no detectable IMP content. Similarly, in rodent skeletal muscle, IMP accumulation was many-fold greater in fast-twitch (FT) white compared with slow-twitch (ST) red muscle (Arabadjis et al. 1993; Tullson et al. 1996).

Additionally, IMP exerts substrate inhibition on AdSS at high IMP concentrations (Goodman and Lowenstein, 1977; Stayton et al. 1983). Thus, the greater IMP accumulation in Type II relative to type I fibres, the greater the potential to retard the recovery of ATP. This trend has been reported following 1.5 min of recovery from an intense 25 s sprint bout (Karatzaferi et al. 2001a). Additionally, Winder et al. (1974) report higher *in vitro* AdSS activity in the more oxidative FT red muscle and lowest in rat FT white muscle. Perhaps, not surprisingly, *in vivo* reamination rates of IMP, following running in rats, are greater in fast-twitch red fibres than in fast-twitch white (Tullson et al. 1996). In mixed muscle in humans, a higher production of IMP in

skeletal muscle results in a recovery time which is simply proportional to the initial concentration of IMP.

The restoration of ATP following intense metabolic stress producing intramuscular IMP accumulation is a function of the PCr resynthesis potential (Tullson et al. 1996; Karatzaferi et al. 2001a). PCr resynthesis rates in oxidative FT red muscle was more rapid compared with the less oxidative FT white and this reflected the capacity for IMP resynthesis exhibited in these muscles (Tullson et al. 1996). Similarly, in humans muscle fibres, retarded recovery rates of PCr and ATP resynthesis has been reported in type II compared with type I fibres (Karatzaferi et al. 2001a)

2.6.6 Influence of Allopurinol

2.6.6.1 Background

Many studies have demonstrated allopurinol to be an effective allosteric inhibitor of xanthine oxidase and reported a pronounced reduction in both the serum uric acid content and urinary uric acid excretion (Edwards et al. 1981; Feigelson et al. 1957; Klinenberg et al. 1965; Rundles et al. 1966; Rundles et al. 1969; Wyngaarden et al. 1965; Yü and Gutman, 1964). Inhibition of xanthine oxidase is effected by both allopurinol and it's metabolic byproduct, oxypurinol (Elion, 1966). Oxypurinol, an analogue of xanthine, is a more effective inhibitor with a longer half-life (Elion, 1966), but is less effective when administered orally, due to its poor gastrointestinal absorption (Chalmers et al. 1968). Allopurinol has since been used therapeutically as a standard treatment for patients with gout. This is a condition of hyperuricemia, in which crystals of uric acid form in the joints due to a low solubility of uric acid at physiological pH.

The levels of resting plasma purine bases do not change with allopurinol therapy (Klinenberg et al. 1965; Yü and Gutman, 1964). However, increases of urinary purine bases have been reported (Edwards et al. 1981; Yü and Gutman, 1964) which is predominantly made up of an elevation in Hx and xanthine (Rundles et al. 1966).

It is anticipated that the reduction in uric acid production via xanthine oxidase would be balanced by an equal rise in purine bases. However, studies have demonstrated the total urinary purine excretion to be 10-60% lower at rest with the increase in purine base excretion substantially lower in magnitude than the decreased uric acid excretion (Yü and Gutman, 1964; Rundles et al. 1966; Klinenberg et al. 1965). This mismatch in purines suggests there are alternative mechanisms influencing their removal from the blood with allopurinol administration. An increased loss of purines via the gut, a decrease in the rate of *de novo* purine biosynthesis and increased purine salvage with allopurinol are possible mechanisms that could explain this imbalance.

Substantial purine loss via the gut occurs in healthy humans. Two thirds of uric acid is excreted through the kidney while one third enters the gut and undergoes uricolysis (Sorensen and Levinson, 1975). The action of allopurinol reduces uric acid production from Hx and although plasma hypoxanthine and xanthine may increase, loss via the gut is minimal as demonstrated by radiolabel tracing where less than 3% of purine bases get across into the gut (Bradford et al. 1968). Similarly with allopurinol, production of uric acid is inhibited. Thus, it could be assumed that with allopurinol the efflux of total purines via the gut will be reduced.

An increase in purine salvage with allopurinol in humans could also provide a mechanism to explain the discrepancy. Evidence from patients deficient in HGPRT, (Lesch-Nyhan syndrome) report elevated purine salvage with allopurinol (Edwards, 1981). An increased purine salvage rate may provide a significant advantage to the

restoration of muscle ATP during periods of recovery following acute metabolic stress.

It is also proposed that the decrease in total purine excretion is due to a reduced purine *de novo* biosynthesis following allopurinol administration (Yü and Gutman, 1964; Klinenberg et al. 1965; Rundles et al. 1966; Fox et al. 1970; Kelley and Wyngaarden, 1970). Substrate limitation could influence a reduction in the rate of purine biosynthesis caused by a reduction of PRPP in competition with the purine salvage pathway. No study has investigated *de novo* biosynthesis with impaired xanthine oxidase activity following allopurinol administration.

2.6.6.2 Allopurinol and purine excretion with exercise

As mentioned previously, intense exercise sufficient to reduce ATP levels in the active muscle, elevates the purine levels in the blood (Stathis et al. 1994) and increases urinary excretion of these metabolites (Nasrallah and Al Khalidi, 1964). All-out exercise provides a large increase in the flux of the AdN degradation pathway and presents the plasma with up to 9% of the resting muscle AdN pool `(Hellsten et al. 1999). With complete inhibition of xanthine oxidase it would be anticipated that there would be large increases in the plasma concentrations, and urinary excretion of plasma purine bases and little change in the plasma uric acid levels or its urinary excretion after exercise. Allopurinol increases the exercise-induced rise in plasma concentration of purines during 15 mins of recovery (30 min from the beginning of exercise time, which includes about 15 min of exercise time; Sutton et al. 1980). However, the recovery time examined by Sutton et al. (1980) was not sufficient to consider the effects on plasma uric acid accumulation. Plasma uric acid increases significantly above resting concentrations after 20-30 min of recovery from maximal

exercise and the time frame may not be sufficient for a peak in the plasma oxypurines (Stathis et al. 1994). Therefore, further research is required to ascertain the influence of allopurinol and exercise on the accumulation of purine degradation products in the plasma and urine following intense exercise.

Furthermore, the potential for an increased capacity for purine salvage with allopurinol administration provides a potential mechanism to maintain the AdN content of muscle during sprint training. Hence investigating the influence of allopurinol during, and in recovery from, a single high intensity "all out" exercise on plasma accumulation and urinary excretion of purines requires further investigation.

2.7 Summary

There is a transient reduction of muscle ATP during maximal short duration exercise and subsequent recovery. The initial reduction is due to an imbalance in ATP hydrolysis and resynthesis pathways and results in intramuscular IMP accumulation. A majority of the IMP is reaminated to ATP, however a small proportion is degraded further to purines, inosine and Hx. The purines not salvaged in the muscle traverse the sarcolemma and accumulate in the plasma and are eventually excreted. These purine bases thus represent a loss of ATP precursor in the muscle that must be restored by *de novo* biosynthesis. Intense intermittent sprint training results in a reduction of resting skeletal muscle ATP content and represents the net loss of purine nucleotide metabolites from the muscle over the training period. This is a consequence of a greater rate of loss of purine metabolites from the muscle compared to the capacity for purine recovery and/or synthesis by the muscle over the training period.

There are a number of areas of purine metabolism in skeletal muscle and sprint training that require further investigation. Those highlighted in this review include the

influence of the number of sprint bouts on the accumulation of purines in the plasma and the extent of purine loss via urinary excretion. The influence of sprint training on the loss of endogenous purines via the urine following a single sprint bout requires investigation. Furthermore, no research is available on the influence of allopurinol on, plasma or urinary purine metabolism during intense exercise and sprint training. Allopurinol has the potential to influence the extent of purine salvage within the muscle and can play a role in the removal of purine metabolites within the plasma due to its pharmacological inhibition of Hx to uric acid. Additionally, sprint training with a pharmacologically enhanced capacity for salvage may influence the extent of the reduction of resting ATP content observed following sprint training.

The major purpose of this thesis was to investigate some of the factors influencing purine nucleotide metabolism in human skeletal muscle and to determine the fate of these purines metabolites downstream of efflux from the muscle into the plasma. In particular, it will focus on the breakdown of muscle PrN degradation during high intensity exercise and the fate of purine bases subsequently produced and the events downstream of their efflux into the plasma, in particular their excretion in the urine. Furthermore, it will investigate the direct influence of allopurinol as a xanthine oxidase inhibitor on purine metabolism in the plasma and the apparent longer term intramuscular influence it has on purine salvge.

The thesis aims to test the following hypotheses;

- Increasing the number of successive bouts in an intermittent exercise protocol will increase the accumulation of plasma purine metabolite accumulation and the extent of purine excretion during subsequent recovery.

- Sprint training will attenuate the concentration of plasma inosine, Hx and uric acid, and subsequently reduce urinary excretion of purine metabolites during recovery from a 30 s sprint bout.
- A combination of allopurinol and repeated sprint exercise will elevate the urinary excretion of purine bases (Hx and inosine), balanced by a decreased urinary uric acid excretion in recovery, resulting in an unchanged total urinary purine excretion during subsequent recovery.
- The underlying influence of allopurinol administration on purine salvage would attenuate the extent of the sprint-induced reduction of ATP observed in resting muscle.

Chapter 3

Methods and Procedures

3.1 Introduction

This chapter describes the general methods and procedures used in all studies presented in this thesis. Details of specific experimental protocols used during the individual studies will be described in the chapter of the appropriate study. The studies in this thesis are listed as follows;

Study 1: Purine loss after repeated sprint bouts in humans.

Study 2: Sprint training reduces urinary purine loss following intense exercise in humans.

Study 3: The influence of allopurinol on urinary purine loss after repeated sprint exercise in humans.

Study 4: The influence of allopurinol and sprint training on purine nucleotide metabolism in humans at rest.

3.2 Participants.

The volunteers in all studies were healthy males between the ages of 18-35 years. They were active but non-specifically trained and were required to be non-smokers. All studies were approved by the Victoria University Human Experimentation Ethics Committee before proceeding. All volunteers were fully informed of the experimental procedures and signed informed consent forms and were free to withdraw from any study at any time. The participants were also instructed to refrain from strenuous exercise, caffeine and alcohol consumption 24 hours prior to the experimental trials. In addition, subjects recorded their diet for the 24 hours prior

to the first experimental trial and were asked to consume similar foods before any subsequent trials. Additionally, they were instructed to refrain from alcohol during any training periods. All experimental trials were conducted in the morning after an overnight fast. During the first 15 min of recovery from experimental trials in studies 2, 3 and 4 subjects ingested 500 ml of water to improve urine volume production. Subjects were also restricted from ingesting food during the first two hours of recovery in all trials and where possible maintained a similar diet during the remaining 22 hours of recovery.

3.3 Measurement of Peak Aerobic Capacity

The peak oxygen consumption (VO_2 peak) of each subject was determined about one week prior to beginning the experimental trials. The exercise protocol involved riding on a cycle ergometer (Lode, Groningen) for three min at three submaximal work rates, subsequently the work rate was increased every min thereafter until volitional exhaustion. Participants were encouraged to maintain a pedalling frequency of 80 revolutions per minute. Expired air was directed, by a Hans Rudolph valve, through a ventilometer (Pneumoscan S30) into a mixing chamber and analysed for oxygen and carbon dioxide content by gas analysers (Applied Electrochemistry S-3A O_2 and CD-3A CO_2). These analysers were calibrated before each test using commercially prepared gas mixtures. Oxygen consumption was calculated by a microprocessor using standard equations.

3.4 Sprint Testing Protocols

The sprint cycling performance tests bouts were performed on an air-braked ergometer (study 1: Series A, Repco, Melbourne, Australia; study 2, 3 and 4: a cycle

ergometer custom made for the Victorian Institute of Sport) modified to enable computerised determination of peak power, mean power and fatigue index. The power output of the air-braked cycle ergometer is proportional to the cube of the wheel velocity, which was measured using a tachometer (Hall-effect device and a cog at the wheel hub). The random error produced when using air-braked cycle ergometers in "all out" sprint tests has been reported to be as low as 1% (Paton and Hopkins, 2001). Subjects were instructed to remain seated and pedal as fast as possible for the duration of the test. In multiple sprint protocols all recovery was passive rest. Subjects were familiarised with the respective sprint tasks for each study by performing the sprint bout (without sampling procedures) at least one week prior to the trials.

3.5 Blood sampling and treatment.

Blood was sampled from an antecubital vein, via an indwelling catheter, at rest, and during recovery following performance tests. For repeated sampling of blood, the catheter was kept patent by periodic flushing with small amounts of heparinised saline. The blood was immediately placed into lithium heparin tubes and spun in a centrifuge. Subsequently, 100 µl of plasma were added to 200 µl of ice-cold 3 M perchloric acid, spun and the supernatant was stored at -80° C before analysis for lactate. The remaining plasma was stored in liquid nitrogen for later analysis of inosine, Hx and uric acid. Prior to analysis 100 µl of plasma was thawed and deproteinised 50 µl of 1.5 M perchloric acid and subsequently neutralised with 37 µl of 2.1 M potassium hydrogen carbonate immediately prior to analysis.

3.5.1 Blood and plasma analysis

Plasma lactate was determined in duplicate, using an enzymatic spectrophotometric technique (Lowry and Passonaeu, 1972). Plasma Hx, inosine and uric acid were determined on neutralised perchloric acid extracts, using a modification of the reverse phase high performance liquid chromatography (HPLC) technique described by Wynants and Van Belle (1985) using a Waters 600 chromatography system (Waters Chromatography, Milford, MA, USA). A Hibar Lichrosphere 100CH-18/2 (Merck) (240 x 4mm) analytical column was used to perform the analysis. The mobile phases used for separation consisted of 0.15 M ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 6.00 and a mixture of acetonitrile and methanol (50/50, v/v). The eluent was monitored at 254 nm.

3.6 Urine sampling and treatment

Urine was collected between 12-24 hours prior to any exercise testing protocol. In addition, urine was collected for the first 2 hours and the subsequent 6, and 16 hour periods following the exercise testing protocols employed. Urine volumes were determined and a sample was frozen at -80° C for subsequent analysis. A proportion of the urine was deproteinised with 1.5 M perchloric acid and subsequently neutralised with 2.1 M potassium hydrogen carbonate immediately prior to analysis of Hx, inosine. Another portion was thawed and analysed for uric acid.

3.6.1 Urine analysis

Urinary uric acid concentration was determined by an enzymatic colorometric method using a Beckman Synchron CX® system. Urinary Hx and inosine were

determined on neutralised perchloric acid extracts, using a modification of the reverse phase HPLC technique as described above in the plasma analysis (Section 3.5.1).

3.7 Muscle sampling and treatment

Muscle biopsies were obtained from the vastus lateralis under local anasthesia (1% xylocaine). An incision was made at the site in the skin (one incision for each sample per sprint test) and muscle samples were obtained from the vastus lateralis muscle. With multiple samples in a trial samples were taken distal to proximal (3 cm apart) and the proximal incision was approximately 10 cm to the lateral epicondyle of the femur, using the percutaneous needle technique (Bergström, 1962) modified for suction (Evans et al. 1982). Leg selection was random and in the second trial the contralateral leg was biopsied. Muscle samples were frozen in liquid nitrogen within 5 s of obtaining biopsies. This small time delay in freezing would have little consequence on the metabolite levels of resting muscle samples. However, it may play a small role in the post exercised samples, in particular, the PCr and Cr levels where estimates of PCr resynthesised in a 5 s delay are significant (Sant' Ana Pereira et al. 1996). This is an inevitable consequence of the methodology employed.

3.7.1 Muscle extraction and analysis

Muscle samples were weighed at -30^{o}C and freeze-dried (Edwards, Modulyo) for at least 48 hours, dissected free of connective tissue and blood under a dissection microscope and then crushed and powdered at room temperature. The powder was mixed and approximately 2 mg was extracted with pre-cooled 0.5 M PCA/1mM ethylenediaminetetra-acetic acid (EDTA). The extract was subsequently neutralised with cold 2.1 M potassium hydrogen carbonate (KHCO3; Harris et al. 1974). The

neutralised extracts were assayed enzymatically for PCr, Cr, ATP and Lactate by fluorometric analysis (Turner Digital Filter Fluorometer, Model 112) according to the methods described by Lowry and Passoneau (1972). ATP, ADP, AMP, IMP, inosine and Hx were determined using reverse-phase HPLC. This technique was modified from the methods described by Wynants and Van Belle (1985) using a Waters 600 chromatography system (Waters Chromatography, Milford, MA, USA) and is described above in section 3.5.1. Muscle ATP, ADP, AMP, IMP, creatine and PCr were corrected to the highest individual total creatine (PCr + Cr) values for samples in each trial (note, not corrected across training). All muscle metabolite concentrations are expressed per kilogram of dry weight $\{(kg\ dw)^{-1}\}$.

