EFFECTS OF EXERCISE, RENAL DISEASE, AND DIGOXIN ON SKELETAL MUSCLE Na^+,K^+-ATPase AND RELATED EFFECTS ON PLASMA K^+ AND MUSCLE PERFORMANCE.

Submitted by

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ABSTRACT

In skeletal muscle, the Na^+,K^+-ATPase enzyme regulates trans-membrane Na^+ and K^+ fluxes during contractions, and therefore also affects muscle excitability and plays an important role in delaying muscle fatigue. Consequently, any modulation of Na^+,K^+-ATPase content or activity has the potential to affect muscle fatiguability. Thus, this thesis investigated three factors thought to impair or down-regulate the skeletal muscle Na^+,K^+-ATPase – acute exercise, renal disease and digoxin. The related effects on plasma [K^+] during exercise and on muscle performance were also examined. This thesis firstly investigated the acute effects of brief intense exercise on muscle Na^+,K^+-ATPase content and maximal activity (Study 1). Study 2 investigated the effects of end-stage renal disease on plasma [K^+] regulation during exercise; examined the relationship between impaired [K^+] regulation and muscle performance, and investigated the effects of endurance training in these patients. Study 3 investigated the impacts of end-stage renal disease and renal transplantation on skeletal muscle Na^+,K^+-ATPase and its relationship with muscle performance. Finally, Study 4 investigated the effects of chronic digoxin administration on skeletal muscle Na^+,K^+-ATPase content and maximal activity and on muscle performance in healthy humans.

Study 1. Acute, fatiguing exercise has been shown to reduce skeletal muscle in-vitro maximal Na^+,K^+-ATPase activity, with potentially detrimental effects on muscle fatigue and exercise performance. This study investigated whether this depressed muscle in-vitro maximal Na^+,K^+-ATPase activity with exercise reflected a loss of Na^+,K^+-ATPase units, the time-course of its recovery post-exercise and whether this depressed activity was related to increased Na^+,K^+-ATPase isoform gene expression. Fifteen subjects performed fatiguing, knee extensor exercise at ~40% maximal work

output per contraction. A vastus lateralis muscle biopsy was taken at rest, fatigue, 3 h, and 24 h post-exercise and analysed for maximal Na^+,K^+-ATPase activity via 3-O-methylfluorescein phosphatase (3-O-MFPase) activity, Na^+,K^+-ATPase content via [^{3}H]ouabain binding site content and Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoform mRNA expression by Real-Time RT-PCR. Exercise (352±69 s; mean±SEM) did not affect [^{3}H]ouabain binding sites, but decreased 3-O-MFPase activity by 10.7±2.3% (P<0.05), which had recovered by 3 h post-exercise, without further change at 24 h. Exercise elevated α_1 mRNA by 1.5-fold at fatigue (P<0.05). This increase was inversely correlated with the percentage change in 3-O-MFPase activity from rest to fatigue (%Δ3-O-MFPase$_{rest\text{-}fatigue}$) (r=-0.60, P<0.05). The average post-exercise (fatigue, 3, 24 h) α_1 mRNA was increased 1.4-fold (P<0.05) and approached a significant inverse correlation with %Δ3-O-MFPase$_{rest\text{-}fatigue}$ (r=-0.56, P=0.08). Exercise elevated α_2 mRNA at fatigue 2.5-fold (P<0.05), which was inversely correlated with %Δ3-O-MFPase$_{rest\text{-}fatigue}$ (r=-0.60, P=0.05). The average post-exercise α_2 mRNA was increased 2.2-fold (P<0.05) and was inversely correlated with the %Δ3-O-MFPase$_{rest\text{-}fatigue}$ (r=-0.68, P<0.05). No significant correlations were found between %Δ3-O-MFPase$_{rest\text{-}fatigue}$ and other isoforms. Thus, acute exercise did not affect Na^+,K^+-ATPase content but transiently decreased Na^+,K^+-ATPase activity, which was correlated to increased Na^+,K^+-ATPase gene expression. This suggests a possible signal transduction role for depressed muscle Na^+,K^+-ATPase activity with exercise.

Study 2. Previous studies indicated that plasma K^+ regulation during exercise is impaired in haemodialysis patients (HD) that were anaemic and their plasma K^+ regulation is related to their poor exercise performance. Both treatment of anaemia with erythropoietin (EPO) and exercise training improve exercise performance in HD,

but whether this is associated with improved K^+ regulation is unknown. Therefore, six HD with near-normal [Hb] were tested for aerobic power ($\dot{V}O_{2peak}$) and plasma [K^+] during incremental cycling exercise, for knee-extensor muscle isokinetic peak torque (PT) (0-360°.s^{-1}) and fatigability, and for quality of life (QOL). All tests were conducted at Baseline, after 6 weeks of normal activity (Pre-train) and following 6 weeks of stationary cycle training (Post-train). Six healthy controls (CON) matched for age, sex, body mass, and height, were also tested at Baseline. Plasma [K^+] at rest, during and following the Baseline aerobic power test was higher in HD than CON ($P < 0.05$, main effect). The rise in [K^+] ($\Delta[K^+]$) during exercise was also higher in HD than CON ($P < 0.05$, main effect). The rate of rise in [K^+], and $\Delta[K^+]$ relative to total work done ($\Delta[K^+].work^{-1}$ ratio) during the aerobic power test were also higher in HD, by 93 and 239%, respectively ($P < 0.05$ for both). $\dot{V}O_{2peak}$ and time to fatigue during the aerobic power test were lower in HD at Baseline compared to CON ($\dot{V}O_{2peak}$ 23.5 ± 7.7 vs. 37.4 ± 4.7 ml.kg^{-1}.min^{-1}; time to fatigue 511 ± 139 vs. 950 ± 224 s, respectively, $P < 0.05$). PT was reduced by 27 – 42% in HD at Baseline compared to CON ($P < 0.05$). Exercise training increased time to fatigue by 12% ($P < 0.05$) and reduced the $\Delta[K^+].work^{-1}$ ratio by 31% ($P < 0.05$). The $\Delta[K^+].work^{-1}$ ratio (pooled data, $n = 24$) was inversely related to $\dot{V}O_{2peak}$ ($r = -0.72$, $P<0.001$), time to fatigue ($r = -0.64$, $P<0.001$), and peak work rate ($r = -0.65$, $P<0.001$) during the aerobic power test, and also isometric PT ($r = -0.43$, $P<0.05$). Thus, in HD patients treated with EPO, extrarenal K^+ regulation was still impaired and may contribute to their poor exercise performance compared to healthy controls. Furthermore, their improvement in exercise performance with training is consistent with enhanced K^+ regulation.

Study 3. A potential cause of impaired K^+ regulation in HD is depressed skeletal muscle Na^+,K^+-ATPase activity. However, no studies have investigated this

possibility. Therefore, this study examined whether impaired exercise performance and extrarenal K^+ regulation in HD are associated with abnormal skeletal muscle Na^+,K^+-ATPase. It was further investigated whether these are normalised with improved renal function via renal transplantation (RTx). $\dot{V}O_{2peak}$ and plasma $[K^+]$ were measured during incremental exercise in 10 HD, 9 RTx, and 10 CON. Knee-extensor isokinetic PT and fatiguability during 30 maximal knee-extensions, and thigh muscle cross-sectional area (TMCSA) were also measured. A resting vastus lateralis muscle biopsy was analysed for Na^+,K^+-ATPase maximal activity, content, and isoform (α_1, α_2, α_3, β_1, β_2, and β_3) abundance. $\dot{V}O_{2peak}$ was 35% and 32% lower in HD and RTx than CON, respectively ($P < 0.05$). PT was less in HD and RTx than CON ($P < 0.05$) but did not differ between groups when expressed relative to TMCSA. Muscle fatigability was higher in HD (25 ± 4%) and RTx (24 ± 11%) than CON (15 ± 5%) ($P < 0.05$). Plasma $[K^+]$ was higher in HD than in RTx and CON ($P < 0.05$) at rest, during exercise and at 5 and 10 min post-exercise, however, the $\Delta[K^+].work^{-1}$ ratio was not different between HD, RTx, and CON. Muscle 3-O-MFPase activity was 31% and 28% lower in HD and RTx, respectively than CON ($P < 0.02$), but [^{3}H]ouabain binding site content and isoform abundance did not differ between groups. $\dot{V}O_{2peak}$ (pooled data, $n = 29$) was correlated with 3-O-MFPase activity (r=0.45, P<0.05) and creatinine clearance (CrCl) (r=0.43, P<0.05); and 3-O-MFPase activity with CrCl (r=0.44, P<0.05). Thus, $\dot{V}O_{2peak}$ and muscle Na^+,K^+-ATPase activity were depressed and muscle fatigability increased in HD and RTx. Furthermore, correlations between $\dot{V}O_{2peak,}$ 3-O-MFPase activity and CrCl suggest a link between impaired exercise performance, muscle Na^+,K^+-ATPase activity and renal function.