The variability of the enzymatic analysis procedures was within 5% limits for the coefficient of variation (CV) for most metabolites. PCr was the only exception to this where the CV was approx 7.5%. The variability for HPLC, however, was not as tight with the CV ranging between 5-10% for all measured metabolites.

3.8 Allopurinol administration

In studies that utilised allopurinol, a double blind random procedure was used to allocate subjects to treatment groups. The volunteers ingested either a tablet of calcium carbonate every day for the placebo group or 300 mg doses of allopurinol daily for the experimental group. Pilot testing indicated that calcium carbonate was an appropriate placebo for this study and these data are shown in Appendix A.

3.9.1 Statistical analyses

Where appropriate, the metabolite and performance data were analysed using analysis of variance (ANOVA) with repeated measures (BMDP statistical software).

Simple main effect analyses and Newman-Kuels post hoc tests were used to locate differences when ANOVA revealed a significant interaction. Appropriate T-Tests were also employed for comparisons of two sets of data. The level of probability to reject the null hypothesis was set at $P<0.05$. All values are reported as means ± SE.

3.9.2 Power analysis to determine n (number of participants)

Study 1, A sample size (n) of 7, with a power of 88% and a significance level set at $P<0,05$, was sufficient to detect a 20% increase in plasma Hx concentration observed in an earlier study using a single 30s sprint bout. There are no previous studies reporting urinary purines, with sufficient recovery time, following intense exercise. Hence plasma hypoxanthine concentration was considered to be the most appropriate guide for the power analysis of this study as the metabolic by products measured in the urine are downstream of the accumulation of Hx in the plasma. Data from plasma Hx accumulation during recovery from a 30s bout (Stathis et a. 1994) was used as a guide for this study.

Study 2, Historical data provided little guidance for the power analysis of this study as most experiments investigating allopurinol only report basal urinary measurements. Thus results from the first study were used to determine that n=9 was adequate to achieve a 20 % difference in total urinary purine metabolites after 8x10s sprint bouts. The power was 0.95 with the significance level set at $P<0.05$.

Study 3, The primary metabolite in these studies was the resting ATP content of the skeletal muscle. The number of subjects was shown to be sufficient to detect a

reduction in resting ATP similar to the magnitudes observed in similar training protocols (Hellsten-Westing et al. 1993) with a significance level set at $P<0.05$.

Study 4; With allopurinol treatment, n = 7 per group was sufficient to detect a 10% change in post trained resting ATP, using data from Hellsten-Westing et al. (1993), relative to the placebo group (a power of 77% and a significance set at $P<0.05$).

Chapter 4

Purine loss following repeated sprint bouts in humans

4.1 Introduction

The metabolic demand of intense sprint exercise requires a high skeletal muscle ATP turnover. This high turnover usually results in the failure of ATP resynthesis to match ATP hydrolysis rates thereby causing a reduction in skeletal muscle ATP content (Bogdanis et al. 1998; Gaitanos et al. 1993; McCartney et al. 1986; Nevill et al. 1989; Stathis et al. 1994). During short-duration, high intensity exercise the fall in muscle ATP content is matched by an equimolar increase in inosine monophosphate (IMP) and ammonia (Fig. 2.1, Katz et al. 1986 a; Katz et al. 1986b; Jansson et al. 1987; Stathis et al. 1994). A small proportion of the accumulated IMP can undergo further degradation to produce inosine, which can be subsequently converted to Hx (Bangsbo et al. 1992; Tullson et al. 1995). Both inosine and Hx are either resynthesised to IMP via the purine salvage pathway (Fig. 2.1; Edwards et al. 1979; Manfredi and Holmes, 1984) or leave the muscle and accumulate in the plasma (Balsom et al. 1992a; Balsom et al. 1992b; Harkness et al. 1983; Hellsten-Westing et al. 1991; Hellsten-Westing et al. 1994; Ketai et al. 1987; Sjodin and Hellsten-Westing 1990; Stathis et al. 1994). Importantly, once Hx has diffused from the muscle it appears to be permanently lost as a precursor to muscle ATP synthesis since Hx uptake into the recovering muscle following intense exercise does not occur (Hellsten et al. 1998). Hx and inosine may be removed from the plasma via renal excretion (Nasrallah and Al-Khalidi, 1964; Sutton et al. 1980) or by the liver (Hellsten-Westing et al. 1994). The liver converts Hx to uric acid (Hellsten-Westing et al. 1994; Vincent

et al. 1984) which is released into the circulation and excreted by the kidney (Nasrallah and Al-Khalidi, 1964; Sutton et al. 1980), or may be taken up by the recovering muscle where it is possibly used as an oxygen free radical scavenger (Hellsten 1996), but not for AdN resynthesis. The latter removal pathway only occurs when plasma uric acid levels are high (Hellsten et al. 1998). The elevated plasma accumulation and urinary excretion of inosine, Hx and uric acid following intense exercise represents a loss of ATP from the active and recovering muscle.

The high intensity, intermittent exercise associated with sprint training provides an opportunity for increased flux through the purine nucleotide catabolic pathways which may lead to a chronic reduction of resting muscle ATP content. The effect of sprint training on resting skeletal muscle ATP content is controversial. Several studies have reported no change (Boobis et al. 1983; Nevill et al. 1989; Thorstensson et al. 1975), whereas others have demonstrated a reduced resting skeletal muscle ATP content following sprint training (Hellsten-Westing et al. 1993a; Stathis et al. 1994). The reasons for the discrepant results are not obvious but may be related to variations in training intensity, exercise bout duration and the recovery interval between bouts. Several studies have indicated that a greater production of purine bases occurs when the intensity of exercise is high compared with less intense exercise (Ketai et al. 1987; Sjodin and Hellsten-Westing, 1990). Additionally, an increased sprint duration from 2 to 6 s bouts during intermittent exercise was also associated with an elevated plasma Hx concentration (Balsom et al. 1992a). Furthermore, a reduction in recovery duration between bouts (Balsom et al. 1992b) has also been shown to increase plasma purine base concentration.

The number of sprint bouts completed per training session may also explain, at least in part, why some studies have observed an attenuated resting muscle ATP

content after sprint training. To date however, there are no studies which have examined the influence of the number of sprint bouts on muscle purine loss. Several studies (Gaitanos et al. 1993; McCartney et al. 1986) have reported that most of the fall in muscle ATP content occurs during the initial bouts of intermittent, high intensity exercise with little, if any, change occurring in the latter bouts. If the recovery duration between bouts prevents significant recovery of ATP (less than 3 min; Graham et al 1990; Snow et al. 1998; Stathis et al. 1994) then an increased number of exercise bouts per training session may elevate muscle purine loss. This may occur because the enzymes associated with the degradation pathway of IMP have a longer period in which they are exposed to an elevation of their substrate concentrations. Therefore, the aim of the present study, was to investigate the influence of single and multiple intermittent sprint exercise protocols on the accumulation and excretion of purine bases in the plasma and urine, respectively. It was hypothesised that increasing the number of sprint bouts will result in an elevation of plasma purine concentration and urinary purine excretion following sprint exercise thereby indicating an increased purine loss from the active muscle.

4.2 Methods

4.2.1 Subjects.

Seven active, non-specifically trained males (age: 24.9 ± 1.7 years, weight: 77.3 ± 3.8 kg, VO_2peak: 50.6 ± 3.8 $ml.kg^{-1}.min^{-1}$), volunteered for the study.

4.2.2 Exercise Protocols.

Subjects were asked to perform three separate experimental exercise trials, consisting of either one (B1), four (B4) or eight (B8) 10s "all out" sprint bouts on an

air-braked cycle ergometer (series A, Repco, Melbourne, Australia). Multiple bouts were separated by 50s of passive rest on the cycle ergometer and all trials were assigned in random order. Subjects were familiarised with the intermittent sprint task by performing the B8 protocol, without blood or urine collection procedures, at least one week prior to testing.

4.2.3 Blood and urine sampling, treatment and analysis.

Blood was sampled from an antecubital vein, via an indwelling catheter, at rest, and where appropriate following the first, fourth and eighth bout of the trials. Blood samples (10 mls) were also obtained following 5, 10, 15, 20, 30, 45, 60 and 120 min of passive recovery after the final exercise bout. Plasma inosine, Hx, uric acid and lactate were analysed (section 3.5). Urine was collected prior to and following exercise and analysed for inosine, Hx and uric acid (section 3.6).

4.3 Results

4.3.1 Performance data

Peak power and mean power were not different (P>0.05) between the first bout of all trials and for the fourth bout of the B4 and B8 trials (Table 4.1).

Table 4.1. Peak and mean power during the first, fourth, and eighth bout of 10 s sprint cycling in the three experimental protocols (B1, B4, B8).

	First bout			Fourth bout		Eighth bout
	B1	**B4**	**B8**	**B4**	**B8**	**B8**
Peak Power (W)	1052 ± 54	1049 ± 55	1048 ± 44	956 ± 47	915 ± 43	807 ± 62
Mean Power (W)	821 ± 36	795 ± 55	816 ± 30	699 ± 35	718 ± 42	601 ± 61

Values are means ± SE, n=7.

4.3.2 Plasma metabolites

All of the measured plasma metabolites were similar ($P>0.05$) at rest between the trials. Plasma inosine increased ($P<0.05$) in concentration during recovery in B4 and B8 but remained at pre-exercise levels throughout recovery in B1 (Fig 4.1A). In B4 and B8 the plasma inosine levels peaked at 30 min and returned to basal levels after 60 min. The inosine concentration was higher ($P<0.05$) during most of the recovery period in B8 and B4 compared with B1. Furthermore, the inosine concentration in B8 was greater than B4 after 20, 30 and 45 min of recovery. The plasma Hx concentration (Fig. 4.1B) peaked between 20-30 min following the cessation of exercise and had not returned ($P<0.05$) to basal levels after 60 min of recovery in all trials. In B1, the plasma Hx concentration was similar to pre-exercise levels after 120 min of recovery, however, the Hx levels remained elevated in the other two trials. At most sampling times during recovery the plasma Hx concentration was higher ($P<0.05$) after B8, compared with B4 and B1. Furthermore, the plasma Hx concentration was also greater in B4 compared with B1. Plasma uric acid concentration increased ($P<0.05$) above resting levels after 15 min of recovery in all exercise trials (Fig. 4.1C) and remained above pre-exercise levels for at least 120 min of recovery. The plasma uric acid concentration was higher ($P<0.05$) in B8 compared with B1 at 60 min of recovery but no differences were observed between B4 and the other trials at this time. After 120 min of recovery the uric acid concentration was higher in the B4 and B8 trials when compared to the B1 trial, however no difference was observed between B4 and B8. Plasma lactate concentration increased ($P<0.05$) above resting values during the early stages of recovery in all trials (Fig. 4.2). At most sampling times during the first 45 min of recovery plasma lactate concentrations were progressively higher when a greater number of exercise bouts were performed.

4.3.3 Urine metabolites

The basal rate of urinary Hx excretion was similar (P>0.05) between trials (Fig 4.3A). Increases (P<0.05) in this rate were observed in the two hours following exercise in all trials**.** The rate of Hx excretion during this time period was greater (P<0.05) in B8 compared with B4 and B1. Similarly, this rate was higher (P<0.05) in B4 compared with B1. There were no differences (P>0.05) between trials during the later stages of recovery and these rates were similar to basal rates. Statistical analysis of the urinary uric acid and inosine excretion rates revealed no significant interaction (time x trial) or main effect for trial (Fig 4.3B,C). There was however, a significant main effect for time. This demonstrated that the uric acid and inosine excretion rates were elevated during the two hours immediately after exercise compared with basal rates and those measured in recovery following the initial two hour period. The total amount (μmol) of inosine, Hx and uric acid excreted above basal levels into the urine during the two hour recovery period are reported in Table 4.2. The amount of Hx excreted following B8 was 1.4 and 3 fold greater (P<0.05) than B4 and B1, respectively. The amount of inosine and uric acid excreted were not different between trials. Although there was a trend (P=0.09) for the total urinary purine (inosine + Hx + uric acid) excretion to be greater in B8 compared with the other trials this did not reach statistical significance.

4.3.4 Estimated muscle purine loss

In order to estimate muscle purine loss the following assumptions were made. First, the Hx and uric acid concentration accumulated above basal levels after 120 min of recovery were permanently lost from the muscle adenine nucleotide pool. It should be noted that plasma inosine had returned to pre-exercise levels after two

hours of recovery in all trials (Fig. 4.1A), therefore this metabolite was not included in the calculations. Second, we assumed that Hx and uric acid were evenly ditributed in 25 and 18.5 l, respectively (see discussion for rationale). Using these assumptions we calculated that the estimated muscle purine loss in the B8 trial was 1.4 and 4.9 fold greater ($P<0.05$) than B4 and B1, respectively (see Table 4.2). Estimated muscle purine loss was also greater ($P<0.05$) in B4 compared with B1 (Table 4.2).

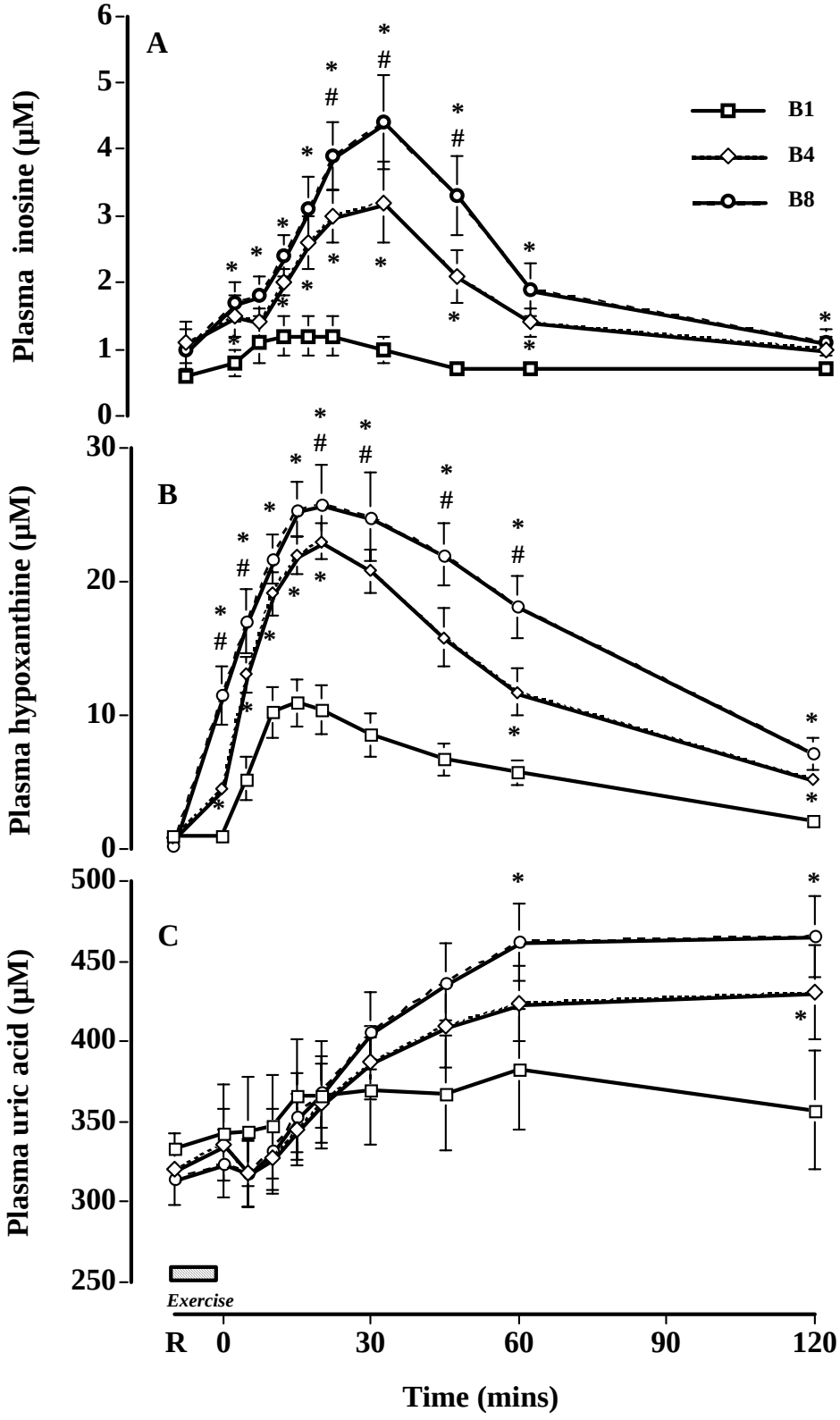


Fig. 4.1. Forearm venous plasma inosine (A), hypoxanthine (B), and uric acid (C) concentration at rest (R) and during 120 min of recovery following one (B1), four (B4) and eight (B8) 10s sprint cycling bouts. Values are means ± SE, n=7. * different from B1, # different from B4 ($P<0.05$).

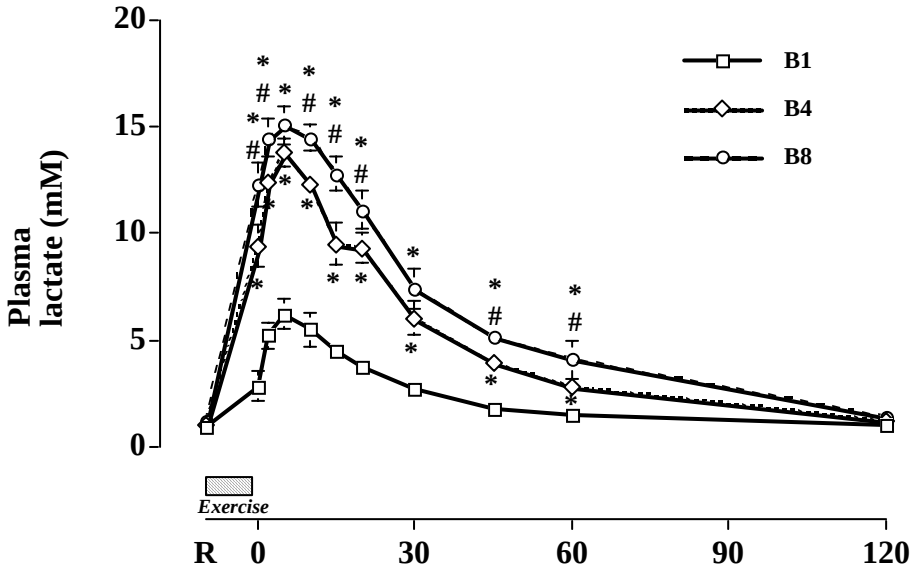


Fig. 4.2. Forearm venous plasma lactate concentration at rest (R) and during 120 min of recovery following one (B1), four (B4) and eight (B8) 10s sprint cycling bouts. Values are means ± SE, n=7. * different from B1, # different from B4 ($P<0.05$).

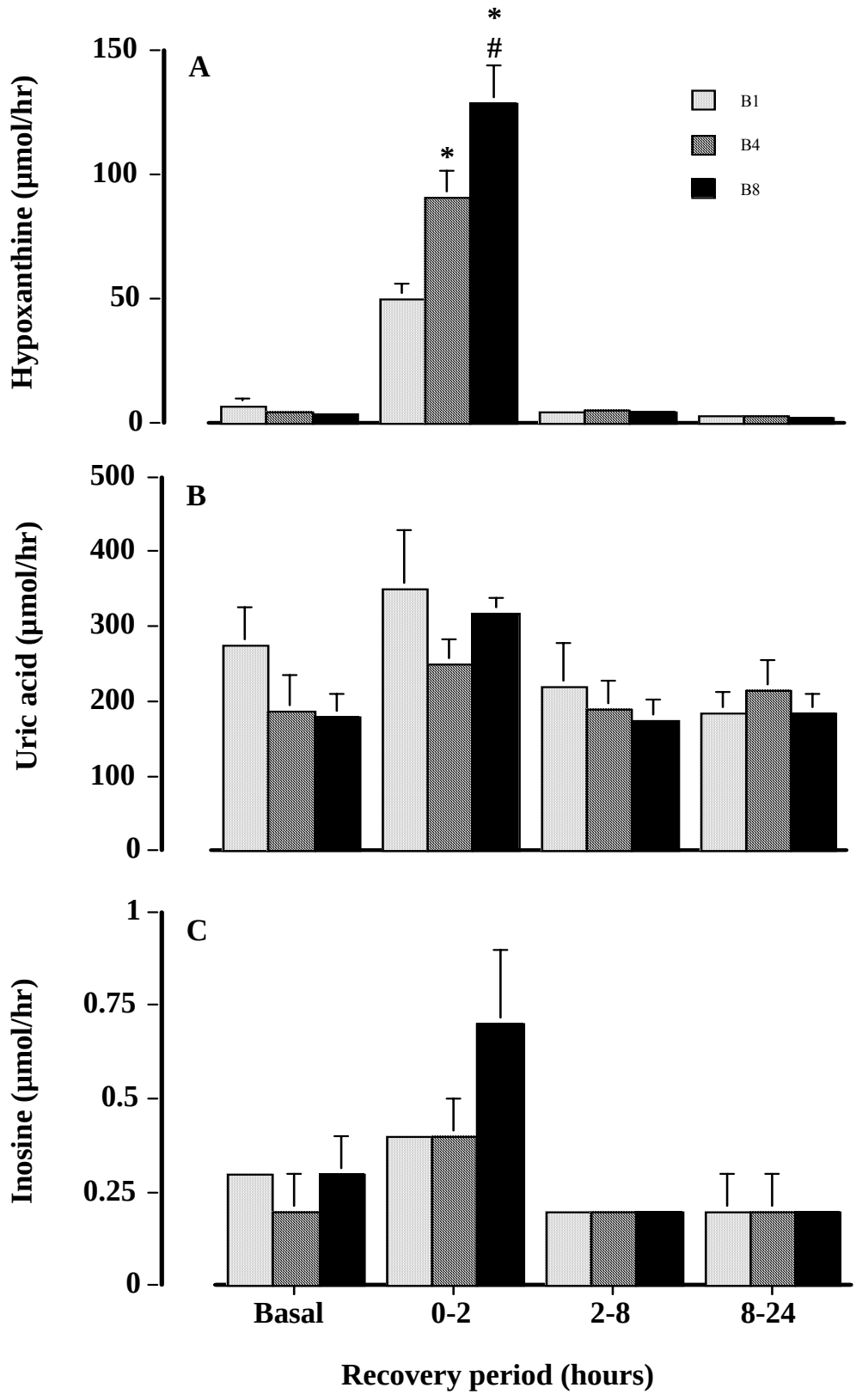


Fig. 4.3. Urinary hypoxanthine (A), inosine (B) and uric acid (C) excretion rate at rest (R) and during the first 2 hours and the subsequent 6 and 1o6 hours of recovery following one (B1), four (B4) and eight (B8) 10s sprint cycling bouts. Values are means ± SE, n=7. * different from B1, # different from B4, † main effect for time ($P<0.05$).

Table 4.2. Calculated purine loss after 2 hours of recovery from one (B1), four (B4) or eight (B8) 10s sprint cycling bouts.

	B1	**B4**	**B8**
Hx lost to urine after 2 hours of recovery	85.4 ± 10.4	172.5 ± 22.0*	249.2 ± 31.1*#
Uric acid lost to urine after 2 hours of recovery	151.1 ± 96.4	129.1 ± 63.1	276.6 ± 66.4
Inosine lost to urine after 2 hours of recovery	0.7 ± 0.1	0.7 ± 0.2	1.3 ± 0.4
Total urinary purine loss after 2 hours of recovery	237 ± 94	302 ± 74	527 ± 77
Accumulated plasma Hx at 2 hours of recovery ($\mu mol.l^{-1}$)	1.1 ± 0.4	4.3 ± 0.4*	6.0 ± 1.1*
Accumulated plasma uric acid at 2 hours of recovery ($\mu mol.l^{-1}$)	24.1 ± 10.3	110.4 ± 7.6*	151.1 ± 8.9*#
Estimated Hx within body‡ fluids	28.2 ± 9.8	106.8 ± 10.3*	149.6 ± 27.0*
Estimated uric acid within extracellular fluid†	447 ± 191	2044 ± 141*	2796 ± 166*#
Estimated muscle purine loss	712 ± 209	2453 ± 181*	3473 ± 178*#

Values are means ± SE, expressed in μmol except where indicated. Accumulated plasma metabolite values were calculated by subtracting the metabolite concentration at rest from metabolite concentration at 2 hours of recovery. ‡ Estimation based on the assumption that Hx was evenly distributed in 25 l (extracellular fluid (ECF) volume + intracellular fluid (ICF) volume-intramuscular water volume). † Estimate based on the assumption that uric acid was evenly distributed in the ECF volume (18.5 l). * different from B1 ($P<0.05$), # different from B4 ($P<0.05$).

4.4 Discussion

The present study demonstrates that increasing the number of sprint bouts performed in an exercise session results in elevated plasma inosine, Hx and uric acid concentrations. Furthermore, increasing the number of sprint bouts resulted in an increased urinary Hx excretion and estimated skeletal muscle purine loss after 2 hours of recovery. Previous studies (Bangsbo et al. 1992; Hellsten-Westing et al. 1994; Sahlin et al. 1991; Stathis et al. 1994) indicate that the major source of plasma inosine, Hx and uric acid, during and in recovery from intense exercise, is the degradation of the ATP stores within contracting skeletal muscle. The present data, coupled with the findings of previous research, suggest that skeletal muscle purine loss is progressively enhanced when the number of 10 s sprint cycling bouts performed in a single session is increased from one to four to eight bouts. Although speculative, a difference in the number of sprint bouts performed per exercise session may explain, at least in part, why some studies have reported an attenuated resting muscle ATP content after sprint training (Hellsten-Westing et al. 1993a; Stathis et al. 1994), while others have not (Boobis et al. 1983; Nevill et al. 1989; Thorstensson et al. 1975).

The increase in plasma inosine, Hx, and uric acid concentration with more sprint bouts observed in the present study must be due to a greater difference between the rates of entry of these metabolites into, and removal from, the circulation. The results from the present study are unable to determine the relative importance of the inosine, Hx and uric acid plasma entry and removal rates between the three trials. However, it is highly likely that an increase in sprint bout number results in an enhanced rate of entry of these metabolites into the plasma. The increased entry rate of inosine and Hx into the plasma is likely to occur for the following reasons. First, it

has been demonstrated that one 10 s sprint bout is sufficient to acutely deplete the contracting muscle ATP pool by about 21% (Bogdanis et al. 1998). Second, a more marked reduction (i.e., 41%) in muscle ATP content has been reported immediately following 10x6 s sprints (Gaitanos et al. 1993). Third, the fall in ATP content is highly likely to be associated with a concomitant increase in intramuscular IMP concentration (Jansson et al. 1987; Stathis et. 1994). Finally, the increased IMP content probably occurs throughout the entire sprint protocol in the present study because the reamination of IMP and the return of ATP stores to resting levels requires several minutes (Graham et al. 1990; Snow et al. 1998; Stathis et al. 1994). Taken together these data strongly indicate that the muscle IMP content is likely to be elevated for a longer period of time in B8 compared with B4, and when B4 is compared with B1. In the current study, the accumulation of IMP in the initial bouts is likely to increase the activity of 5'-nucleotidase (Camici et al. 1985) and consequently the production rate of inosine and Hx (Fig. 2.1). Since the Km of 5'-nucleotidase for IMP is low (i.e., 100 μM, Camici et al. 1985), the enzyme is likely to be saturated with substrate in intensely contracting muscle and during the early stages of recovery. For example, a 10% decrease in muscle ATP content equates to an increase in IMP concentration of about 0.8 $mmol.l^{-1}$ of intracellular water. Consequently, the flux generating step for inosine and Hx formation is at maximal capacity, hence the production of these metabolites is mainly influenced by the exposure time of 5'-nucleotidase to its substrate. Therefore, each additional sprint performed in the present study allows further time for IMP degradation to proceed.

The above evidence suggests that the elevated plasma inosine and Hx concentrations during and following sprint exercise were due to an increased rate of entry into the plasma. Plasma removal rates of these metabolites are also a factor,

however, they cannot explain the rise in plasma concentration in early recovery since removal rates are likely to be increasing during this period. Clearly, the elevation in plasma Hx concentration observed in the present study (Fig. 4.1B) provides indirect evidence that the rate of inosine oxidation to Hx has increased. Some of this conversion may occur in the circulation since purine nucleoside phosphorylase activity has been found in the plasma (Harkness et al. 1983a) and endothelial cells (Rubio and Berne 1980). Another fate of circulating inosine and Hx is their excretion into the urine (Sutton et al. 1980). Our data demonstrated that the rates of urinary inosine and Hx excretion were elevated above basal levels during the intitial two hour period of recovery in all trials (Fig. 4.3 A,B). Furthermore, the rate of Hx excretion during this period was greater when more bouts were performed. Another removal pathway for plasma inosine and Hx is via the liver (Hellsten-Westing et al. 1994). Furthermore, these authors provided evidence that most of the Hx entering the liver was converted to uric acid which was then released into the circulation. A small proportion of the circulating Hx may also be oxidised to form uric acid by capillary endothelial cells (Jarasch et al. 1981). The elevated plasma uric acid concentration during recovery from B8 and B4 compared with B1 observed in the present study (Fig. 4.1C) provides indirect evidence that the rate of Hx removal and uric acid production by the liver was increased during recovery in these two trials. A proportion of the uric acid can also be taken up by the recovering muscle (Hellsten-Westing et al. 1994; Hellsten et al. 1998) where it may be converted to allantoin (Hellsten, 1996). Unfortunately our experimental design does not allow us to account for this uric acid uptake. Nevertheless, our data suggest that plasma inosine and Hx removal rates were elevated above basal levels during recovery from high intensity exercise. In addition, the urinary Hx excretion rate and plasma uric acid data also

indicate that the plasma Hx removal rate was higher when more sprint bouts were performed.

The total urinary purine (inosine + Hx + uric acid) excretion, a marker of whole-body purine loss, tended to be greater ($P<0.09$) in B8 compared with the other two trials during the 2 hour recovery period (Table 4.2). The lack of statistical significance is likely due to a type II error. Based on the elevated plasma Hx and uric acid concentrations at 2 hours of recovery in the B8 and B4 trials, it may be expected that further urinary excretion of these metabolites would occur after the 2 hour collection period. This expectation, however, appears to be unfounded since the urinary excretion rate of Hx and uric acid in the subsequent 6 and 16 hour recovery period (Fig 4.3 A,B) was not different between trials. It is unclear why this was the case. It is possible that we were unable to detect an increased urinary Hx excretion during the latter 22 hours of recovery in B8 and B4 trials since the accumulated plasma Hx after the two hour recovery period was converted to uric acid and excreted in this form. It is also possible that we failed to detect an increased urinary uric acid excretion during the latter stages of recovery in the intermittent sprint trials since the urinary uric acid excretion rate was quite variable. In addition, it cannot be ruled out that some of the uric acid accumulation in the plasma was removed by other means. Gut loss has also been shown to play a significant role in purine excretion (Sorensen and Levison, 1975). There is also the possibility that uric acid is taken up by recovering skeletal muscle (Hellsten-Westing et al. 1994; Hellsten et al. 1998). It should be noted, however, that this process only occurs during the early stages of recovery (e.g., first 20 min) and is therefore unlikely to explain our results.

The estimated muscle purine loss was greater ($P<0.05$) with an increasing number of sprint bouts (Table 4.2). This estimate was based on the amount of inosine,

Hx, and uric acid accumulated in the urine and body fluids after two hours of recovery. Although the exact distribution volume for Hx is unknown it has been demonstrated that Hx uptake by skeletal muscle does not occur, however, Hx is readily taken up by liver and red blood cells (Hellsten et al. 1998). Based on this information a distribution volume of 25 l was used to estimate the amount of Hx remaining in the body fluids (i.e., extracellular fluid volume + intracellular fluid volume - intramuscular fluid volume). Similarly, the distribution volume for uric acid is uncertain but it is known that this metabolite is found throughout the extracellular fluid compartment (Grootveld and Halliwell, 1987). We have assumed that uric acid was evenly distributed within the extracellular fluid volume (18.5 l). The number of tissues involved with uric acid uptake remains unclear, but as mentioned previously recovering muscle may remove uric acid (about 330 $\mu mol.kg^{-1}$ wet weight) when the plasma concentration of this metabolite is high (Hellsten et al. 1998). It is therefore possible that we have underestimated the amount of uric acid accumulated with the body, especially after B8. Nevertheless, the basic finding that muscle purine loss was greater in B8 compared with the other trials should not be affected by this underestimation. The inclusion of the amount of Hx and uric acid in the body fluids in the calculation of muscle purine loss is reasonable since research has shown that these metabolites are not reincorporated into the recovering muscle adenine nucleotide pool (Hellsten et al. 1998). The increase in the estimated muscle purine loss with the increase in number of sprint bouts is consistent with the proposition that more time is available for muscle IMP degradation in the longer trials.