Study 4. The final study investigated the effects of the specific Na^+,K^+-ATPase inhibitor, digoxin on skeletal muscle Na^+,K^+-ATPase content and maximal activity and exercise performance in healthy humans. In digoxin-treated chronic heart failure patients, skeletal muscle Na^+,K^+-ATPase content and exercise performance were each reduced. However, whether chronic digoxin administration reduces skeletal muscle Na^+,K^+-ATPase content and activity and thus impairs maximal exercise performance in healthy humans has not been investigated. Therefore, this final study tested 10 healthy volunteers after taking digoxin (DIG, 0.25 $mg.d^{-1}$) or a placebo (CON) for 14 days in a double-blind, randomised, counterbalanced, crossover design. Subjects cycled for 10 min at 33% $\dot{V}O_{2peak}$, 10 min at 67% $\dot{V}O_{2peak}$, and then continued until fatigue at 90% $\dot{V}O_{2peak}$. A vastus lateralis muscle biopsy was taken at rest, immediately after the 67% $\dot{V}O_{2peak}$ exercise bout, at fatigue, and at three hours post-exercise (+3h) for later analysis of Na^+,K^+-ATPase measures. Four weeks later, the groups changed to the alternative treatment for two weeks and repeated the exercise test and biopsies. Serum digoxin at rest on d 14 was 0.8 ± 0.2 nM (mean ± SD) in DIG and < 0.4 nM (detection limit) in CON. Time to fatigue during exercise at 90% $\dot{V}O_{2peak}$ was not different between CON and DIG (254 ± 125 and 262 ± 156 s, respectively). Neither [^{3}H]ouabain binding nor 3-O-MFPase activity differed between DIG and CON. At fatigue, [^{3}H]ouabain binding tended to be higher than rest (P < 0.06) and was higher than +3 h by 16% (P < 0.05). Exercise did not affect 3-O-MFPase activity. Thus, chronic digoxin administration elevated serum digoxin to low therapeutic levels, but did not alter skeletal muscle Na^+,K^+-ATPase content or maximal activity and did not impair exercise performance in healthy subjects. Conversely, exercise caused a transient increase in Na^+,K^+-ATPase content but no change in Na^+,K^+-ATPase maximal activity. These results suggest that chronic

administration of a typical clinical digoxin dose does not affect skeletal muscle Na^+,K^+-ATPase or exercise performance in healthy humans. This might possibly be due to inadequate digitalisation in healthy subjects with a large muscle mass.

Conclusions.

Acute Exercise. Studies 1 and 4 report conflicting effects of acute exercise on skeletal muscle Na^+,K^+-ATPase content and maximal activity. In an attempt to resolve this issue, the maximal Na^+,K^+-ATPase activity at fatigue induced by single-leg knee-extensions (~6 min, Study 1) or incremental cycling exercise (~24 min, Study 4), was expressed as a fraction of the resting activity for both studies. These pooled results indicated that acute exercise increased skeletal muscle Na^+,K^+-ATPase content by 8.5 ± 18% ($P < 0.05$) and depressed maximal activity by 7.5 ± 13% ($P < 0.05$). The latter potentially contributed to muscle fatigue. *Renal Disease.* Skeletal muscle Na^+,K^+-ATPase activity was depressed in HD and RTx and this was related to reduced exercise performance. Additionally, exercise performance and the plasma K^+ response to exercise were improved with training in HD. The latter is consistent with a training-induced increase in skeletal muscle Na^+,K^+-ATPase activity. *Digoxin.* Chronic digoxin administration did not significantly depress skeletal muscle Na^+,K^+-ATPase activity or content and, consistent with this, exercise performance was also unchanged. Finally, when data from Studies 1, 3, and 4 were pooled, a significant positive correlation was found between skeletal muscle maximal Na^+,K^+-ATPase activity and $\dot{V}O_{2peak}$ ($r = 0.49$, $P < 0.001$, $n = 50$). These combined results suggest a link between skeletal muscle Na^+,K^+-ATPase activity and exercise performance.

DECLARATION

I, Aaron Christian Petersen, declare that the PhD thesis entitled *Effects of Exercise, Renal Disease, and Digoxin on Skeletal Muscle Na^+,K^+-ATPase and Related Effects on Plasma K^+ and Muscle Performance* is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature Date Monday, April 2nd, 2007

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ABBREVIATIONS

[ ]	concentration
[^{3}H]ouabain binding	tritiated ouabain binding
3-O-MFP	3-O-methylfluorescein phosphate
3-O-MFPase	3-O-methylfluorescein phosphatase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CAPD	continuous ambulatory peritoneal dialysis
Cl^-	chloride ion
CON	healthy control subjects
CRF	chronic renal failure
CSA	cross-sectional area
EDLF	endogenous digitalis-like factor
E_m	resting muscle membrane potential
EPO	erythropoietin
ESRD	end-stage renal disease
Hb	haemoglobin
Hct	haematocrit
HRQOL	health-related quality of life
K^+	potassium ion
$[K^+]_{a-v}$	arterio-venous plasma K^+ concentration difference
$[K^+]_e$	extracellular K^+ concentration
$[K^+]_I$	muscle interstitial K^+ concentration
$[K^+]_i$	intracellular K^+ concentration
$[K^+]_v$	venous plasma K^+ concentration

$\Delta[K^+]$	rise in plasma K^+ concentration
$\Delta[K^+].work^{-1}$ ratio	$\Delta[K^+]$ relative to work done
kDa	kilo daltons
Mg^{2+}	magnesium ion
mRNA	messenger ribonucleic acid
MVC	maximal voluntary contraction
mo	months
Na^+	sodium ion
$[Na^+]_I$	muscle interstitial Na^+ concentration
$[Na^+]_i$	intracellular Na^+ concentration
Na^+,K^+-ATPase	sodium-potassium adenosine 5'triphosphatase
NKCC	$Na^+,K^+,2Cl^-$ cotransporter
PCr	phosphocreatine
P_i	inorganic phosphate ion
p-NPP	*p*-nitrophenyl phosphate
p-NPPase	*p*-nitrophenyl phosphatase
Pre-D	pre-dialysis
PT	peak torque
QOL	quality of life
RBC	red blood cells
RTx	renal transplantation
Lac^-	lactate ion
t-tubules	tranverse tubules
$\dot{V}O_{2peak}$	peak oxygen consumption
wk	weeks

PUBLICATIONS AND PRESENTATIONS

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PUBLICATIONS

1. Murphy, K.T., R.J. Snow, **A.C. Petersen**, R.M. Murphy, J. Mollica, J.S. Lee, A.P. Garnham, R.J. Aughey, J.A. Leppik, I. Medved, D. Cameron-Smith and M.J. McKenna (2004). Intense exercise up-regulates Na^+,K^+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *J Physiol (Lond)* 556: 507-519.

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CONFERENCE PRESENTATIONS

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CHAPTER 1. INTRODUCTION

The Na^+,K^+-ATPase enzyme in skeletal muscle counters the excitation-induced Na^+ and K^+ fluxes and is thus critical in maintaining transmembranous [Na^+] and [K^+] gradients, action potential propagation and facilitating ongoing muscle contraction. Consequently, any modulation of muscle Na^+,K^+-ATPase content or activity has the potential to influence muscle fatiguability.

Acute exercise may alter both Na^+,K^+-ATPase activity and content. Acute, fatiguing exercise has repeatedly been shown to reduce maximal in-vitro Na^+,K^+-ATPase activity (for references see (Aughey *et al.* 2005)), however the time course and subsequent recovery of this depression is equivocal. Inhibition of Na^+,K^+-ATPase activity increased Na^+,K^+-ATPase mRNA expression in rat liver (Pressley *et al.* 1988). Whether exercise-induced Na^+,K^+-ATPase inhibition also exerts a similar effect in skeletal muscle is unknown. Upregulation of muscle Na^+,K^+-ATPase content with acute exercise is suggested by increased muscle [^{3}H]ouabain binding after ~10 h running (Overgaard *et al.* 2002), but other studies have found no immediate change following exercise (for references see (Aughey *et al.* 2005)). Therefore Study 1 investigated the effects of acute exercise on skeletal muscle Na^+,K^+-ATPase activity, content, and mRNA .

Skeletal muscle Na^+,K^+-ATPase might be impaired in renal disease. This was suggested by impaired plasma K^+ regulation during exercise in haemodialysis patients (HD) who were anaemic, which was related to their impaired exercise performance (Kettner Melsheimer *et al.* 1987, Sangkabutra *et al.* 2003). Treatment of anaemia with erythropoietin (EPO) improved K^+ regulation in HD (McMahon *et al.* 1999). However, whether K^+ regulation is still impaired compared to healthy controls (CON) is unknown. Exercise training improves K^+ regulation in healthy subjects (Green *et al.*

1993, McKenna *et al.* 1993) and also exercise performance in HD (Painter *et al.* 1986b). If training also improves K^+ regulation in HD, this may contribute to their improved exercise performance, however this remains to be determined. Thus, Study 2 determined whether K^+ regulation is impaired in EPO-treated HD compared to CON, and whether exercise training can improve the impaired K^+ regulation and exercise performance observed in HD.