If it is assumed that skeletal muscle comprises 40% of body weight and 55% of the muscle mass is located in the legs (Snyder et al. 1975), then the mean weight of both leg muscles in our subjects is 3.95 kg dry mass (assuming 4.3 kg wet mass/kg

dry mass). Taking into account the amount of purines lost into the urine and an estimation of the amount of purines remaining in the body fluids after two hours of recovery, it can be calculated that 0.18, 0.62, 0.88 mmol of purine per kg dry mass was lost from the recovering leg muscle in B1, B4 and B8, respectively. The calculated muscle purine loss occurring during B1 represents about 3.7% of the fall in the contracting muscle ATP content assuming a resting ATP content of 23 $mmol.kg^{-1}$ dm and a decreased ATP content of 21% (Bogdanis et al. 1998). In B8, the calculated purine loss is about 9.3% of the decrease in the muscle ATP pool (assuming resting ATP = 23 $mmol.kg^{-1}$ dm and decreased ATP content of 41%; Gaitanos et al. 1993). These data are supported by Bangsbo et al. (1992) who calculated from arterio-venous and blood flow measurements that Hx loss from recovering skeletal muscle was at least 5% of the fall in the muscle ATP pool. Based on our data we estimate that the daily performance of the B8 protocol results in a muscle purine loss of 0.88 $mmol.kg^{-1}$ dm. This may be an underestimate since uric acid uptake by recovering muscle and loss via the gut was not considered. Based on Hellsten et al. (1998) measurements of recovering skeletal muscle uric acid uptake of 330 $\mu mol.kg^{-1}$ wet weight following intermittent arm and leg exercise, we estimate that the upper limit of muscle purine loss to be about 2.2 $mmol.kg^{-1}$ dm. This is likely to be the upper limit since studies examining intermittent leg exercise alone (Bangsbo et al. 1992; Hellsten et al. 1998) did not observe muscle uric acid uptake. Interestingly, the estimated purine loss (0.88-2.2 $mmol.kg^{-1}$ dm) should be more than adequately met by *de novo* adenine nucleotide synthesis, assuming that human skeletal muscle *de novo* adenine nucleotide synthesis rates are similar to rat mixed skeletal muscle (i.e., 3.6 $mmol.kg^{-1}$ $dm.day^{-1}$; Tullson et al. 1988). Unfortunately, there are no data published on human skeletal muscle *de novo* adenine nucleotide synthesis rates. Future research needs to

establish this rate to ascertain the capacity of *de novo* adenine nucleotide synthesis to prevent a fall in the adenine nucleotide pool despite an elevated muscle purine loss during sprint training.

4.4.1 Conclusion

In conclusion, this study demonstrated that there was an elevated plasma inosine and Hx concentration during recovery with an increasing number of sprint bouts. Furthermore, plasma uric acid concentration was greater in B8 and B4 compared with B1 after 120 min of recovery. The elevated plasma inosine and Hx levels occuring with more sprint bouts is best explained by an increased production and release of these metabolites from the recovering skeletal muscle. Where as, the increased plasma uric acid concentration in B8 and B4 compared with B1 is probably the result of an increased hepatic uric acid release into the circulation, subsequent to an enhanced liver Hx uptake and oxidation to uric acid. The present study also demonstrated a tendency for urinary purine excretion to be higher during the first two hours of recovery in B8 compared with the other trials. In addition, the magnitude of estimated skeletal muscle purine loss was increased as more sprint bouts were performed. These data provide convincing, indirect evidence that muscle purine base production and loss was enhanced as a function of increasing sprint bout number. However, it must also be noted that rather than the number of bouts being the primary factor in the reduction of skeletal muscle AdN content, it is likely to be the aggregate of the metabolic stress imposed on the muscle. This finding indicates that the extent of muscle adenine nucleotide degradation is not only dependent upon exercise intensity, exercise bout duration and recovery interval, it is also dependent on the number of bouts performed in an exercise session.

Chapter 5

Sprint Training Reduces Urinary Purine Loss Following Intense Exercise in Humans

5.1 Introduction

The ATP content of untrained human skeletal muscle is about 20-25 mmol.kg^{-1}dry mass (d.m.), and several reports have demonstrated that intense intermittent sprint training can lower the resting content by up to 20% (Harmer et al. 2000; Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994). The factors responsible for the set point of resting muscle AdN levels are unknown, however, it is influenced by the balance between loss of purine base from the muscle and its production via *de novo* purine synthesis. Intense exercise results in a transient reduction of ATP and concomitant accumulation of IMP (Stathis et al 1994) and most of the IMP is rapidly resynthesised to ATP during recovery. However, a small portion of IMP is further degraded and results in the production of the purine bases inosine and Hx (Stathis et al. 1994). Unlike the sarcolemma bound IMP, purine bases not recovered intramuscularly via purine salvage, efflux the muscle and accumulate in the plasma (Bangsbo et al. 1992; Hellsten et al. 1999; Tullson et al. 1995). Once in the plasma purine bases are not recovered by skeletal muscle (Hellsten et al. 1999). The magnitude of purine loss can be between 5% and 9% of the resting muscle ATP content (Bangsbo et al.1992; Hellsten et al. 1999). The plasma purines can be excreted (Chapter 4; Nasrallah and Al Khalidi, 1964; Sutton et al. 1980;), or converted to uric acid (Hellsten et al. 1994) and subsequently excreted (Chapter 4; Nasrallah and Al Khalidi, 1964; Sorensen and Levinson, 1975; Sutton et al. 1980).

Exercise intensity and duration can influence the plasma purine accumulation in recovery (Sjödin and Hellsten-Westing, 1990; Hellsten et al. 1999), as does the frequency and number of intermittent sprint bouts (Chapter 4; Balsom et al 1992a; Balsom et al. 1992b). The number of repetitions in a sprint challenge also influences the excretion of urinary purines during recovery (Chapter 4).

Sprint training reduces the accumulation of IMP (Harmer et al. 2000; Parra et al 2000; Stathis et al. 1994) and inosine (Stathis et al. 1994) in the muscle following an intense sprint. Furthermore, sprint training attenuates the plasma inosine, Hx and uric acid accumulation during recovery from an intense exercise bout (Stathis et al. 1994). No study has examined the effect of sprint training on urinary purine excretion following a maximal sprint bout is unknown.

The aim of this study was to investigate the effects of an intense seven-day sprint training program on the loss of endogenous purine metabolites in the urine during recovery from an intense sprint bout. It was hypothesised that sprint training will result in a reduced ATP degradation and an attenuated accumulation of muscle IMP and inosine during a 30s maximal sprint. This will subsequently attenuate the concentrations of plasma inosine, Hx and uric acid, and lead to a subsequent reduction in urinary excretion of purine metabolites during recovery from a 30 s sprint bout. Furthermore, we aimed to confirm previous findings of training induced reductions of muscle ATP degradation, IMP and inosine accumulation during a 30 s sprint. In addition to this, we aimed to investigate plasma inosine, Hx and uric acid in recovery from, a 30 s sprint in the sprint-trained state.

5.2 Methods

5.2.1 Subjects

Seven active, non-specifically trained males (age: 23.1 ± 1.8 years, weight: 76.1 ± 3.1 kg, VO_2peak: 56.3 ± 4.0 $ml.kg^{-1}.min^{-1}$), volunteered for the study.

5.2.2 Sprint performance test.

Performance tests (PT) were 30s maximal sprints completed before and after a training regime. Fatigue index (FI) was calculated using the equation FI = (peak power – power at the end of 30 s exercise)/ peak power x 100.

5.2.3 Training protocol.

Sprint training was performed at least 4 days after the first PT, and involved two sessions per day for a week. Each session was separated by at least 6 hours. A training session involved 15 x 10s maximal cycling bouts on an air-braked cycle ergometer with 50-s of rest between bouts.

5.2.4 Muscle sampling, treatment and analysis.

Muscle was sampled from the vastus lateralis (section 3.7) at rest, immediately upon cessation of the sprint and following 10 min of passive recovery. Muscle samples were analysed for ATP, ADP, IMP, inosine, Hx, PCr, Cr, Lac. Post-Training biopsy was obtained between 24-36 hours following the cessation of the last training session.

5.2.5 Blood and urine sampling, treatment and analysis.

Blood was sampled at rest, and where appropriate following PT and after 5, 10, 15, 20, 30, 60 and 120 min of passive recovery after the final exercise bout. Plasma inosine, Hx, uric acid and lactate were analysed (section 3.5.1). Urine was collected prior to and following exercise and analysed for inosine, Hx and uric acid (section 3.6.1).

5.3 Results

5.3.1 Performance variables.

No change in the peak power (1085.1 ± 40.3 v 1081.7 ± 38; untrained vs trained), mean power (617.9 ± 20.4 v 630.4 ± 10.1) or fatigue index (58.9 ± 1.6% v 57.5 ± 1.4%) was observed with the sprint training protocol employed in this study.

5.3.2 Muscle metabolite concentrations.

The muscle metabolite concentrations at rest, immediately post-exercise and 10 min into recovery are summarised in Table 5.1. No differences were observed with resting muscle metabolites before and after training. The resting ATP concentrations showed a tendency to be lower after training using different analytical methods ($p=0.06$ and 0.13, enzymatic and HPLC methods, respectively). Exercise decreased muscle ATP concentration in both the pre- and post-training PT but the concentration upon termination of exercise was significantly higher in the post-trained compared with the pre-trained state (Table 5.1). Similarly, sprint exercise resulted in an accumulation of IMP and inosine, however, the end exercise concentration of IMP and inosine were lower ($P<0.05$) with training (Table 5.1). The PCr, Cr and lactate levels after exercise were unaffected by the training.

The ATP content was partially restored 10 min into recovery from post-exercise values, but were still lower than pre-exercise concentrations, irrespective of training status. Additionally, the concentration at 10 min recovery was not different between trials. Similarly, the IMP content reduced during recovery but had not returned to resting levels in both trained and untrained states. Muscle inosine continued to increase during recovery in both trials, however the content was lower in the trained compared with the pre-trained value (Table 5.1). The PCr concentration was significantly higher in the post-trained, compared with the pre-trained state after 10 min of recovery. The PCr at 10 min recovery following training was not different to pre-exercise levels, whereas it was significantly lower in the untrained state. Muscle lactate and Cr concentrations during recovery were not affected by training.

Table 5.1: Effect of seven days of sprint training on skeletal muscle metabolites at rest, after a 30s sprint and 10 min recovery.

		Untrained			Trained	
	Rest	Exercised	10 min recovery	Rest	Exercise	10 min recovery
ATP	24.05±1.24	13.35±1.62 a	18.70±2.03 a,b	21.16±0.80	16.12±1.56* a	19.09±0.85 a,b
ATP-hplc	22.01±1.28	12.49±1.27 a	17.47±1.34	19.65±0.80	15.40±1.24* a	17.49±0.78 a,b
ADP	1.60±0.22	1.57±0.21	1.63±0.26	1.64±0.23	1.70±0.25	1.50±0.23
AMP	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.01	0.05±0.01
IMP	0.04±0.02	7.08±1.19 a	3.97±0.37 a,b	0.04±0.02	3.06±0.41* a	0.42±0.11 * a,b
Inosine	0.01±0.00	0.13±0.02	0.96±0.12 a,b	0.02±0.01	0.06±0.01*	0.42±0.07 *a,b
Hypoxanthine	<0.01	<0.01	0.04±0.00 a,b	<0.01	<0.01	0.03±0.01 a,b
PCr	82.0±3.7	27.3±4.4 a	71.9±3.8 a,b	87.4±3.7	31.5±4.1 a	83.8±4.3* b
Creatine	45.3±3.2	100.1±4.0 a	55.5±4.8 a,b	48.4±5.1	104.4±3.3 a	52.1±7.0 b
Lactate	5.51±0.63	91.90±7.51 a	49.37±6.46 a,b	5.53±0.99	83.94±5.32 a	39.24±2.53 a,b
AdN	23.67±1.34	14.13±1.35 a	19.16±1.56 a,b	21.36±0.96	17.17±1.41 * a	19.05±0.96 a,b
AdN + IMP + Inosine	23.72±1.34	21.33±0.61	24.10±1.40	21.41±0.97	20.28±1.21	19.89±0.87

Values are means ± SE; n=7; units are mmol.(kg dry mass)$^{-1}$ * different from untrained, $p<0.05$; a, different from Rest value $p<0.05$; b, different from Exercise value $p<0.05$.

Muscle ADP and AMP concentrations were not different following PT and 10 min recovery and were not different across training. The mean total creatine concentration was higher in the post-trained state (123.1 ± 5.2 vs 131.5 ± 6.4; $P<0.05$, untrained vs. trained). There was no difference in the dry weight: wet weight ratio of the muscle samples following training (24.6 ±0.7 vs 24.7 ± 0.6, untrained vs trained, $P> 0.05$).

5.3.3 Plasma metabolite concentrations.

The resting concentration of all measured metabolites were similar pre- and post-training (Fig 5.1, 5.2). Plasma lactate was not different at any time-point of recovery with training (Fig 5.1). Training resulted in significantly lower levels of inosine in the 15-30 min (Fig 5.2A) and hypoxanthine in the 15-60 min (Fig 5.2B) period of recovery, whilst uric acid was lower with training from 60 min to the end of measurement (120 min; Fig 5.2C).

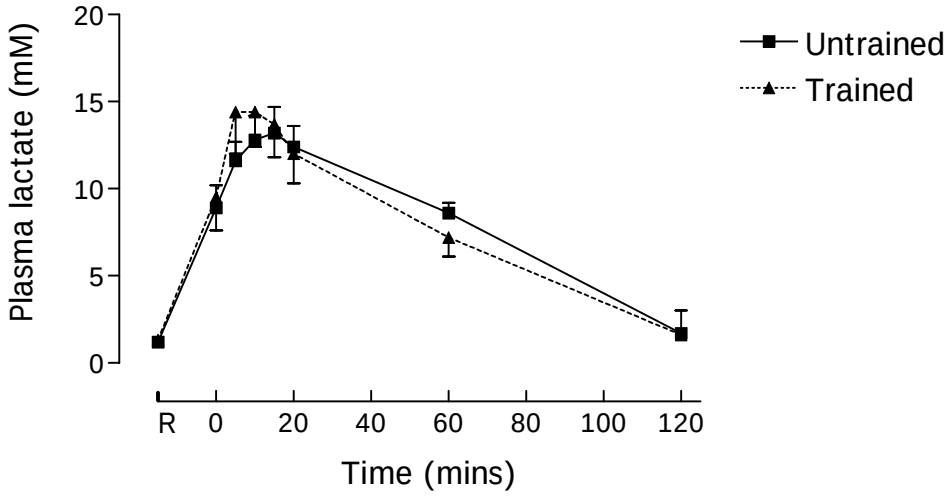


Fig 5.1. Forearm venous plasma lactate concentrations at rest (R) and during recovery from a 30s sprint bout before and after one week of sprint training. Values are means ± SE, n=7.

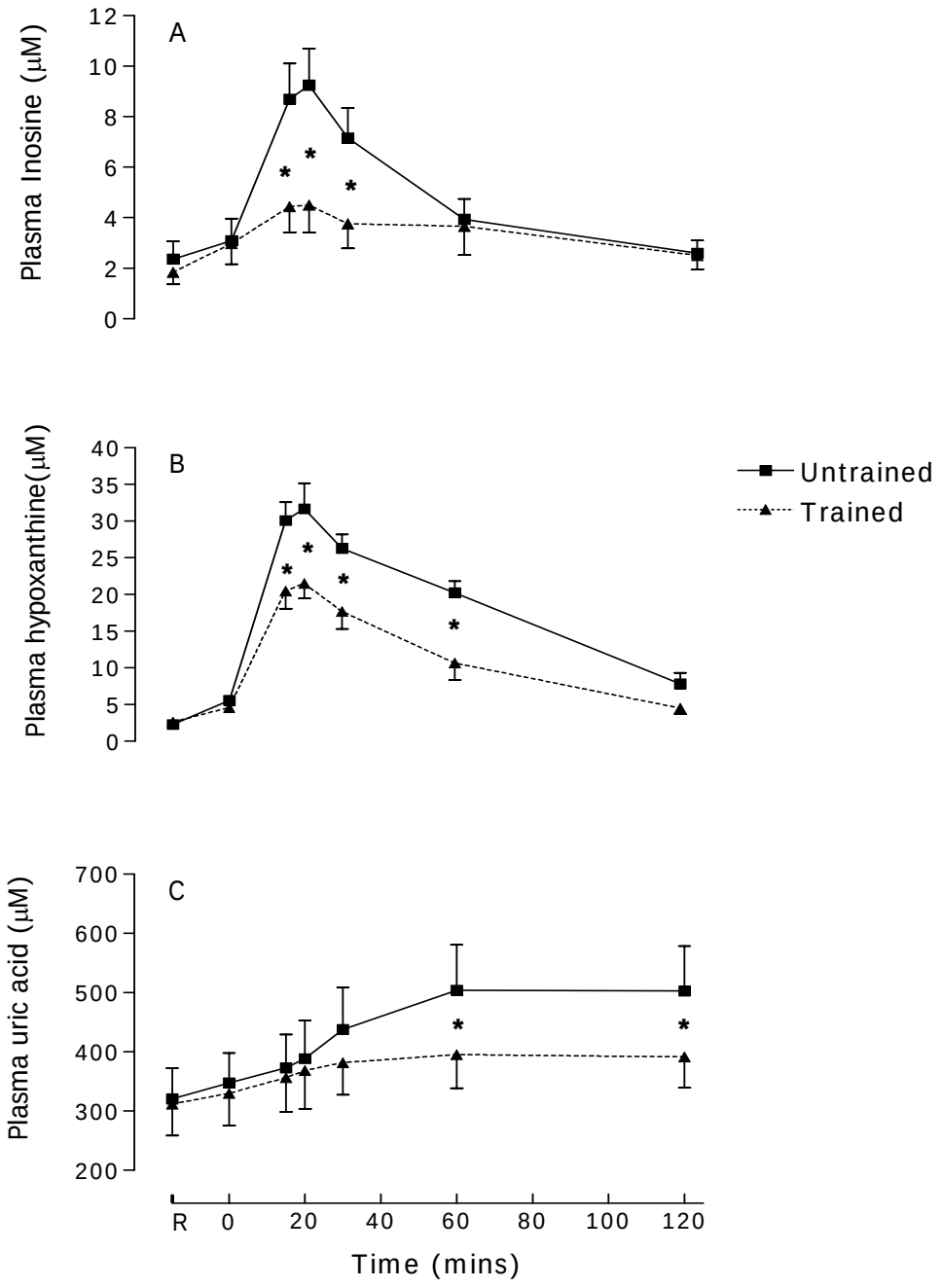


Fig. 5.2. Forearm venous plasma inosine (A), hypoxanthine (B), and uric acid (C) concentrations at rest (R) and during recovery from a 30s sprint bout before and after one week of sprint training. Values are means ± SE, n=7, * different from untrained; $P<0.05$. (note difference in scale).

5.3.4 Urinary excretion.

Urinary excretion of inosine, Hx and uric acid was elevated ($p<0.05$) from basal levels during the first 2 hour period after exercise but were not different with training (not shown). No differences in excretion (including basal) were observed in the trained compared with the untrained state during 24 hours recovery (Fig 5.3). However, there was a strong tendancy ($p=0.05$) for a lower uric acid excretion in the 24 hour recovery period following PT with training (Fig 5.3). The difference in the excretion of purine metabolites (inosine + Hx + uric acid) relative to basal levels, were not different in the first 2 hours of recovery (775±133 v 739±108 μmoles, untrained v trained). However, there was a tendency ($p=0.12$) for an attenuated excretion with training after 8 hours recovery, 1378±213 v 981± 141 μmoles and a reduced ($p<0.05$) excretion in the 24 hour recovery period 1603±267 v 1237±264 μmoles (Fig 3), untrained vs trained, respectively.

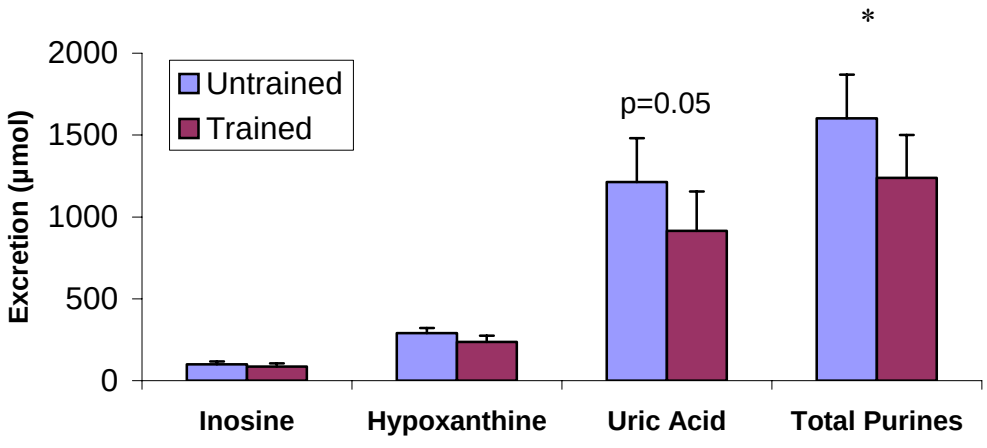


Fig. 5.3. Exercise induced urinary inosine, hypoxanthine, uric acid and total purine excretion after 24 hours of recovery from a 30s sprint bout before and after one week of sprint training. Values are means ± SE, n=7. * different from untrained, $P<0.05$.

5.4 Discussion

Sprint training reduced the muscle and plasma purine concentrations (Table 5.1 and Fig 5.2) and the excretion of urinary purines during recovery from a 30s sprint bout (Fig 5.3). This reduction likely represents a training-induced adaptation to minimise the loss of purines from skeletal muscle. Such an adaptation is advantageous in reducing the subsequent metabolic cost of AdN replacement by the purine *de novo* biosynthesis pathway.

As expected sprint training reduced the extent of ATP degradation (44.6%) and the IMP and inosine accumulation (56.7% and 66%, respectively) in the muscle during a 30 s sprint. A similar finding has been reported in other studies (Harmer et al. 2000; Parra et al. 2000; Stathis et al. 1994) and demonstrates an improved balance between the rates of ATP degradation and resynthesis with sprint training. Furthermore, sprint training attenuated plasma inosine, Hx and uric acid concentrations during the first two hours of recovery following the sprint (Fig 5.3). A lower plasma Hx following training has been reported in an earlier training study (Stathis et al. 1994) and is best explained by a reduction in the magnitude of purine efflux from the muscle, although no experimental evidence is available to confirm this possibility.

Urinary purine excretion in the 24 hours after the sprint was reduced following sprint training (Fig 5.3) and is best explained by a lower muscle purine base production, efflux and accumulation into the plasma. The lower urinary purine excretion may also be influenced by a training induced change in the loss of purines via the gut (Sorensen and Levinson, 1975). Sorensen and Levinson (1975) demonstrated that in normal untrained individuals one third of basal uric acid excretion occurs via the gut and, as yet, no studies have investigated the influence of intense exercise and/or sprint training on this avenue of purine loss.

The reduction in 1) muscle purine accumulation, 2) plasma inosine, Hx and uric acid concentrations (Fig 5.2; Stathis et al. 1994) and 3) the reduced urinary excretion (Fig 5.2) during recovery from a 30 s sprint, indicate that sprint training reduces purine loss from the muscle following intense exercise.

There was a strong tendency for a lower resting ATP concentration in the muscle after sprint training (Table 5.1; p=0.06 and 0.13, enzymatic and HPLC analysis, respectively). Earlier studies have reported reduced resting muscle ATP concentration after sprint training using the sprint training protocol employed in this study (Hellsten-Westing et al. 1993a; Hellsten et al. 2004). The statistical power of the current study may result in a type 2 error (n=7). This is supported by a significant reduction in resting ATP following a similar training protocol with nine (Hellsten-Westing et al. 1993a) and eight (Hellsten et al. 2004) participants.

The imbalance between ATP loss and its resynthesis is considered to be responsible for the tendency of a reduced resting muscle ATP content. Assuming the elevation in purine excretion during recovery represent the loss of endogenous purines from the active muscle, the loss of purine metabolites in 24 hours recovery from an intense sprint bout represents around 2% of the resting ATP content in the untrained and trained state. This was estimated by calculating the extent of purine excretion relative to the active muscle. If skeletal muscle is 40% of body weight and the legs comprise 55% of the total muscle mass (Snyder et al. 1975), then the mean weight of both legs is 3.89 kg d.m. (assuming 23% dry weight). The urinary purine loss is probably an underestimate, as uric acid can also cross into the gut (Sorensen and Levinson, 1975). As well as reducing the production of purines in the muscle, the conservation of muscle purine nucleotides with sprint training could also be enhanced by an increased capacity of intramuscular purine recovery after sprint training. Evidence of an

elevated activity of the purine salvage enzyme HGPRT following sprint training (Hellsten-Westing et al. 1993b) supports this possibility and provides a mechanism to further reduce the loss of purines from the muscle following exercise in the trained state.

Seven days of intense sprint training resulted in no differences in mean power, peak power or the fatigue index during a 30 sec maximal sprint cycle bout. These results are consistent with those reported after a similar training protocol (Hellsten-Westing et al. 1993a). The training protocol may limit any performance improvement because there is inadequate recovery time between sessions to produce optimal training adaptation. In support of this, Parra et al. (2000) demonstrated that a training programme using the same training load but over a longer duration improved performance in a 30 s sprint test.

An interesting point that this study has exposed is that the loss of endogenous purines in recovery following a 30 s sprint exercise bout is substantial, irrespective of training status. Sprint training does attenuate the loss of endogenous purines via the urine and, all factors remaining equal, a proportionally similar reduction presumably occurs also in the loss from the muscle post-training. This by implication indicates a significant loss of purine nucleotide degradation products from the muscle following intense exercise, albeit lower with sprint training. Hence there is a consequencial metabolic cost of to restore the AdN content of the muscle via *de novo* synthesis compared with purine salvage pathway.

5.4.1 Conclusion

In conclusion, the present study demonstrated that sprint training reduced purine base accumulation in the muscle and plasma, and decreased urinary excretion of

endogenous purines (inosine, Hx and uric acid) following maximal sprint exercise. An attenuated loss of muscle purine nucleotide degradation products after a sprint bout reduces the extent of replacement of the muscle nucleotide pool via the metabolically expensive purine *de novo* synthesis pathway.

Chapter 6

The influence of allopurinol on urinary purine loss after repeated sprint exercise in man

6.1 Introduction

The metabolic demand of intense sprint exercise produces a large efflux of purine bases from the muscle into the blood during recovery following exercise (Chapter 4; Hellsten-Westing et al. 1994; Stathis et al. 1994). The inability of ATP resynthesis to match the rate of ATP hydrolysis and the subsequent action of myokinase and AMP deaminase results in a degradation of the skeletal muscle ATP content (Bogdanis et al. 1998; Jansson et al. 1987; Stathis et al. 1994) and a stoichiometric increase in IMP (Jansson et al. 1987; Stathis et al. 1994). The accumulated IMP can undergo further degradation to produce inosine, which is converted to Hx (Tullson et al. 1995). Both inosine and Hx can be resynthesised to IMP via the purine salvage pathway (Edwards et al. 1979, Manfredi et al. 1984) or leave the muscle and accumulate in the plasma (Chapter 4; Bangsbo et al. 1992; Harkness et al. 1983; Hellsten-Westing et al. 1994). Once in the plasma Hx can no longer be a precursor for purine salvage by the muscle as no Hx uptake has been measured in muscle following intense exercise (Hellsten et al. 1998). The extent of the purine base efflux from the muscle following intense exercise has been reported to be equivalent to 9% of the resting pre-exercise ATP content (Ch 4; Hellsten et al. 1999) and represents a large increase in purine load in the plasma. Once in the plasma Hx can be converted to xanthine and uric acid in the liver (Hellsten-Westing et al. 1994; Vincent et al. 1984) as a result of the action of xanthine dehydrogenase/oxidase (Hellsten, 1994). Uric acid is the end point metabolite of purine metabolism and it

accumulates in the plasma following intense exercise (Sjödin et al. 1990; Stathis et al. 1994). The major mechanism responsible for the removal of inosine, Hx, xanthine and uric acid from the plasma is renal excretion (Chapter 4; Nasrallah and Al-Khalidi, 1964; Sorensen and Levinson, 1975). Uric acid can also efflux via the gut (Sorensen and Levinson, 1975) and, to a lesser extent, when plasma uric acid levels are high (Hellsten et al. 1998), circulating uric acid may also be taken up by the recovering muscle where it is used as an oxygen free radical scavenger (Hellsten, 1996). Hypoxanthine loss via the gut is small (Bradford et al. 1968) suggesting that the major mechanism for removal of plasma purine bases (Hx and xanthine) is via the kidney.

The renal transport mechanism of uric acid involves glomerular filtration, near complete pre-secretory reabsorption, tubular secretion and post-secretory reabsorption (Hiroshige et al. 1994, Yeun et al. 1995). Manipulation with uricosuric and antiuricosuric agents demonstrate differences in renal transport mechanisms of Hx, xanthine and uric acid by the kidney (Auscher et al. 1978; Cacini, 1982; Hiroshige et al. 1994; Yamamoto et al. 1993; Yeun et al. 1995). Considering that intense exercise results in the accumulation of these metabolites in the plasma (Chapter 4; Stathis et al. 1994; Sutton et al. 1980) any manipulation of the purine composition delivered to the kidney could be expected to influence the urinary excretion pattern and the magnitude of total urinary purine excretion.

Allopurinol, a structural analogue of Hx, and its major metabolic product oxypurinol are both potent inhibitors of xanthine oxidase (Hoey et al. 1988). The inhibition of xanthine oxidase reduces the oxidation of Hx to uric acid and it is for this reason allopurinol is commonly used in the treatment of hyperuricemia and gout (Emmerson, 1984). Given the prevalence of allopurinol usage it is perhaps surprising

that only two studies have examined the influence of allopurinol on purine metabolism following high intensity exercise in humans (Hadano et al. 1987; Sutton et al. 1980). Unfortunately, these studies have several limitations. For example, Sutton et al. (1980) reported that allopurinol had no effect on the exercise-induced uric acid excretion. However, this finding must be treated cautiously since blood sampling only occurred for 30 min after exercise, while urine sampling was only taken up to 15 min post exercise. This is probably insufficient to observe peak changes in uric acid concentration in these fluids (Chapter 4; Sjödin et al. 1990). Sutton et al. (1980) also reported that allopurinol resulted in an increase in plasma oxypurine (Hx and inosine) concentration following exercise, however no statistical comparison was made with the control condition. Interestingly, these authors also reported that urinary oxypurine excretion increased significantly after 15 min of recovery in the control, but was unchanged in the allopurinol condition. This finding was attributed to a decrease in renal clearance of the oxypurines in the allopurinol trial. The results from the Hadano et al. (1987) study are difficult to interpret since they compared plasma purine concentrations and urinary purine excretion rates in subjects ingesting allopurinol in the resting condition with those recovering from intense cycling exercise. Such an experimental design without the comparison with control conditions does not provide information on the influence of allopurinol ingestion on urinary purine loss following high intensity exercise.

Edwards et al. (1979) concluded that allopurinol may play a potential role in improving intramuscular purine salvage. Although not within the scope of this study, it would stand to reason that an increase in purine salvage capacity within the muscle would subsequently reduce the net efflux from skeletal muscle recovering from

intense exercise and attenuate the extent of purine handling downstream. Hence allopurinol would potentially reduce the extent of purines excreted in the urine.

The present study investigated the effects of an increased plasma purine load following intense exercise, combined with the effects of allopurinol to examine the purine handling functions of the kidneys and whole body purine excretion. It is hypothesised that allopurinol will attenuate the exercise induced elevation of urinary purines observed during recovery following repeated sprint exercise.

6.2 Methods

6.2.1 Subjects.

Seven active, non-specifically trained males (age: 24.9 ± 3.0 years, weight: 82.8 ± 8.3 kg, VO_2peak: 48.1 ± 6.9 $ml.kg^{-1}.min^{-1}$), volunteered for the study.

6.2.2 Exercise Protocols.

Subjects were asked to perform two exercise trials separated by at least one week. The trials were allocated using a double blind random crossover design. The exercise protocol in each of these trials consisted of 8 x 10s "all out" sprint bouts on an air-braked cycle ergometer (series A, Repco, Melbourne, Australia) modified to enable computerized determination of peak and mean power. In the placebo and experimental trials the subjects ingested a tablet of calcium carbonate or a 300 mg dose of allopurinol, respectively, once a day for the five days preceding each trial.

6.3 Results

6.3.1 Exercise performance.

There were no differences in peak or mean power between the trials. Both peak (1074 ± 50 vs 1072 ± 46 W; allopurinol vs placebo) and mean power (866 ± 36 vs 848 ± 35.3) were highest in the first sprint and decreased significantly ($P<0.05$) by the third sprint with a further significant reduction during sprint five. No further decreases in performance were observed during the final three sprints.

6.3.2 Plasma metabolites

Plasma inosine concentration increased ($P<0.05$) above resting levels in both trials and peaked around 20-30 min into recovery, before returning to pre-exercise levels by 120 min (Fig. 6.1A). There was no difference in plasma inosine concentration between trials. Plasma Hx concentrations were similar at rest and increased ($P<0.05$) immediately following exercise in both trials (Fig. 6.1B). They remained above basal levels even after 120 min of recovery. The Hx concentrations were higher with allopurinol treatment compared with placebo from 10 min until at least 120 min of recovery ($p<0.05$). Peak Hx levels in the allopurinol trial were almost twice that of the placebo trial. Exercise elevated the uric acid concentrations above resting concentrations at 15, and 30 min in the placebo and allopurinol trials, respectively (Fig. 6.1C) and did not return to basal concentrations in both trials by 120 min. Plasma uric acid was lower in the allopurinol trial at rest ($P<0.05$) and remained markedly lower throughout recovery compared with the placebo trial. Plasma lactate concentrations were 0.84 ± 0.1 and 1.0 ± 0.1 $mmol.l^{-1}$ (allopurinol vs control) at rest increased markedly following the sprint bouts in both trials peaking at 5 min after the last exercise bout (17.8 ± 1.3 vs 17.1 ±1.0 $mmol.l^{-1}$; allopurinol vs

placebo) with no differences in concentrations observed between treatments at any time point during the 2 hour recovery period (data not shown).