The mechanisms underlying impaired K^+ regulation in HD may be due to abnormal skeletal muscle Na^+,K^+-ATPase, as found in uraemic rats (Bofill *et al.* 1994, Druml 1988) but not yet investigated in humans. Whether abnormalities exist in skeletal muscle Na^+,K^+-ATPase in renal transplantation recipients (RTx) is also unknown. RTx display reduced exercise performance compared to CON (van den Ham *et al.* 2005), however whether their K^+ regulation during exercise is also impaired is unknown. Therefore, Study 3 examined whether impaired K^+ regulation in HD is associated with abnormal skeletal muscle Na^+,K^+-ATPase. It was further investigated whether these are normalised with improved renal function in RTx.

Digoxin is a specific Na^+,K^+-ATPase inhibitor. In digoxin-treated chronic heart failure patients, skeletal muscle Na^+,K^+-ATPase content and exercise performance were each reduced. However, whether chronic digoxin administration reduces skeletal muscle Na^+,K^+-ATPase content, activity, and exercise performance in healthy humans has not been investigated. Furthermore, chronic digoxin administration upregulated guinea-pig myocardial Na^+,K^+-ATPase (Bonn *et al.* 1978), but whether this occurs in human skeletal muscle is equivocal. Therefore, Study 4 examined the effects of skeletal muscle Na^+,K^+-ATPase inhibition by chronic digoxin administration on exercise performance in healthy humans.

This thesis comprises a literature review, four experimental studies, and concludes with a general discussion, conclusions, and recommendations for future research

CHAPTER 2. LITERATURE REVIEW

This literature review consists of three sections. The first section describes the role of skeletal muscle Na^+ and K^+ fluxes in muscle fatigue and the role of skeletal muscle Na^+,K^+-ATPase in countering these fluxes and subsequent effects on muscle fatigue. It also describes the structure, function, and acute and chronic regulation of skeletal muscle Na^+,K^+-ATPase. Section II focuses on the impaired exercise performance typically observed in patients with end-stage renal disease treated by haemodialysis (HD) or renal transplantation (RTx). The factors contributing to their poor exercise performance are discussed with particular emphasis on the role of skeletal muscle Na^+,K^+-ATPase and extrarenal K^+ regulation. Section III details the specific aims and hypotheses of each study within this thesis.

This literature review focuses primarily on data from human skeletal muscle. Where this is not available or to provide supporting evidence, data from other tissues or from animal studies is used.

SECTION I: ROLE OF Na^+, K^+ AND Na^+,K^+-ATPase IN MUSCLE FATIGUE

2.1 ROLE OF Na^+ AND K^+ IN MUSCLE FATIGUE

The ability to sustain muscle contractions depends on repeated action potential generation and propagation along the sarcolemma and into the transverse tubules (t-tubules). Action potential generation and propagation are dependant on steep trans-sarcolemmal $[Na^+]$ and $[K^+]$ gradients. However, action potentials are caused by small Na^+ and K^+ fluxes across the sarcolemma, thus reducing both the $[Na^+]$ and $[K^+]$ gradients and potentially compromising the capacity to generate further action potentials. Thus, reviewing the roles of Na^+ and K^+ changes during contraction and their regulation is important in understanding muscle fatigue.

2.1.1 Na^+ and K^+ concentration and fluxes during muscle contractions

2.1.1.1 Na^+ concentration and fluxes

In human skeletal muscle at rest, the interstitial [Na^+] ($[Na^+]_I$) has been determined between 132 - 143 mM (Sjøgaard 1983, Sjøgaard *et al.* 1985, Street *et al.* 2005). The intracellular [Na^+] ($[Na^+]_i$) has been measured between 6 – 32 mM (Bergström *et al.* 1971, Juel 1986, Sjøgaard 1983, Sjøgaard *et al.* 1985), however the majority of evidence suggests that the range is between 6 – 13 mM at rest (Bergström *et al.* 1971, Juel 1986, Sjøgaard 1983, Sjøgaard *et al.* 1985). At rest, passive Na^+ fluxes are mediated primarily by the voltage-sensitive Na^+ channels (Hille 2001). During excitation, the voltage-sensitive Na^+ channels open to allow a rapid influx of Na^+, thus depolarising the cell. In stimulated rat skeletal muscle, this influx was calculated between 2 – 11 nmol.g wet weight^{-1} (Gissel *et al.* 2000, Nielsen *et al.* 1997). Depolarisation increases sodium channel mean open time and sodium conductance (Franke and Hatt 1990), and thus sodium conductance is likely to be increased during contraction, but this is yet to be investigated.

Following intense cycling exercise, vastus lateralis muscle Na^+ content was increased by 68 – 208% (Sjøgaard 1983, Sjøgaard *et al.* 1985), in keeping with increases in $[Na^+]_i$ of 77% - 240% (Bergström *et al.* 1971, Sjøgaard *et al.* 1985). A small increase (4%) in $[Na^+]_I$ was also noted, however this was attributed to diffusion of extracellular fluid in to the muscle (Sjøgaard 1983). Thus, increased $[Na^+]_i$ but unchanged $[Na^+]_I$, results in a decrease in the trans-sarcolemmal Na^+ driving gradient ($[Na^+]_I/[Na^+]_i$), potentially decreasing the driving force for the inward Na^+ current during action potentials (Cairns *et al.* 2003).

During electrical-stimulation of isolated muscles, there was a positive correlation between progressive force reduction and increasing $[Na^+]_i$ in the rat (Nielsen *et al.*

1996) and an inverse correlation between action potential amplitude and $[Na^+]_i$ in the frog (Balog *et al.* 1996). Also, by using carbacholine to elevate $[Na^+]_i$ by 36%, tetanic force was reduced by 69% in isolated rat muscles (Macdonald *et al.* 2005). Furthermore, in skinned rat muscle fibres, it was reported that the ability of the t-system to support closely spaced action potentials was significantly hindered by a rise in $[Na^+]_i$ (Nielsen *et al.* 2004b). Decreasing the Na^+ driving gradient by reducing $[Na^+]_I$ from 147 to 80 mM, doubled the rate of decline in peak torque during intermittent tetanic stimulation in mouse soleus (Cairns *et al.* 2003).

2.1.1.2 K^+ concentration and fluxes

At rest, the interstitial $[K^+]$ ($[K^+]_I$) in human skeletal muscle has been determined at ~ 4.5 mM (Mohr *et al.* 2004, Nielsen *et al.* 2004a, Nordsborg *et al.* 2003, Sjøgaard 1983, Sjøgaard *et al.* 1985), whereas the intracellular $[K^+]$ ($[K^+]_i$) was reported between 160 - 168 mM (Sjøgaard 1983, Sjøgaard *et al.* 1985).

Passive K^+ fluxes are mediated by K^+ channels, primarily the voltage-sensitive inward rectifier, and delayed rectifier, the Ca^{2+}-sensitive and the ATP-sensitive K^+ channels (Clausen 2003). During excitation, repolarisation of the cell membrane is achieved by a rapid efflux of K^+ through the voltage-sensitive K^+ channels (Hille 2001). Action potentials result in a net myocytic efflux of K^+. In humans, it has been calculated that each muscle action potential results in a K^+ efflux of ~ 1.7 – 2.0 nmol.g muscle wet weight^{-1} (Hallén 1996, Sjøgaard 1990). ATP-sensitive K^+ channels may also contribute to K^+ efflux during muscle contraction, possibly due to localised ATP depletion (Spruce *et al.* 1987). Thus, sarcolemmal K^+ conductance is dramatically increased in contracting muscle. K^+ released from contracting muscle cells enters the interstitial space and diffuses into the capillaries where it is transported in the venous circulation via plasma and red blood cells and taken up by inactive muscle or excreted

by the kidneys (Sejersted *et al.* 2000). Thus, the $[K^+]$ is in the order of: intracellular > interstitial > venous > arterial.

In humans, exercise reduced muscle K^+ content from 466 to 430 mmol.kg dry weight^{-1} and $[K^+]_i$ from 162 mM to 129 mM (Sjøgaard *et al.* 1985). Recent studies using micro-dialysis probes in the interstitial space have reported $[K^+]_I$ up to 10 - 12 mM during contractions (Green *et al.* 2000b, Juel *et al.* 2000b, Nielsen *et al.* 2004a, Nordsborg *et al.* 2003). Although it has not been measured, it is likely that the $[K^+]$ within the t-tubules would be even higher during exercise due to their large surface area, small volume and diffusion limitations. Femoral venous $[K^+]$ reached 7.8 – 8.2 mM after 30 s of maximal cycling exercise, whereas arterial $[K^+]$ reached 6.9 – 7.0 mM (Kowalchuk *et al.* 1988, McKenna *et al.* 1997).

Exercise-induced increases in $[K^+]_I$ and decreases in $[K^+]_i$ have deleterious effects on muscle contractility. In skinned rat muscle fibres, a reduction in $[K^+]_i$ from 113 mM to 60 mM caused an ~ 30% decrease in twitch force (Nielsen *et al.* 2004b). Similarly, by increasing $[K^+]_I$ to 10 mM, the isometric twitch and tetanic force in isolated rat soleus muscles decreased by 40 – 45%, and at $[K^+]_I$ of 12.5 mM, tetanic force was reduced by 96% (Clausen *et al.* 1993).

2.1.1.3 Synergistic effects of Na^+ and K^+ fluxes

Increases in $[K^+]_I$ and decreases in the Na^+ driving gradient occur simultaneously during contraction, and their combined depressive effect on force development is greater than their individual effects. Individually, increasing $[K^+]_I$ from 4 to 9 mM, or reducing the Na^+ driving gradient by decreasing $[Na^+]_I$ from 147 to 85 mM, did not affect tetanic force in isolated rat soleus muscle (Overgaard *et al.* 1999). However, the combination of 9 mM $[K^+]_I$ and 85 mM $[Na^+]_I$ induced a 50% decrease in tetanic force (*ibid*). Also, 7 mM $[K^+]_I$ had no effect on tetanic force at 120 mM $[Na^+]_I$ in frog

sartorius muscle, but caused a 31% decrease in tetanic force at 100 mM $[Na^+]_I$ (Bouclin *et al.* 1995).

2.1.2 Mechanism of Na^+ and K^+ flux induced fatigue

Resting human muscle membrane potential (E_m) is approximately -90 mV at rest (Cunningham *et al.* 1971, Fitts *et al.* 1996, Sjøgaard *et al.* 1985) and is predominantly dependant on the trans-sarcolemmal $[Na^+]$,$[K^+]$ and $[Cl^-]$ gradients (Hodgkin *et al.* 1959a, Sejersted *et al.* 2000). During exercise, the trans-sarcolemmal $[K^+]$ gradient is markedly reduced, resulting in membrane depolarisation. In humans, changes in $[Na^+]_i$, $[K^+]_i$, $[Na^+]_I$, and $[K^+]_I$ during intense knee extensor exercise were calculated to induce a 15 mV membrane depolarisation in the vastus lateralis muscle (Sjøgaard *et al.* 1985). E_m depolarisation is associated with a reduction in the action potential amplitude (Balog *et al.* 1994, Hodgkin *et al.* 1952), which, if severe enough, could prevent the action potential from reaching the threshold value of 0 mV, thus preventing action potential generation and therefore muscle contraction (Fitts *et al.* 1996). Strong evidence to support this hypothesis comes from the finding of reduced contractility and action potential amplitude, and increased action potential threshold and numbers of inexcitable fibres in isolated mouse EDL and soleus muscles exposed to a reduced Na^+ driving gradient (Cairns *et al.* 2003). Additionally, it is hypothesised that contraction induced changes to ion gradients, E_m and action potentials will be more pronounced in the t-tubular system than the sarcolemma (Fitts *et al.* 1996, Overgaard *et al.* 1999) and are therefore more likely to result in excitation failure. At present, this remains a hypothesis due to difficulty in detecting such changes in the t-tubules. Ionic perturbations in the t-tubules will depend on the distribution of ion channels and pumps. This has not been thoroughly investigated, but there is some evidence to suggest that compared to the sarcolemma, the t-tubules have a higher

density of Na^+ channels (Moore *et al.* 1983), lower (Kristensen *et al.* 2006) or similar (Moore *et al.* 1983) density of K^+ inward rectifier channels, lower Na^+,K^+,$2Cl^-$ co-transport density (Kristensen *et al.* 2006) and similar Na^+,K^+-ATPase density (see 2.2.4). K^+ permeability of resting rat sternomastoid muscles was found to be similar between the t-tubules and sarcolemma (Dulhunty, 1979), thus K^+ conductance is also likely to be similar. However, t-tubular K^+ conductance has not been determined. Nonetheless, numerous studies have indirectly implicated failure of action potential propagation in the development of fatigue in humans. M-wave amplitude and/or area were depressed at fatigue after sustained (Bellemare *et al.* 1988, Fuglevand *et al.* 1993) and repeated isometric contractions (Fowles *et al.* 2002a). Evidence suggested that impairment of action potential propagation contributed to fatigue after fatiguing drop-jumps (Strojnik *et al.* 1998), concentric knee-extensions (Babault *et al.* 2006) and isometric contractions (Babault *et al.* 2006, Milner-Brown *et al.* 1986). Studies in isolated animal muscles strongly suggest a link between membrane excitability and muscle function. During electrical stimulation of isolated rat soleus muscle, a linear correlation was found between M-wave area and tetanic force (Overgaard *et al.* 1999), while the reduction in tetanic force was associated with decreases in deep M-wave amplitude and area (Harrison *et al.* 1999). In frog semitendinosus muscle, high-frequency stimulation was associated with a reduction in force and a 9 mV depolarisation (Balog *et al.* 1994). Conversely, M-wave area was not changed after prolonged cycling (Sandiford *et al.* 2004) or running (Millet *et al.* 2002) in humans, and soleus muscle action potential amplitude was even increased after 5 min tetanic stimulation (Hicks *et al.* 1989).

In summary, considerable evidence implicates depressed muscle membrane [Na^+] and [K^+] gradients with muscle fatigue and these are likely due to exert a depressive effect

on membrane excitability. Membrane inexcitability has also been linked with muscle fatigue in both human and animal studies.

2.1.3 The role of the Na^+,K^+-ATPase in skeletal muscle fatigue

The Na^+,K^+-ATPase counters the excitation-induced Na^+ and K^+ fluxes which cause depolarisation and is thus critical in maintaining transmembranous [Na^+] and [K^+] gradients, action potential generation and facilitating ongoing muscle contraction.

2.1.3.1 Na^+,K^+-ATPase activation and muscle contractility

In isolated rat soleus muscles exposed to 10 mM [K^+], tetanic force was reduced by 58 - 77% (Nielsen *et al.* 1998, Overgaard *et al.* 2001). Subsequent activation of the Na^+,K^+-ATPase by stimulation of the muscle at 1 min intervals restored tetanic force to 81 – 97% of control levels (Nielsen *et al.* 1998, Overgaard *et al.* 2001). Restoration of force has also been reported in isolated rat soleus muscles inhibited by high [K^+], after Na^+,K^+-ATPase activation by the addition of adrenaline, salbutamol, amylin, CGRP, and insulin (Andersen *et al.* 1993, Clausen 2000, Clausen *et al.* 1993). In isolated rat soleus muscles in which the Na^+,K^+-ATPase had been stimulated by adrenaline, salbutamol or insulin, the decline in force during subsequent exposure to high $[K^+]_e$ was significantly retarded (Clausen *et al.* 1991).

Thus, activation of the Na^+,K^+-ATPase appears to be vitally important in the maintenance of muscle contractility during exposure to high [K^+], such as that which occurs during exercise.

2.1.4 Na^+,K^+-ATPase content and exercise performance

Changes in the content or activity of available Na^+,K^+-ATPase units could potentially alter the muscles capacity to regulate transmembrane [Na^+] and [K^+] gradients and thus muscle contractility.

2.1.4.1 Na^+,K^+-ATPase upregulation and exercise performance

Exercise training increases skeletal muscle Na^+,K^+-ATPase content (section 2.2.7.1.1) and should thus be expected to improve muscle Na^+ and K^+ regulation, and muscle excitability during exercise. In elite alpine skiers, the increase in Na^+,K^+-ATPase content following strength training was correlated to the improvement in an endurance test (Medbø *et al.* 2001). In elite junior cross-country skiers, Na^+,K^+-ATPase content was correlated with $\dot{V}O_{2peak}$, treadmill running performance, and to the rank of the subjects cross-country skiing performance (Evertsen *et al.* 1997). Furthermore, Na^+,K^+-ATPase content was correlated to maximum isometric strength (Klitgaard *et al.* 1989). Conversely, seven weeks of sprint training increased Na^+,K^+-ATPase content by 16% and attenuated the exercise-induced rise in plasma K^+, however isometric muscle function and the improvement in work-output following training were not correlated with Na^+,K^+-ATPase content (McKenna *et al.* 1993). Thus, upregulation of Na^+,K^+-ATPase content via training has been linked with improvements in exercise performance, thus emphasising it's importance in delaying muscle fatigue. However this relationship is not always apparent, possibly due to the multi-factorial and complex nature of muscle fatigue.