6.3.3 Urinary Metabolites.

Allopurinol had no effect on basal urinary excretion of inosine Hx, or uric acid (Fig 6.2 A, B & D). In contrast, basal urinary excretion of xanthine was greater ($P<0.05$) in the allopurinol compared with placebo (Fig. 6.2C). The excretion rate of all measured urinary metabolites in both trials increased ($P<0.05$) in the two hour period following sprinting, with the notable exception of uric acid in the allopurinol trial which remained similar to basal levels. The Hx and xanthine excretion rates in this period were greater ($P<0.05$) in the allopurinol trial compared with placebo, and remain higher during the 2-8 hr period after sprint exercise. Although a similar pattern was observed, urinary inosine excretion was not different in the first 2 hours ($P=0.06$) or the 2-8 hr period of recovery from exercise. In contrast, the excretion rate of uric acid in the placebo trial was markedly elevated in the 0-2 hr and 2-8 hr periods following exercise ($P<0.05$) in comparison with the allopurinol trial. In the 8-24 hour period of recovery the excretion rates of inosine, Hx, xanthine and uric acid were similar between trials and had returned to basal values. A significant interaction ($P<0.05$) for total purine excretion was observed (Fig 6.2E), however post-hoc analysis only revealed a tendency ($P=0.08$) for an elevated excretion in the allopurinol trial in the 0-2 hr and 2-8 hr recovery periods.

Since urinary excretion of all purines had returned to basal levels by the end of the first 8 hours of recovery the exercise-induced urinary purine loss was calculated using the first 8 hours of urinary data. Total urinary inosine, Hx and xanthine loss during this period were ~ 2, 4.5 and 10 fold greater ($P<0.05$) in the allopurinol trial

(Table 6.1). In contrast, the amount of uric acid excreted above basal levels was 3 fold lower ($P<0.05$) in the allopurinol trial compared with placebo (Table 6.1). The total urinary excretion of purines (inosine + Hx + xanthine + uric acid) was 2 fold higher ($P<0.05$) in the allopurinol trial compared with placebo (Table 6.1).

Table 6.1. Urinary purine loss above basal levels during the first 8 hours of recovery from 8x10 s sprints after 5 days of prior ingestion of allopurinol or a placebo.

	Placebo	**Allopurinol**
	μmol	μmol
Inosine lost to urine	41 ± 7	94 ± 25*
Hx lost to urine	421 ± 43	2232 ± 419*
Xanthine lost to urine	52 ± 23	493 ± 111*
Uric acid lost to urine	1137 ± 360	380 ± 222*
Total urinary purine loss	1651 ± 410	3198 ± 639*

Values are means ± SE, n=7. * different from placebo ($P<0.05$).

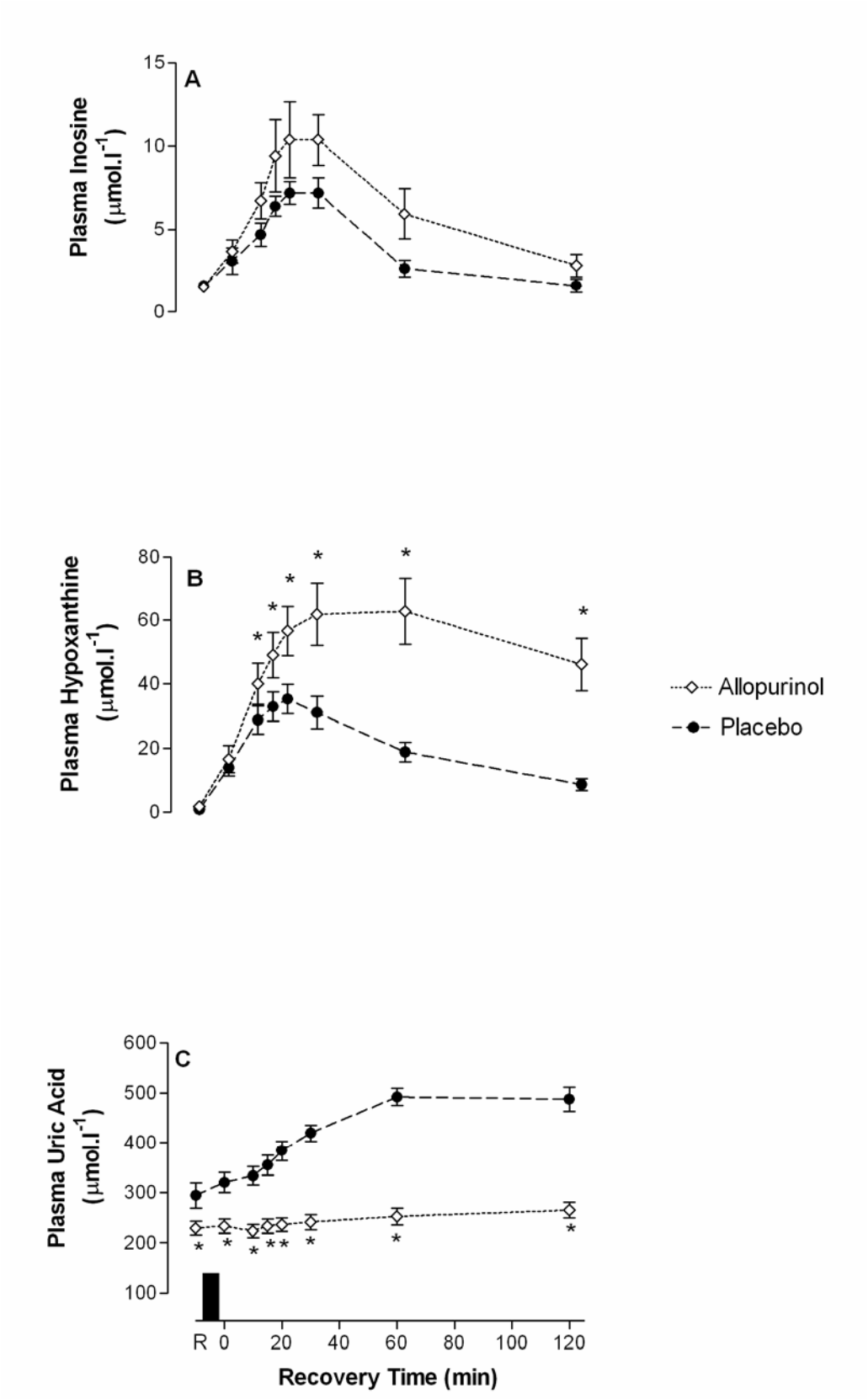


Fig. 6.1. Forearm venous plasma inosine (A), hypoxanthine (B), and uric acid (C) concentrations at rest (R) and during recovery from eight 10s intermittent sprint bouts with allopurinol or placebo ingestion. Values are means ± SE, n=7, * different from placebo; $P<0.05$. (note difference in scale).

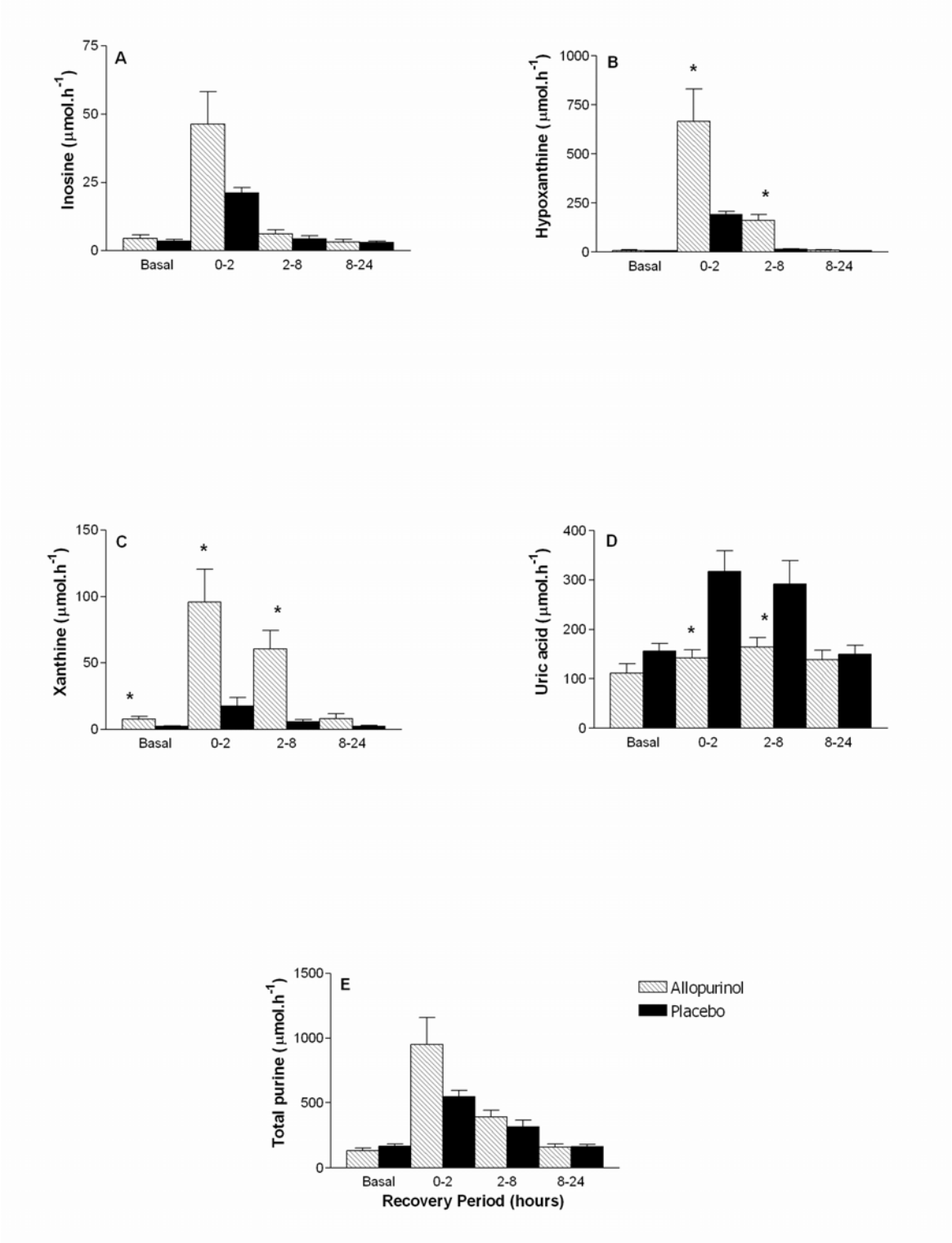


Fig. 6.2. Urinary inosine (A), hypoxanthine (B) xanthine (C) uric acid (D) and total purine (E) excretion rate at rest (basal) and during the first 2 hours and the subsequent 6 and 16 hours of recovery following eight 10s intermittent sprint bouts with allopurinol or placebo ingestion. Values are means ± SE, n=7. * different from placebo, $P<0.05$. (note difference in scale).

6.4 Discussion

This study is the first to demonstrate a substantially greater loss of total urinary purines following intermittent sprint exercise with allopurinol treatment. Consistent with the characteristic pharmacological effects of allopurinol we report markedly attenuated urinary uric acid excretion (33%), and enhanced urinary Hx (530%) and xanthine (950%) excretion during recovery from sprint exercise (Table 6.1). Exercise performance was not different between the trials and plasma lactate concentrations were unaffected by the treatment, demonstrating that 5 days of allopurinol ingestion does not influence intermittent sprint performance. This is supported by the work of others (Sutton et al. 1980), and indicates that the metabolic stress and subsequent purine base production within the contracting musculature was likely to be similar between trials.

As previously reported allopurinol induced resting hypouricaemia but had no effect on plasma inosine or Hx concentrations (Fig 6.1; Kelley, 1975; Sutton et al. 1980). Sprint exercise increased ($P<0.05$) plasma inosine, Hx and uric acid concentrations during recovery in both trials (Fig 6.1). This is likely due to an increase in circulating purines as a result of the production and subsequent efflux of purines from the muscle following intense exercise, rather than a decrease in plasma purine removal rates (Bangsbo et al. 1992; Hellsten-Westing et al. 1994; Hellsten et al. 1998). As mentioned above, the metabolic stress is likely to be similar in both trials and hence the magnitude of purine base efflux across the sarcolemma is probably the same following exercise. However, consistent with the inhibitory effect of allopurinol (Kelley, 1975), the inhibition of xanthine oxidase influenced the mix of the circulating purines and resulted in greater rises in plasma inosine (Fig 6.1A) and Hx (Fig 6.1B) and blunted the rise in uric acid concentrations (Fig. 6.1C) during

recovery from sprint exercise. The increase in plasma metabolite concentrations in the placebo trials has been observed previously during similar experimental conditions (see chapter 4) but there are no comparable studies with the allopurinol intervention trial.

The exercise-induced rise of plasma Hx and inosine, and the augmentation of this rise with allopurinol ingestion have been previously reported (Sutton et al. 1980). However, the uric acid results of the present study are in conflict with Sutton et al. (1980) who reported no increase in plasma uric acid in either trial following exercise. It is unclear why no increase was observed in the placebo trial since the exercise was intense enough to produce a 30% fall in the contracting muscle total AdN post-exercise and a 250% increase in plasma oxypurine concentration during recovery (Sutton et al. 1980). A likely explanation is that they did not measure the plasma metabolites after 30 mins post exercise where changes in uric acid levels above rest are more likely to occur (Sjödin and Hellsten-Westing, 1990).

The small (15%) increase in plasma uric acid concentration after 2 hours recovery (Fig. 6.1C) and the elevated urinary xanthine excretion during the first 8 hours of recovery in the allopurinol trial (Fig. 6.2C) indicate that the inhibition of xanthine oxidase was incomplete. This may also help explain the elevated excretion of xanthine observed with allopurinol (Fig 6.2B). Furthermore, basal level urinary uric acid excretion was maintained during the 24 hr recovery period in the allopurinol trial, suggesting that there was some production of uric acid despite the presence of the drug. It should be noted that dietary sources of purine possibly help maintain basal levels of plasma uric acid with allopurinol administration (Clifford et al. 1976). The small increase in plasma uric acid during recovery in the allopurinol trial may also be caused by a decreased removal rate. In support of this possibility, a decreased

urinary excretion of uric acid has been demonstrated during the first 45 min of recovery from intense exercise (Nichols et al. 1951).

Allopurinol had no effect on urinary basal excretion rates of inosine, Hx or uric acid, however, xanthine excretion was increased by the drug (Fig. 6.2). Our inability to measure a decrease in basal urinary uric acid excretion with allopurinol is in contrast with previous research (Kelley, 1975). The increase in basal xanthine excretion confirms the data of Klinenberg et al. (1965) who reported that approximately half of the increase in basal oxypurine excretion following allopurinol treatment was attributed to xanthine. However, although the plasma xanthine concentration is generally low at rest and suggests that circulating levels are likely to be small, it must be noted that the excreted xanthine may have originated from existing stores.

Consistent with previous research (Chapter 4; Harkness et al. 1983; Sutton et al. 1980), sprint exercise increased the excretion rates of purines (Fig 6.2). The rates of urinary loss, especially inosine and Hx, are higher than we have previously observed following a similar exercise protocol (Chapter 4). However, allopurinol further enhanced total urinary purine loss following intermittent, sprint exercise (Table 6.1) as a result of differences in the excretion of the individual purine bases. The differences in the excretion rates of Hx, xanthine and uric acid (Fig 6.4) in the first 8 hours of recovery from exercise after taking allopurinol reflect the altered plasma purine concentrations and the subsequent change in purine mix delivered to the kidney. Although not measured in this study due to analytical and technical difficulties arising from contamination by allopurinol and downstream breakdown products. Recent research has demonstrated that plasma xanthine is elevated after exercise with allopurinol to similar levels to that of Hx (Kaya et al. 2006).

Furthermore, there was a tendency (p=0.06) for an elevated excretion of inosine with allopurinol and like Hx and xanthine, is probably a reflection of the concentration changes in the plasma. Although the difference in the plasma was not significant (p=0.13), Sutton et al. (1980) has demonstrated a higher plasma inosine concentration with allopurinol treatment following exercise.