2.1.4.2 Na^+,K^+-ATPase inhibition and exercise performance

The importance of the skeletal muscle Na^+,K^+-ATPase in minimising muscle fatigue has been shown in isolated rat soleus muscles, in which inhibition of the Na^+,K^+-ATPase by ouabain increased the rate of force decline during tetanic stimulation by 3-fold (Nielsen *et al.* 1996). The effects of Na^+,K^+-ATPase inhibition on *in-vivo* muscle performance have not been thoroughly investigated however. Repeated running performance was worsened after digoxin treatment of 0.5 mg, three times daily, for a total of seven or 10 doses (Bruce *et al.* 1968). Exercise performance

was reduced in patients with chronic heart failure (CHF) treated with a non-specified dose of digoxin (Sullivan *et al.* 1988). CHF patients treated with digoxin for 9 – 108 months with 125 – 187.5 $\mu g.d^{-1}$ had reduced skeletal muscle Na^+,K^+-ATPase content (Nørgaard *et al.* 1990, Schmidt *et al.* 1993b), and $\dot{V}O_{2peak}$ was correlated with Na^+,K^+-ATPase content in another group of CHF patients (Green *et al.* 2001). Digoxin administration of 1 $mg.d^{-1}$ for three days resulted in serum [digoxin] of 1.2 $nmol.L^{-1}$ and a 9% occupancy of skeletal muscle Na^+,K^+-ATPase in CHF, thus further reducing Na^+,K^+-ATPase content, and increased the K^+ loss from exercising muscles by 138%, but time to fatigue was not altered (Schmidt *et al.* 1995). Thus, inhibition of Na^+,K^+-ATPase content did not impair exercise performance in CHF. However, exercise performance is severely impaired in CHF patients due to numerous myopathies (Gosker *et al.* 2000) that may have outweighed the effects of a further small reduction in Na^+,K^+-ATPase content on muscle fatiguability. In healthy humans, daily digoxin treatment of 0.5 mg for two weeks, giving a serum [digoxin] of 1 $nmol.L^{-1}$, had no effect on $\dot{V}O_{2peak}$ or isokinetic leg strength (Sundqvist *et al.* 1983), but Na^+,K^+-ATPase activity or content were not measured. As two weeks may be sufficient time for compensatory upregulation of Na^+,K^+-ATPase activity to occur (Bonn *et al.* 1978), Na^+,K^+-ATPase activity or content may not have been inhibited at the time of testing. Thus, it is yet to be determined whether inhibition of the Na^+,K^+-ATPase reduces maximal exercise performance in healthy humans. This was investigated in Chapter 6. Skeletal muscle Na^+,K^+-ATPase activity is depressed with acute exercise (section 2.2.6.1.3). Acute exercise during digoxin administration may therefore further depress skeletal muscle Na^+,K^+-ATPase activity, with potentially confounding effects on exercise performance. This was also investigated in Chapter 6.

2.2 SKELETAL MUSCLE Na^+,K^+-ATPase

2.2.1 Structure

The Na^+,K^+-ATPase is a P-type ATPase whose minimal functional unit is an αβ heterodimer comprised of a catalytic α subunit and a glycosylated β subunit (Figure 2.1) (Levenson 1994, Lingrel 1992). A third γ subunit of the Na^+,K^+-ATPase has also been identified recently (Crambert *et al.* 2003).

2.2.2 Function

2.2.2.1 Na^+ and K^+ transport

The primary function of Na^+,K^+-ATPase in skeletal muscle is to maintain $[Na^+]$ and $[K^+]$ gradients across the cell membrane and, via its electrogenic effect, thus maintain membrane potential. To do this the Na^+,K^+-ATPase hydrolyses an ATP molecule and the energy from this is used to transport three Na^+ ions out of the cell and two K^+ ions into the cell with each cycle (Figure 2.2) (Clausen 1998).

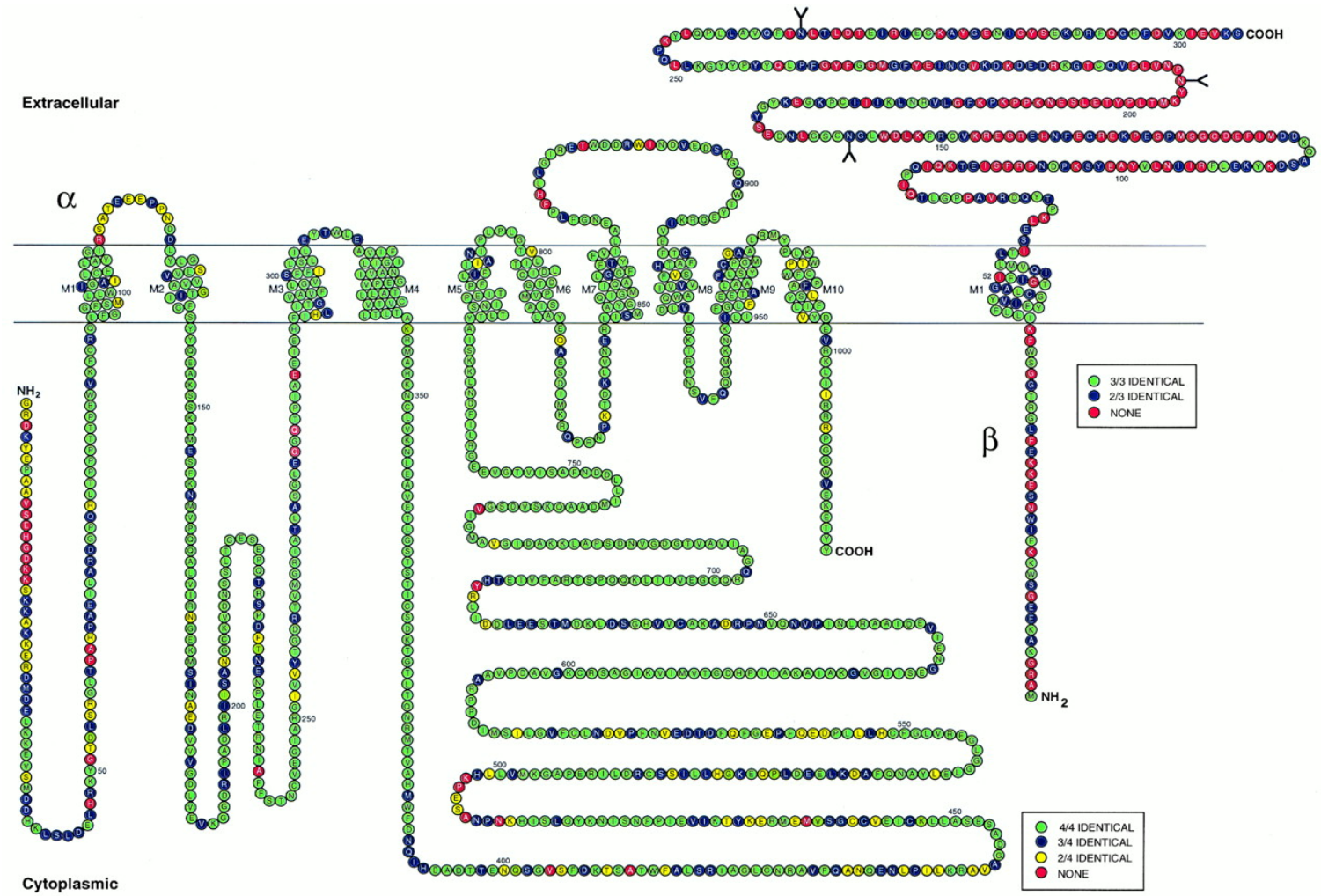

Figure 2.1. Scheme of the membrane topology of the α- and β-isoforms of the Na^+,K^+-ATPase.

Sequences of rat α_1 and β_1-isoforms are shown. Residues are coloured to indicate the amino acid homology among the different α-isoforms (α_1, α_2, α_3, and α_4) or β-isoforms (β_1, β_2, and β_3). From (Blanco *et al.* 1998).

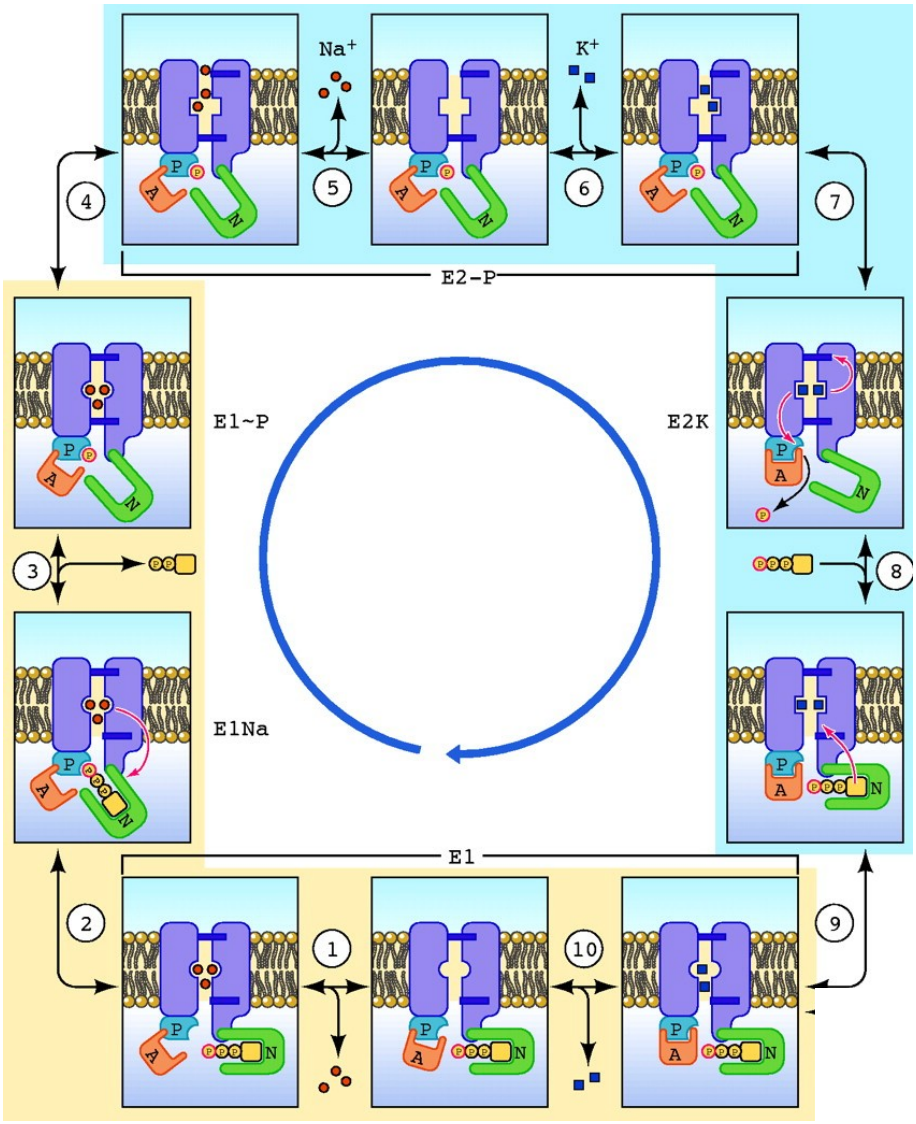

Figure 2.2. Scheme of the cation pumping cycle of the Na^+,K^+-ATPase.

A, actuator domain involved in dephosphorylation; P, phosphorylation domain; N, nucleotide domain containing ATP binding site. Step 1, (E1 state) with ATP present in its binding site, three Na^+ ions enter through the open internal gate; Step 2, Na^+ ion entry produces a conformational change; Step 3, the internal gate closes and ADP is released (E1-P state); Step 4, the outside gate opens (E2-P state); Step 5, Na^+ ions are relaeased to the extracellular side; Step 6, K^+ ions enter the open outside gate; Step 7, K^+ entry closes the extracellular gate and causes dephosphorylation (E2-K state); Step 8, ATP binds (E1 state); Step 9, internal gate opens; Step 10, K^+ is released to cytoplasmic side. From (Horisberger 2004).

2.2.2.2 Signal transduction

In addition to Na^+/K^+ exchange, the Na^+,K^+-ATPase also acts as a signal transducer (for review, see (Xie *et al.* 2002)). Experiments performed mainly in cardiac, renal or hepatic tissue, or HeLa cells, have shown that inhibition of Na^+,K^+-ATPase by ouabain, or by incubation in low K^+ medium, increases Na^+,K^+-ATPase content or mRNA abundance (Pressley *et al.* 1988, Vaughan *et al.* 1972, Xie *et al.* 2002). This may occur via direct protein-protein interaction between Na^+,K^+-ATPase and it's neighbouring proteins, triggering a signalling cascade culminating in increased gene transcription (Figure 2.3) (Xie *et al.* 2002). Alternately, ionic disturbances caused by depressed Na^+,K^+-ATPase activity may stimulate synthesis of new Na^+,K^+-ATPase enzymes to re-establish favourable Na^+ and K^+ gradients (Bundgaard *et al.* 1997a, Wolitzky *et al.* 1986). Consistent with this, exposure to 13mM $[K^+]$ elevated α_1 mRNA by 160%, although 6-8 fold increases in $[Na^+]_i$ did not change Na^+,K^+-ATPase isoform mRNA expression (Murphy *et al.* 2006a). Acute exercise inhibits skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity (section 2.2.6.1.3) and also increases the mRNA expression for each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2, and β_3 isoforms (Murphy *et al.* 2004). Thus, acute inhibition of Na^+,K^+-ATPase activity with exercise might then be linked to increased Na^+,K^+-ATPase gene expression and consequently also increased Na^+,K^+-ATPase content with chronic training (section 2.2.7.1.1). This suggests a possible signal transduction role for the exercise-induced inhibition of maximal in-vitro Na^+,K^+-ATPase activity in skeletal muscle. However, whether such a role exists in skeletal muscle is unknown and was thus investigated in Chapter 3.

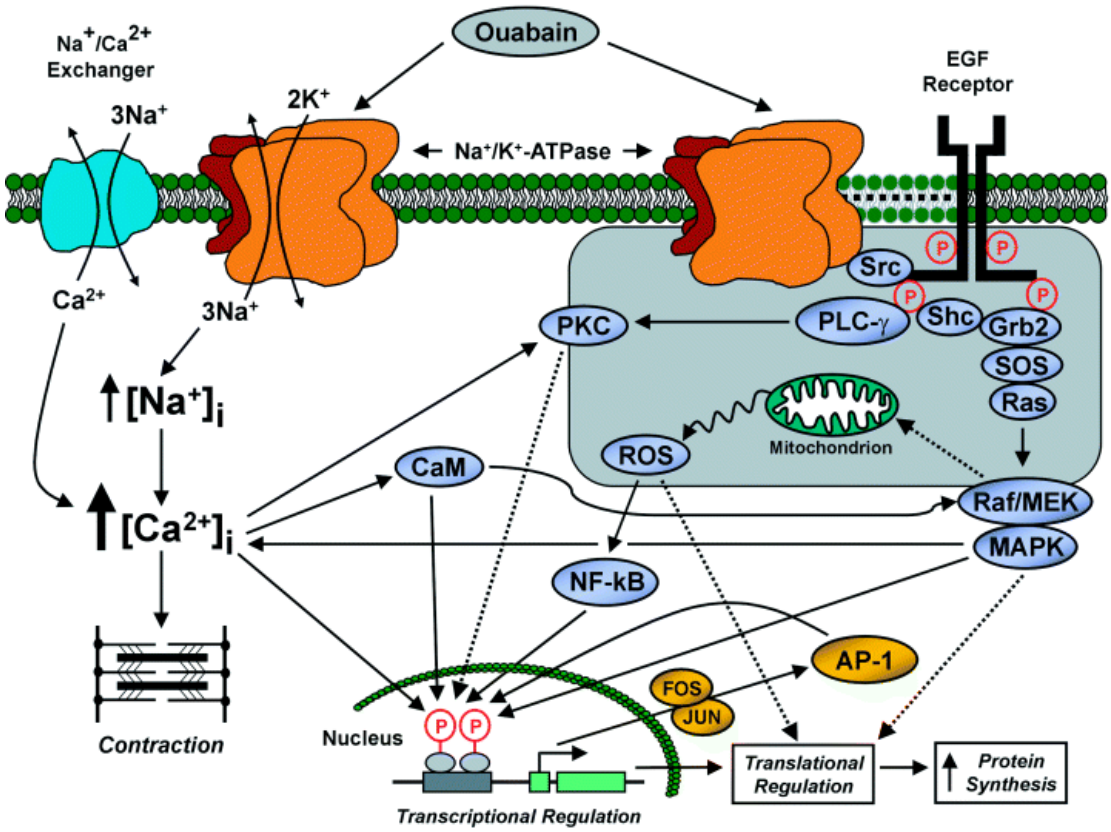

Figure 2.3. The signal transducing function of the Na^+,K^+-ATPase and its consequences in cardiac myocytes.

From (Xie *et al.* 2002).

2.2.3 Isoforms

The α subunit (110-112 kDa) consists of a NH_2-terminal segment with four trans-membrane spanning domains and a carboxyl-terminal segment containing six trans-membrane spanning domains (Figure 2.1) (Blanco *et al.* 1998). The α subunit contains the functional properties of the Na^+,K^+-ATPase, including the binding sites for K^+, Na^+, Mg^{2+}, and ATP and also for cardiac glycosides (Lingrel 1992, Lingrel *et al.* 1998). At present, four different α subunits (α_1, α_2, α_3, α_4) have been identified (Shamraj *et al.* 1994, Shull *et al.* 1986), with α_1, α_2, and α_3 protein being expressed in human skeletal muscle (Murphy *et al.* 2004). The gene transcript for α_4 has also been

identified in human skeletal muscle (Keryanov *et al.* 2002, Nordsborg *et al.* 2005b) however α_4 protein expression could not be detected (Nordsborg *et al.* 2005b).