The fact that the reduction in urinary uric acid excretion was not balanced proportionally to the increase in the aggregate of urinary purine bases suggests that other factors are influenced by allopurinol. One limitation of sampling venous blood and urine is that tissue other than muscle may contribute to the purine content. However, no evidence is available to demonstrate any influence of purine metabolism, other than xanthine oxidase inhibition, in other tissues with allopurinol administration. The processes that may be influenced by allopurinol are the renal handling mechanism(s) of purine excretion, haemodynamic factors and the loss of purine bases via the gut. Although this study cannot provide any evidence on the precise mechanism(s), haemodynamic factors are not likely to play a role as previous studies show no effect of allopurinol (Hestin and Johns, 1999) or oxypurinol (Dillon et al. 1993) on the blood pressure or renal blood flow in rats. Perhaps the best explanation for the increased excretion of purines in the drug trial is the influence of the different renal handling mechanisms by which the kidney excretes these metabolites (Aucher et al. 1978; Cacini, 1982; Harkness et al. 1983; Yamamoto et al. 1988; Yamamoto et al. 1993). Renal uric acid excretion involves filtration at the glomerulus, near complete reabsorption at the proximal tubule, secretion into the proximal tubule and further reabsorption (Yamamoto et al. 2000). At present, the evidence indicates that renal excretion of Hx is mainly dependent upon glomerular filtration (Harkness et al. 1983; Yamamoto et al. 1988), whereas xanthine excretion is

dependent upon glomerular filtration, and tubular secretion (Auscher et al. 1978). The fractional clearance of uric acid is approximately one seventh of Hx and xanthine (Yamamoto et al. 2001) and neither is altered by allopurinol (Moriwaki et al. 2002). Thus the increased urinary excretion of purines after allopurinol ingestion and intense exercise could simply be due to an altered mix of plasma purines being presented to the kidney. Furthermore, Yamamoto et al. (1993) have also demonstrated that hyperlactatemia inhibited urinary excretion of uric acid but does not influence that of Hx or xanthine. This would further exacerbate the potential for increased loss of purines in the allopurinol trial via the kidneys. Plasma lactate concentration increased following both trials and the recovery profile was not different between trials during the 2 hours post exercise. From this it is assumed that the inhibition of uric acid excretion by lactate will be similar in both trials.

Assuming the total purine production and efflux from the muscle was the same between the trials, and that the action of xanthine oxidase effectively produces a 1:1 stoichiometric replacement of uric acid with Hx, then the twofold greater total urinary purine excretion after 8 hours recovery in the allopurinol trial needs to be accounted for. The fate of these unaccounted purines in the placebo trial is not clear but there are several possibilities. The difference in uric acid and Hx exchange across the gut may provide an explanation for this difference. One third of the resting circulating uric acid is excreted via the gut compared with two thirds excreted in the urine (Sorensen and Levinson, 1975), whilst the efflux of Hx into the gut has been measured at 3% (Bradford et al. 1968). Hence, a change in composition of plasma purines with allopurinol will favour a reduced uric acid loss in the gut effectively shunting the purines to the kidney for excretion. If the ratios of excretion across the gut stay the same under all plasma concentrations, the uric acid excretion via the gut in the 8

hours post exercise reduce from 568 μmol to 190 μmol and this only accounts for approximately one third of the difference in total purine loss observed between trials. Uric acid excretion is an exponential function of the plasma concentration (Simkin, 1979) and the results in this study are consistent with this except that there is no difference in basal excretion in the allopurinol trial when the plasma concentration is significantly lower. Thus the relationship between plasma concentration and excretion rates of uric acid is not a factor in the large unaccounted purine excretion. A further possibility lies in the potential pool of uric acid in the plasma still to be excreted at 8 hours recovery in the placebo trial. This is difficult for us to ascertain in this study as we did not measure the plasma concentrations at 8 hours and the excretion data taken between 8-24 hours is not different between the trials. Hence we cannot confirm this as a potential source for the greater urinary purine base excretion with allopurinol.

A two-fold increase in exercise-induced urinary purine excretion following allopurinol (Table 6.1) raises an interesting question if a shift from fecal to urinary excretion pathways is not a factor. On the premise that total urinary purine loss post exercise equates to endogenously produced purines, intramuscular purine concentrations may be depleted as a consequence of allopurinol ingestion combined with intense repeated exercise. The greater fractional clearance of Hx and xanthine (Yamamoto et al. 2001) combined with elevated plasma Hx and xanthine concentrations observed with allopurinol ingestion may simply enhance the loss of purines via the kidney by bulk flow.

The results introduce an interesting conundrum when allopurinol and intense intermittent exercise are combined. The consequences of an elevated loss of urinary purines, should the proportional loss via the gut not change, increases the requirement for the “metabolically expensive” *de novo* replacement of muscle purines. However,

the widespread therapeutic use of allopurinol without incident over several decades suggests that there are likely to be adaptations that attenuate the loss or maintain the content of muscle purines with chronic intense exercise training and allopurinol ingestion. Enhanced purine salvage (Edwards et al. 1981) with allopurinol or an increased HGPRT enzymatic activity, as observed with training (Hellsten-Westing et al. 1993b) may explain the mechanism of replacement and subsequent maintenance of purine nucleotide content of skeletal muscle in patients during allopurinol therapy. Additionally, intense repeated exercise during sprint training has produced reductions in muscle nucleotides at rest (Stathis et al. 1994) and further investigation with allopurinol and chronic exercise is needed.

6.4.1 Conclusion

In conclusion, the present study demonstrated that allopurinol augments the exercise-induced rise in plasma Hx concentration but markedly attenuated the increase in plasma uric acid levels. These changes were reflected in higher Hx and xanthine, and lower urinary uric acid excretion rates following exercise with allopurinol administration. The increase in urinary Hx and xanthine excretion markedly exceeded the fall in urinary uric acid loss and as a consequence total urinary purine loss was two-fold greater after intense exercise with allopurinol treatment. These results suggest that altering the mix of plasma purines filtered by the kidney combined with a higher fractional clearance of Hx will change the magnitude of total urinary purine loss. This is likely related to the different renal transport mechanisms that have been previously described for each of the purine molecules. Additionally, the changes in plasma purine composition may reduce intestinal purine loss since efflux mechanisms appear to differ from one purine to another at the gut. If this

occurred, the elevated total urinary purine excretion with allopurinol treatment after exercise may also be explained, at least in part, by a greater delivery of purines to the kidney.

Chapter 7

The Influence of Allopurinol and Sprint Training on Purine Nucleotide Metabolism in Human Skeletal Muscle.

7.1 Introduction

Intense sprint exercise can produce transient 30-40% reductions in skeletal muscle ATP content (Jansson et al. 1987; Stathis et al. 1994) virtually matched by a concomitant increase in muscle IMP levels (Jansson et al. 1987; Stathis et al. 1994). Most of the IMP, which cannot efflux the muscle, is resynthesised to ATP during recovery upon cessation of exercise (Sahlin et al. 1978). However, a small proportion can be further degraded to inosine and Hx (Fig 2.1; Stathis et al. 1994; Tullson et al. 1995), which may leave the muscle and accumulate in the plasma or be converted to uric acid (Stathis et al. 1994; Hellsten et al 1999). The extent of purine efflux amounts to between 5-9% of the resting ATP pool (Bangsbo et al. 1992; Hellsten et al. 1999) or 46% of the magnitude of ATP reduction during exercise (Hellsten et al. 1999). Studies measuring uptake and release across muscle beds have indicated that skeletal muscle does not take up purines from the plasma (Hellsten et al. 1998; Hellsten 1999). Thus inosine, Hx and uric acid are ultimately excreted via the kidney or gut (Chapter 4; Sorensen and Levison, 1975). Purine salvage occurs within the muscle cell (section 2.3.3.3), thus restoration of ATP, lost from the muscle due to purine base efflux must occur via *de novo* synthesis.

Sprint training may reduce AdN content of resting skeletal muscle (Harmer et al. 2000; Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994). This is attributed to a greater loss of purines relative to replacement via *de novo*

biosynthesis over the duration of the training period (Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994).

The capacity for intramuscular purine salvage is enhanced following sprint training via an increased HGPRT activity (Hellsten-Westing et al. 1993b) and likely contributes to a reduction of muscle inosine and plasma inosine and Hx accumulation during recovery, following a sprint bout (Chapter 5; Stathis et al. 1994). This adaptation enhances the potential of purine nucleotide retention within the muscle by reducing the extent of purine base efflux and accumulation in the plasma following maximal exercise (Stathis et al. 1994). This alleviates the requirement for ATP replacement via the slow and metabolically expensive *de novo* biosynthesis pathway (Newsholme and Leech, 1983).

Allopurinol, an allosteric inhibitor of xanthine oxidase, produces hypouricemia and a reduced urinary uric acid excretion at rest (Rundles and Wyngaarden, 1969). However, allopurinol administration does not balance the reduction of urinary uric acid with elevated Hx and xanthine excretion and results in a 10-60 % reduction in total purine excretion (Kelley et al. 1969; Rundles and Wyngaarden, 1969; Yü and Gutman, 1964). This latter finding may be explained by an enhanced purine salvage rate with allopurinol administration (Edwards et al. 1981). Although the evidence presented by Edwards et al. (1981) is indirect, the results in Chapter 6 further support their data. The elevated purine excretion following allopurinol is brought about by an increased renal purine load, due to an increased metabolic stress with intense exercise substantially elevating purine efflux from the muscle (Hellsten et al 1999; Stathis et al. 1994), and a seven fold higher excretion of the purine bases relative to uric acid (Yamamoto et al. 2001; Moriwaki et al. 2002). Although speculative the abovementioned excretion fractions during basal conditions would be expected to

favor elevated purine excretion with a reduced plasma uric acid concentration following allopurinol treatment. The inhibition of xanthine oxidase by allopurinol occurs downstream of the efflux of Hx from the muscle into the plasma. Therefore, the aforementioned elevation of allopurinol on purine salvage at rest may influence the recovery of intra-muscular ATP content during sprint training and help maintain ATP resting concentrations.

No study has investigated the influence of allopurinol and sprint training on resting AdN content, plasma purine concentration or urinary excretion of purines. This study aimed to investigate the effect of intense sprint training and allopurinol administration on skeletal muscle AdN content, plasma purine concentration and urinary purine excretion at rest. It is the hypothesis of this study that allopurinol administration concurrent with sprint training would attenuate the extent of the sprint-induced reduction of ATP observed in resting muscle.

7.2 Methods.

7.2.1 Subjects.

Twelve active, non-specifically trained males, whose subject characteristics are shown in Table 7.1, were randomly separated in double-blind fashion into an allopurinol or placebo group. There was no significant difference in characteristics between the groups.

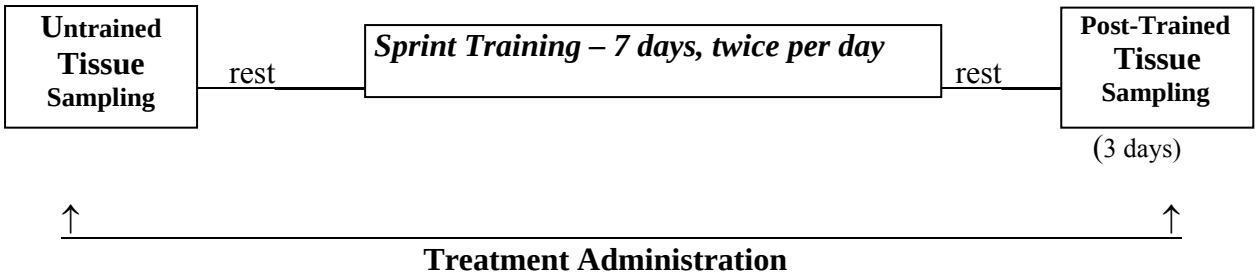
Table 7.1: Subject Characteristics

	Placebo (n=7)	Allopurinol (n=5)
Age (years)	23.1 ± 1.8	24.6 ± 2.2
Weight (kg)	76.1 ± 3.1	80.8 ± 4.4
VO_2peak ($ml.kg^{-1}.min^{-1}$)	56.3 ± 4.0	50.8 ± 3.2

7.2.2 Experimental Protocol.

Each subject performed an initial peak oxygen consumption test (VO_2peak) and after a week they underwent a pre-training sampling of muscle, blood and urine. Following a recovery period the subjects began 7 days of sprint training. Approximately 24-36 hours from the completion of the final training session participants presented for post-training sample collection. Administration of allopurinol or placebo commenced 3 days prior to the sprint training regimen and continued until the completion of post-training sampling. See Fig 7.1 for a summary of the protocol.

Figure 7.1: Experimental Protocol Summary



7.2.3 Peak oxygen consumption (VO_2peak).

The VO_2peak of each subject was determined approximately one week prior to beginning the experimental trials. (Section 3.3).

7.2.4 Sprint training protocol.

Sprint training was performed at least 4 days after the first PT, two sessions per day for a week with each session separated by at least 6 hours. A training session involved 15 x 10s maximal cycling bouts on an air-braked cycle ergometer with 50-s of rest between bouts.

7.2.5 Treatment administration.

Following the pre-training data collection trial the participants were allocated to placebo or allopurinol groups in double-blind fashion. The placebo group ingested a tablet of calcium carbonate, whereas the allopurinol group ingested a 300 mg dose of allopurinol. Treatments were taken once a day for at least three days prior to, during sprint training and up until the morning of post-training tissue collection.

7.2.6 Muscle sampling, treatment and analysis.

Muscle was sampled from the vastus lateralis (Section 3.7) at rest. Muscle samples were analysed for ATP, ADP, IMP, inosine, Hx, PCr, Cr, Lac (Section 3.7.1).

7.2.7 Blood and urine sampling, treatment and analysis.

Blood was sampled at rest. Plasma inosine, Hx, uric acid and lactate were analysed (section 3.5.1). Urine was collected at rest and analysed for inosine, Hx and uric acid (section 3.6.1).

7.3 Results

7.3.1 Muscle metabolite content.

The resting muscle metabolite content before and after training, are summarised in Table 7.2. There was a reduction in the resting AdN and ATP content after training when the combined resting data of the two treatment groups was compared across training. However, there were no differences in the measured metabolite levels between the placebo and allopurinol groups (Table 7.2).

Table 7.2: Muscle metabolites at rest before and after sprint training and allopurinol treatment.

	Untrained		Trained	
	No Treatment	No Treatment	Placebo	Allopurinol
ATP †	24.05±1.24	23.14±1.43	21.16±0.8	19.99±0.69
ATP-hplc †	22.01±1.28	21.44±1.32	19.65±0.80	18.44±1.01
ADP	1.60±0.22	1.85±0.38	1.64±0.23	1.53±0.18
AMP	0.05±0.01	0.06±0.01	0.05±0.01	0.07±0.01
IMP	0.04±0.02	0.06±0.01	0.04±0.02	0.03±0.01
Inosine	0.01±0.00	0.03±0.01	0.02±0.01	0.04±0.02
Hypoxanthine	<0.01	<0.01	<0.01	<0.01
PCr	82.0±3.7	87.7±5.3	87.4±3.7	82.3±4.2
Creatine	45.3±3.2	45.3±5.8	48.4±5.1	60.5±10.7
Lactate	5.5±0.6	6.5±1.49	5.5±1.0	7.0±2.8
AdN †	23.67±1.34	23.36±1.65	21.36±0.95	20.05±0.98

Values are means ±SE; n=7 Placebo, n=5 Allopurinol; units are mmol.(kg dry mass)$^{-1}$; AdN = ATP+ADP+AMP; †, significant effect for training, different to untrained, $p<0.05$.

Table 7.3: Plasma inosine, hypoxanthine and uric acid concentrations at rest prior to, and following sprint training and allopurinol treatment.

	Untrained		Trained	
	No Treatment	No Treatment	Placebo	Allopurinol
Inosine	2.4 ± 0.7	1.6 ± 0.5	1.8 ± 0.5	1.5 ± 0.4
Hypoxanthine	2.3 ± 0.6	3.7 ± 2.1	2.6 ± 0.6	3.8 ± 2.2
Uric Acid	367.1 ± 27.7	316 ± 21.1	338.6 ± 27.3	226 ± 121.9 *

Values are means ± S.E.; units are μM; * significantly different from untrained, $p<0.05$.

7.3.2 Plasma metabolites

There were no differences in any resting plasma metabolites between the groups in the untrained state (prior to treatment, Table 7.3). The resting plasma uric acid content was lower in the allopurinol treated group following training. However, there were no differences in resting plasma inosine and Hx following training between the two groups (Table 7.3).

7.3.3 Urinary metabolites

No difference was observed between the groups for any of the measured urinary metabolites in the pre-trained state (with no treatment) or the post-trained state (Table 7.4).

Table 7.4: Basal urinary inosine, hypoxanthine and uric acid excretion rates prior to, and following, sprint training and allopurinol treatment.

	Untrained		Trained	
	No Treatment	No Treatment	Placebo	Allopurinol
Inosine	11.0 ± 1.8	15.6 ± 8.1	8.4 ± 1.8	8.4 ± 1.5
Hypoxanthine	3.5 ± 0.8	3.7 ± 1.7	2.1 ± 0.4	4.0 ± 1.3
Uric Acid	110.0 ± 16.8	96.0 ± 12.9	87.1 ± 12.5	62.0 ± 15.0

Values are means ± S.E.; units are $\mu mol.hr^{-1}$.