The core protein of the β subunit has a molecular mass of 30 - 35 kDa (Arystarkhova *et al.* 1997, Wang *et al.* 1996), however it has several N-linked glucose molecules which increase its apparent molecular mass to 40 – 60 kDa (Blanco *et al.* 1998). The β subunit spans the membrane only once and is necessary for the structural and functional maturation of the α subunit, as well as for localisation to the sarcolemma (Ackermann *et al.* 1990, Noguchi *et al.* 1990). Three different β isoforms (β_1, β_2, and β_3) have been identified, with each of these being expressed in human skeletal muscle (Murphy *et al.* 2004).

The γ subunit (~ 6.5 kDa) is one of seven members of the FXYD protein family (FXYD2) (Sweadner *et al.* 2000). FXYD proteins 1 - 4 and 7, have been identified as auxiliary subunits of the Na^+,K^+-ATPase and modulate its Na^+, K^+ and ATP affinities (Crambert *et al.* 2003, Horisberger 2004). Skeletal muscle contains the FXYD1 protein (phospholemman) (Bogaev *et al.* 2001), which appears to affect Na^+,K^+-ATPase activity (Reis *et al.* 2005).

2.2.3.1 Isoform affinities

2.2.3.1.1 Ouabain

In humans, the affinities of the different α-isoforms for ouabain are relatively similar, with the K_d for the α_1, α_2, and α_3 isoforms being 4.9, 22, and 4.6 nM, respectively (Crambert *et al.* 2000, Müller Ehmsen *et al.* 2001, Wang *et al.* 2001). In rat muscle, the α_1 isoform is considered to be ouabain-resistant, as its affinity for ouabain is 100- and 1000-fold lower, respectively than the α_2 and α_3 isoforms (O'Brien *et al.* 1994). Thus studies measuring [^{3}H]ouabain binding in rat muscle will detect the α_2 isoform.

2.2.3.1.2 Na^+

The intracellular $K_{0.5}$ of the human Na^+,K^+-ATPase α_1, α_2, and α_3 isoforms for Na^+ was 8.3, 12.8, and 24.7 mM, respectively (Crambert *et al.* 2000). As $[Na^+]_i$ is only 6 - 13 mM at rest (section 2.1.1.1), there is considerable potential for Na^+,K^+-ATPase stimulation by increased $[Na^+]_i$. Indeed, in isolated rat soleus muscle, increasing $[Na^+]_i$ by 58% via tetanic stimulation increased ouabain suppressible Na^+ efflux by 50% (Everts *et al.* 1994).

2.2.3.1.3 K^+

The extracellular $K_{0.5}$ of the human Na^+,K^+-ATPase α_1, α_2, and α_3 isoforms for K^+ was similar for each isoform, being 0.9, 1.3, and 0.9 mM, respectively (Crambert *et al.* 2000). Thus, at a resting $[K^+]_I$ of 4.0 mM, the extracellular K^+ site of the Na^+,K^+-ATPase will be ~80% saturated, indicating that elevated $[K^+]_I$ plays only a minor role in Na^+,K^+-ATPase stimulation (Sejersted *et al.* 2000).

2.2.3.1.4 ATP

The affinity of the human α isoforms for ATP has not been determined. In the rat, the intracellular $K_{0.5}$ for ATP of the α_1, α_2, and α_3 isoforms was similar for each, at 0.43, 0.54, and 0.21 mM, respectively (Jewell *et al.* 1991).

2.2.4 Localisation

In skeletal muscle, the Na^+,K^+-ATPase is located in the sarcolemma, t-tubules, and also at some intracellular sites in an isoform-specific manner (Clausen 2003). Few studies have investigated Na^+,K^+-ATPase localisation in human skeletal muscle due to problems associated with isolating membrane fractions and the large volume of tissue needed. An early study in frog sartorius muscle, found ~ 80% of the Na^+,K^+-ATPase in the sarcolemma, with the remaining 20% in the t-tubules (Venosa *et al.* 1981). As the surface area of the t-tubules is 4 – 5 times greater than that of the sarcolemma, this

indicates that the t-tubular density of Na^+,K^+-ATPase is only 4 - 5% of that of the sarcolemma. However, this estimate of t-tubular Na^+,K^+-ATPase density is likely to be too low as the glycerol pre-treatment method used may not have completely disrupted the t-tubular connections to the sarcolemma, thus including t-tubular Na^+,K^+-ATPase in the quantification of sarcolemmal Na^+,K^+-ATPase (Clausen 2003). Other studies have shown the ratio of t-tubule-to-sarcolemma Na^+,K^+-ATPase per unit of tissue to be between 1.3 and 0.58 (Barchi *et al.* 1977, Jaimovich *et al.* 1986, Mitchell *et al.* 1983). Thus, in the entire cell, most of the Na^+,K^+-ATPase are probably situated in the t-tubules (Clausen 2003), however this has yet to be verified in human muscle. Several studies have reported the existence of intracellular pools of Na^+,K^+-ATPase (Hundal *et al.* 1994, Lavoie *et al.* 1997, Lavoie *et al.* 1996, Marette *et al.* 1993). However, the membrane fractionation techniques employed in these studies typically result in very low recovery of plasma membrane protein (Hansen *et al.* 1996), and thus may not be representative of the entire Na^+,K^+-ATPase pool. Furthermore, [^{3}H]ouabain binding site content was not different when performed on intact and cut muscles specimens (Clausen *et al.* 1974, McKenna *et al.* 2003b), suggesting no intracellular Na^+,K^+-ATPase pool. Therefore, the existence of intracellular Na^+,K^+-ATPase is equivocal.

2.2.4.1 Isoform specific localisation

Protein expression of each of the α_1, α_2, β_1, β_2, and β_3 isoforms has been detected in rat skeletal muscle (Arystarkhova *et al.* 1997, Hundal *et al.* 1993, Tsakiridis *et al.* 1996). Isoform expression appears to be muscle fibre-type specific in rats. The α_1 and α_2 isoforms were equally distributed in fast-glycolytic and slow-oxidative fibres (Hundal *et al.* 1993, Thompson *et al.* 1996a). Conversely, another study reported higher α_1 and α_2 abundance in rat soleus and red gastrocnemius compared to EDL and

white gastrocnemius (Fowles *et al.* 2004). In rats, β_1 isoform abundance was ~ 5-fold higher in soleus compared to EDL (Fowles *et al.* 2004, Hundal *et al.* 1993) and higher in red than white gastrocnemius (Fowles *et al.* 2004). In contrast, β_2 isoform abundance was ~3-fold higher in fast-glycolytic than slow-oxidative muscle fibres (Fowles *et al.* 2004, Hundal *et al.* 1993).

Subcellular localisation studies, using membrane fractionation of rat skeletal muscle, have shown that the α_1 isoform was almost exclusively located in the sarcolemma (Hundal *et al.* 1992, Lavoie *et al.* 1995, Tsakiridis *et al.* 1996), whereas the α_2 isoform abundance was ~3-fold higher in an intracellular fraction than in a sarcolemmal fraction (Hundal *et al.* 1992, Hundal *et al.* 1994). The β_1 and β_2 isoforms were located predominantly in the intracellular fraction (Hundal *et al.* 1992). Immunohistochemical techniques have shown that the α_2 isoform is also located in the t-tubules and was colocalised with the dihydropyrodine receptor (Williams *et al.* 2001).

In human soleus muscle, α_1 was found to be located almost exclusively in a plasma membrane fraction, with only ~ 4% located in intracellular membrane fractions (Hundal *et al.* 1994). Similarly, α_3 was mainly located in the plasma membrane, whereas α_2 was more evenly distributed, with the plasma membrane containing only 25% more than the intracellular membranes (*ibid*). The authors did not probe for β_3 and were unable to detect β_2, however β_1 was approximately three times more abundant in the plasma than intracellular membrane (*ibid*). Care should be taken regarding the interpretation of these results as it has been shown that intracellular membranes derived by this specific differential centrifugation technique may contain t-tubules (Nielsen *et al.* 2003).

2.2.5 Quantification of Na^+,K^+-ATPase

2.2.5.1 Na^+,K^+-ATPase content

The most accepted method for quantification of Na^+,K^+-ATPase content is by determination of vanadate-facilitated [^{3}H]ouabain binding site content (Nørgaard 1986, Nørgaard *et al.* 1983). This assay is based on the finding that ouabain binds to the Na^+,K^+-ATPase with a 1:1 stoichiometry (Hansen 1984). Vanadate (VO_4) binds to the Na^+,K^+-ATPase and locks it in a configuration capable of binding ouabain (Hansen *et al.* 1979), thus enabling the [^{3}H]ouabain binding site determination of skeletal muscle biopsy samples. Due to the low affinity of the rat α_1 isoform for ouabain (section 2.2.3.1.1), the [^{3}H]ouabain binding site technique will only detect the α_2 isoform. However, in human skeletal muscle all Na^+,K^+-ATPase α isoforms are detected.

In healthy human skeletal muscle samples [^{3}H]ouabain binding site content ranges from 223 to as high as 425 pmol.(g wet weight)$^{-1}$ with a median value of 306.5 pmol.(g wet weight)$^{-1}$ (Table 2.1).