7.4 Discussion

No difference was observed in resting muscle ATP content following allopurinol administration and sprint training compared with sprint training alone (Table 7.2). This finding did not support the hypothesis that allopurinol would better maintain resting muscle ATP content after sprint training (Table 7.2). Power analysis

of the study indicates that seven participants were sufficient to observe a 10% difference with allopurinol (power of 77% with significance set at $P<0.05$). Although the attrition of 2 participants in the allopurinol trial occurred, it is reasonable to consider that there is likely to be little difference observed between the treatment groups. A lower resting ATP content was observed when the data of both groups were pooled and compared across sprint training (Table 7.2). This is consistent with previous studies and demonstrates that the sprint-training program resulted in a net loss of purines from the muscle over the training period (Harmer et al. 2000; Hellsten-Westing et al. 1993a; Hellsten et al. 2004; Stathis et al. 1994). The similar extent of net ATP degradation with allopurinol after sprint training can be explained by two possible alternatives, an increase in the extent of muscle purine salvage and purine loss from the muscle, or unchanged rates of purine salvage and muscle purine loss. Considering the former scenario, it is possible for allopurinol administration to increase both the extent of muscle purine loss with intense exercise (Chapter 6), and the rate of intramuscular purine salvage (Edwards et al. 1981) with training. An elevated plasma Hx and urinary excretion of purines, particularly Hx, with allopurinol administration has been reported earlier (Chapter 6) and lends support to this possibility. Alternatively, the latter scenario could arise as the potential for any allopurinol-induced increase in purine salvage capacity may be stymied by a lack of substrate availability during the sprint-training period. PRPP is a substrate common to both purine salvage and *de novo* synthesis pathways (Newsholme et al. 1983) and it has been reported that *de novo* synthesis is significantly greater during recovery from sprint training with ribose feeding (Hellsten et al. 2004). Ribose is a precursor of PRPP, which lends support to the possibility that substrate limitation may determine the rate of purine salvage during sprint training irrespective of other influences.

With the exception of basal uric acid excretion rates in the trained state, all resting plasma concentrations and urinary excretion rates of inosine, Hx and uric acid are consistent with the inhibitory effects of allopurinol on xanthine oxidase. The basal uric acid excretion is not different between the treatment groups in the sprint-trained state (Table 7.4). As mentioned above, allopurinol reduces urinary uric acid and produces hypouricaemia (Rundles and Wyngaarden, 1969), however, this was in untrained individuals. No difference was observed in basal urinary excretion with sprint training (Chapter 5) and no other study has reported basal uric acid concentrations after sprint training. Hence sprint training may influence urinary uric acid excretion. However, it is also possible that urinary and gut uric acid excretion dynamics may be altered by training and/or allopurinol and requires further investigation.

7.4.1 Conclusion

In conclusion this study demonstrated that although sprint training reduced the resting ATP content of skeletal muscle allopurinol had no influence on resting ATP content of skeletal muscle following sprint training.

Chapter 8

Summary and Conclusions

8.1 Introduction

This thesis investigated factors that influenced human PrN metabolism during and following intense exercise. Specifically, it focused on 1) the influence of the number of intermittent sprint bouts on the plasma purine accumulation and excretion in the urine, 2) the influence of sprint training on the loss of urinary purines after intense maximal exercise, 3) the influence of allopurinol on the accumulation of plasma purines and the excretion of urinary purines after intense exercise and 4) the influence of sprint training and allopurinol on PrN content of resting muscle.

8.2 Purine loss after repeated sprint exercise

Total purine loss from the muscle was estimated from the rise in plasma purine accumulation and the increase of urinary purine excretion above basal levels. These estimates reveal that increasing the number of consecutive intermittent sprint bouts (i.e. 1, 4, 8) resulted in a greater accumulation of purines in the plasma and a greater urinary purine excretion after 2 hr of recovery. The best explanation for these findings is that an increase in the number of bouts in succession increases the duration in which the contracting muscle is metabolically stressed. Consequently, muscle IMP remains elevated for longer allowing for greater purine production.

8.3 The effect of sprint training on urinary purine loss following a sprint bout.

This study confirmed and extended earlier work by Stathis et al. (1994) who found that there was a sprint-trained reduction of inosine content in skeletal muscle during recovery from a sprint bout and a reduction in plasma Hx during recovery from a maximal sprint. The results of this study further extend the aforementioned findings showing an attenuation of endogenous plasma purines (inosine, Hx and uric acid) in the plasma and reduced urinary excretion of total endogenous purines during recovery from a maximal 30s sprint bout in the sprint trained state. These data indicate that there is a substantial sprint-trained induced reduction in the extent of the loss of purines from the muscle after intense exercise. Although speculative, this adaptation should reduce the need to replace PrN via the metabolically expensive *de novo* synthesis pathway.

8.4 The influence of allopurinol on urinary purine loss following repeated sprint exercise.

This study investigated the influence of the xanthine oxidase inhibitor allopurinol on urinary purine loss after an intense exercise bout. The expected pharmacological action of allopurinol increased plasma Hx and reduced plasma uric acid concentrations following exercise and were similarly reflected in the urinary excretion patterns. Interestingly, the total urinary purine excretion did not indicate a direct replacement of uric acid by Hx. The total purine excretion was 2 fold higher with allopurinol ingestion compared with the placebo following a similar exercise performance test. The results indicate that an altered purine mix was presented to the kidney. This combined with a higher renal fractional clearance of Hx may explain the

elevated total urinary purine excretion observed with allopurinol treatment and exercise. Although speculative, allopurinol may decrease the extent of intestinal purine loss. This may also partly explain the increase in total purine excretion measured in the urine.

8.5 The influence of allopurinol and sprint training on purine nucleotide metabolism in man.

This study demonstrated that allopurinol had no effect on attenuating the reduction in muscle ATP content following sprint training. Two possible explanations can be provided to account for this result. The first is that allopurinol did not influence purine salvage during training and the balance between the degradation and loss of purines from the muscle relative to the salvage rate are not changed. The alternate explanation is that irrespective of the effect of allopurinol on purine salvage, there is insufficient substrate (ribose/PRPP) to supply the increased salvage capacity. Hence, any potential for an increase in purine salvage rates with allopurinol are redundant due to the lack of available substrate.

8.6 Conclusions

The major conclusions of this thesis were:

1. Increasing the number of intermittent sprint bouts increases the plasma inosine, Hx, and uric acid concentration during recovery.
2. Increasing the number of intermittent sprint bouts increases uric acid excretion in the urine after 2 hours recovery.
3. The estimated purine loss from the muscle, increased as the number of intermittent sprint bouts increased.

4. Sprint training reduces the estimated endogenous purine loss from the muscle, measured from the increase in endogenous purines in the plasma and urine, after an intense sprint bout.
5. Allopurinol increases the urinary purine loss following a bout of intermittent sprint exercise.
6. Allopurinol does not influence the attenuation of resting muscle ATP content observed following intense sprint training.

8.7 Recommendations for future research.

The studies in this thesis raise further questions regarding the effect of exercise, sprint training and allopurinol administration on skeletal muscle purine nucleotide metabolism. The elevated loss of purines from the body via the urine as the number of exercise repeats increased provides an indication, albeit indirect, of a greater muscle purine loss. However, more direct measurement of muscle purine loss with an increasing number of exercise bouts are required to get a more accurate picture of the muscle purine loss. Furthermore, purine loss via the gut has not been measured during or following intense exercise and the re-distribution of purine excretion between the gut and kidney is potentially altered by the big increase in purine load in the plasma following exercise and needs further research. Basal measurements have been reported, however, it is unknown whether the same proportional loss via the gut and kidney is maintained with exercise or recovery from exercise.

Further investigation is also required into the flux of purines across the interface between the muscle and the interstitial fluid during exercise and recovery. The potential for one-way flux of purines from the muscle is supported with evidence of concentrative nucleoside membrane transporters and a-v differences across muscle

beds. However, non-physiological plasma concentrations have demonstrated the capacity of skeletal muscle uptake of purine bases in rats. Future studies need to examine the nature of purine base movement (uni or bi-directional) across the sarcolemma and the role it has in PrN conservation within skeletal muscle. In addition to this, studies examining the efflux of muscle purine metabolites following intense exercise in the sprint-trained state would also provide useful information into training effects on skeletal muscle.

Further investigation into the capacity of the purine *de novo* synthesis and salvage pathways in skeletal muscle with training following the loss of purines after intense exercise is required. This will help determine whether the muscle adapts with sprint training to improve metabolic efficiency with training and also whether there are limitations in the normal recovery processes. Furthermore, further studies are warranted into limitations to the activities of both purine *de novo* synthesis and salvage, particularly pertaining to the potential limitation of substrate supply (ribose) in recovery. The activity of HGPRT is higher following sprint training and indicates an increased capacity of purine salvage, although evidently it is insufficient to maintain pre-training resting AdN content in skeletal muscle following sprint training in some studies. The influence of allopurinol on purine salvage also needs to be studied via more direct methods, possibly the influence of allopurinol on HGPRT activity. There is a possibility that the limitation may be due to another factor also involved in regulating intramuscular purine salvage. For example a limited substrate supply (ie ribose) unable to capitalise on the elevated HGPRT rate to recover muscle PrN content during intense sprint training needs to be investigated. Thus, the mechanism and the time course of replacement of PrN precursor in the muscle require further investigation.

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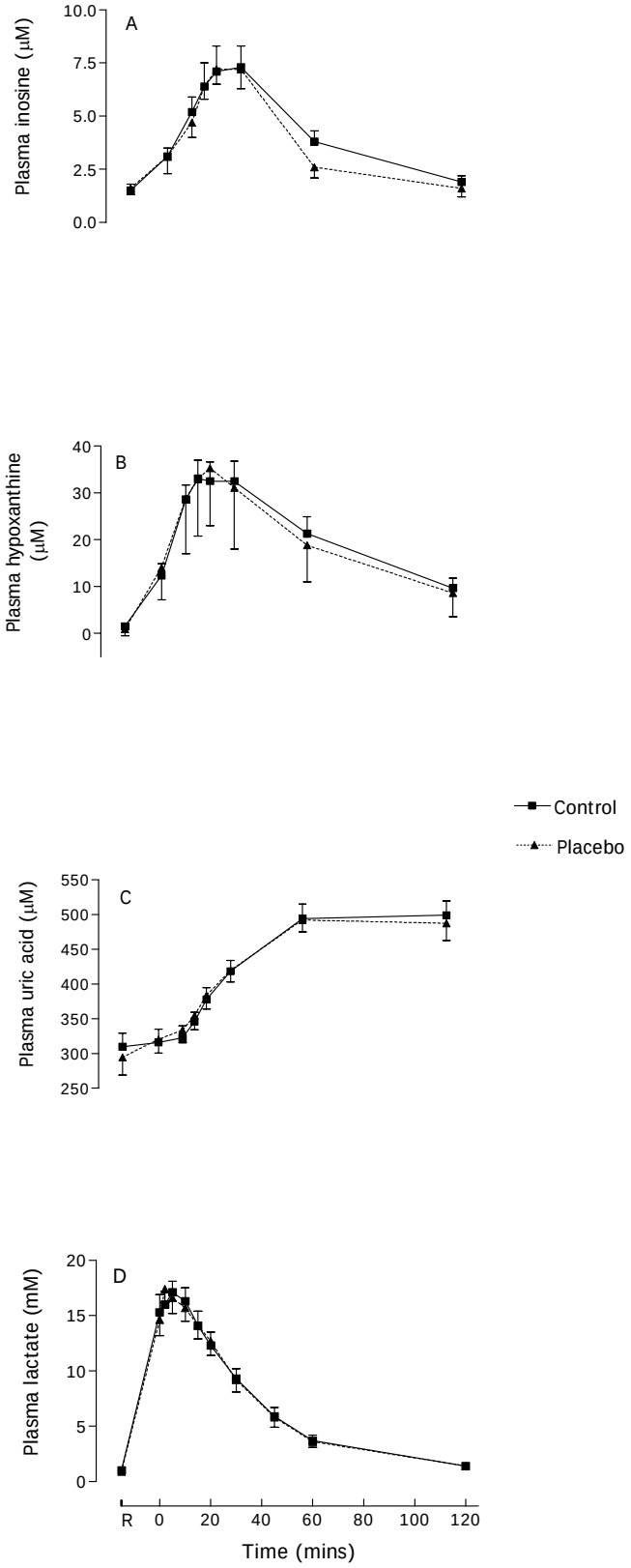
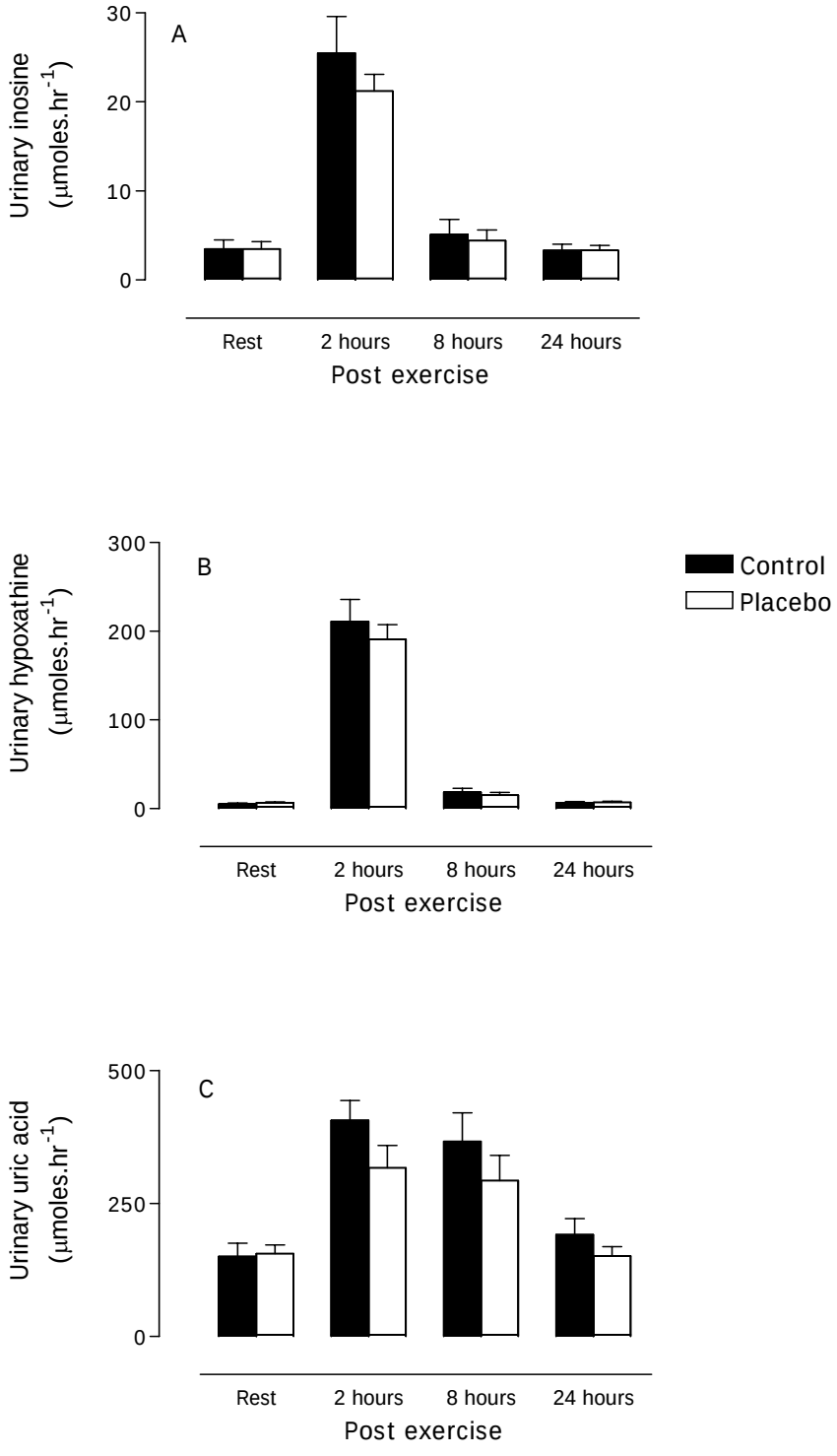
Appendix A

The use of calcium carbonate as an effective placebo.

The use of calcium carbonate as an effective placebo for the studies involving allopurinol administration was tested in a pilot study. Subjects were asked to perform two exercise trials separated by at least one week. The trials were allocated using a double blind random crossover design. The exercise protocol in each of these trials consisted of 8 x 10s "all out" sprint bouts on an air-braked cycle ergometer. In the placebo trials the subjects ingested a tablet of calcium carbonate once a day for the five days preceding the trial. In the control trial the subject presented to the lab for the experimental trial. Plasma and urine samples were collected at rest and during recovery from the experimental trial (Appendix Fig 1 and 2.)



Appendix Fig 1; Forearm venous plasma inosine (A), hypoxanthine (B), uric acid (C) and lactate (D) concentrations at rest (R) and during recovery from eight 10s intermittent sprint bouts with allopurinol or placebo ingestion. Values are means ± SE, n=7, * different from placebo; $P<0.05$. (note difference in scale).



Appendix Fig 2: Urinary inosine (A), hypoxanthine (B) and uric acid (C) excretion rate at rest (basal) and during the first 2 hours and the subsequent 6 and 16 hours of recovery following eight 10s intermittent sprint bouts with allopurinol or placebo ingestion. Values are means ± SE, n=7. * different from placebo, $P<0.05$. (note difference in scale).