2.2.5.1.1 Sex specific content

Few studies have investigated a possible sex difference in Na^+,K^+-ATPase content. Most studies have reported no difference in skeletal muscle [^{3}H]ouabain binding site content between males and females (Green *et al.* 2001, Murphy *et al.* 2007, Murphy *et al.* 2006a, Nørgaard *et al.* 1984a). However, in one study on well-trained cross-country skiers, skeletal muscle [^{3}H]ouabain binding site content was 18% higher in males than females (Evertsen *et al.* 1997).

2.2.5.1.2 Fibre-type specific content

Skeletal muscle Na^+,K^+-ATPase content does not appear to be fibre-type dependent in humans, however this has yet to be comprehensively investigated. [^{3}H]ouabain

binding site content was not different between muscles of differing fibre-type composition (Dørup *et al.* 1988) and no relationship was found between muscle [^{3}H]ouabain binding site content and fibre-type composition (Fraser *et al.* 2002, Madsen *et al.* 1994). Furthermore, there was no difference in the immunocytochemical staining of Na^+,K^+-ATPase between slow- and fast-twitch muscle fibres in humans (Benders *et al.* 1992). Conversely, a significant correlation was found between the proportion of type II fibres and [^{3}H]ouabain binding sites in human vastus lateralis muscle (McKenna *et al.* 2003a).

In 4 and 8 week old rats, [^{3}H]ouabain binding was ~ 20% higher in the EDL than soleus muscles (Bundgaard *et al.* 2002, Clausen *et al.* 2004). Conversely, in 10 week rats, [^{3}H]ouabain binding site content was either higher (Dørup *et al.* 1997) or not different (Chin *et al.* 1993) in EDL than soleus. Furthermore, Na^+,K^+-ATPase content was not related to myosin heavy chain composition in 16 week rats (Fowles *et al.* 2004). Thus, in young rats, Na^+,K^+-ATPase content appears to be muscle fibre-type specific. However, in adult rats and humans, the evidence is inconclusive.

Table 2.1. [^{3}H]ouabain binding site content in healthy human skeletal muscle.

Reference	Muscle	[^{3}H]ouabain binding site content (pmol.(g wet weight)$^{-1}$)
(Medbø *et al.* 2001)	VL	425 ± 11
(Djurhuus *et al.* 1998)	VL	377*
(Benders *et al.* 1992)	VL	360 ± 31
(Green *et al.* 2000a)	VL	348 ± 12
(Evertsen *et al.* 1997)	VL	343 ± 11 (Men)
(Green *et al.* 1993)	VL	339 ± 16
(McKenna *et al.* 1993)	VL	333 ± 19
(Leivseth *et al.* 1994)	Delt.	333 ± 20
(Nordsborg *et al.* 2005a)	VL	326 ± 30
(Aughey *et al.* 2006)	VL	318 ± 37
(Leppik *et al.* 2004)	VL	317 ± 37
(Nordsborg *et al.* 2005a)	Delt.	312 ± 17
(Fraser *et al.* 2002)	VL	311 ± 41
(Kjeldsen *et al.* 1984b)	VL	308 ± 13
(Aughey *et al.* 2005)	VL	307 ± 41
(Madsen *et al.* 1994)	VL	307 ± 43
(Klitgaard *et al.* 1989)	VL	306 ± 26
(Green *et al.* 1999a)	VL	289 ± 22 (Group 1)
		278 ± 31 (Group 2)
(Evertsen *et al.* 1997)	VL	281 ± 14 (Women)
(Haller *et al.* 1998)	VL	281 ± 20
(Nørgaard *et al.* 1984a)	VL	278 ± 15

(Ravn *et al.* 1997)	VL	276 ± 11
(Kjeldsen *et al.* 1990b)	VL	276 ± 19
(Green *et al.* 2001)	VL	268 ± 19 (Men)
(Nørgaard *et al.* 1984a)	RA	265 ± 20
(Dørup *et al.* 1988)	VL	258 ± 16
(Gullestad *et al.* 1995)	VL	258 ± 13
(Nørgaard *et al.* 1984a)	IC	253 ± 11
(Green *et al.* 2001)	VL	243 ± 13 (Women)
(Murphy *et al.* 2006b)	VL	225 ± 17
(Schmidt *et al.* 1994)	VL	223 ± 13

Data are means ± SE; * SE not reported; VL, vastus lateralis; Delt., deltoid; IC, intercostal; RA, rectus abdominus.

2.2.5.2 Na^+,K^+-ATPase activity

Quantification of Na^+,K^+-ATPase activity can be achieved by measurement of radiolabelled ion fluxes (Clausen *et al.* 1987a), ouabain-suppressible inorganic phosphate (P_i) production (Bonting *et al.* 1961), or activity of K^+-dependent phosphatases (Fraser *et al.* 1998, Huang *et al.* 1975, Hundal *et al.* 1994). The measurement of radiolabelled ion fluxes is only suitable for whole muscle fibres and thus cannot be used to measure Na^+,K^+-ATPase activity in human biopsy samples. The determination of Na^+,K^+-ATPase activity by measurement of ouabain-suppressible P_i production is unreliable (Nørgaard 1986), being only a fraction of total P_i production in crude skeletal muscle homogenates (Nørgaard 1986). The measurement of K^+-dependent phosphatase activity uses a substrate such as p-nitrophenyl phosphate (p-NPP) (Hundal *et al.* 1994) or 3-O-methylfluorescein

phosphate (3-O-MFP) (Huang *et al.* 1975). The 3-O-MFPase assay requires 50 – 100 times less tissue than the p-NPPase assay (Nørgaard 1986), making it the preferred assay for analysis of human muscle biopsy samples. The 3-O-MFPase assay has been optimised for human muscle samples (Fraser *et al.* 1998) and its specificity for the Na^+,K^+-ATPase has been demonstrated by its complete inhibition by ouabain and its activation by K^+ (Fraser *et al.* 1998, Nørgaard *et al.* 1984b) and that it has been correlated to [^{3}H]ouabain binding site content (Fraser *et al.* 2002).

In healthy human skeletal muscle, maximal in-vitro 3-O-MFPase values are typically between 200 – 300 (median 277) nmol.min^{-1}.(g wet weight)$^{-1}$ although values as low as 95 nmol.min^{-1}.(g wet weight)$^{-1}$ have been reported (Table 2.2).

Table 2.2. Maximal in-vitro 3-O-MFPase activity in resting healthy human skeletal muscle.

Reference	**Maximal 3-O-MFPase activity ($nmol.min^{-1}.(g\ wet\ weight)^{-1}$)**
(Fraser *et al.* 1998)	292 ± 10
(Fraser *et al.* 2002)	311 ± 41
(Fowles *et al.* 2002b)	203 ± 32*
(Green *et al.* 2004)	95 ± 9*
(Leppik *et al.* 2004)	289 ± 8
(Sandiford *et al.* 2004)	253 ± 13*
(Aughey *et al.* 2005)	284 ± 7
(Nordsborg *et al.* 2005a)	176 ± 10
(Aughey *et al.* 2006)	277 ± 5
(McKenna *et al.* 2006a)	205 ± 8
(Murphy *et al.* 2006b)	283 ± 31

Data are means ± SE. * calculated from data expressed as $nmol.h^{-1}.(mg\ protein)^{-1}$ assuming a muscle protein content of 15%.

2.2.5.2.1 Sex specific activity

To date, only one study has investigated the effect of sex on Na^+,K^+-ATPase activity in humans and reported no difference between males and females (Murphy *et al.* 2007).

2.2.5.2.2 Fibre-type specific activity

Whether human skeletal muscle Na^+,K^+-ATPase activity is fibre-type dependent is unknown. In soleus muscle from adult rats however, maximal in-vitro 3-O-MFPase

activity was ~150% higher than in the EDL and red gastrocnemius muscles, and 270% higher than in the white gastrocnemius (Fowles *et al.* 2004).

2.2.6 Acute regulation

Na^+,K^+-ATPase activity is influenced by numerous factors, including muscle contraction, ionic and hormonal regulation, and cardiac glycosides, each of which are briefly discussed below.

2.2.6.1 Contractile activity

2.2.6.1.1 Contraction-induced activation

At rest, the Na^+,K^+-ATPase operates at only a few percent of its theoretical maximal rate (Clausen *et al.* 1987a, Everts *et al.* 1994). However, excitation of the muscle membrane results in dramatic increases in Na^+,K^+-ATPase activity (Clausen *et al.* 1987a, Everts *et al.* 1994, Everts *et al.* 1988b, McKenna *et al.* 2003b). Even at low stimulation frequencies Na^+,K^+-ATPase activity is still markedly elevated, with $^{22}Na^+$ efflux being increased by 50% after 60 s stimulation at only 2 Hz (Everts *et al.* 1994). The magnitude of the excitation induced Na^+,K^+-ATPase activation appears to be dependent on both the stimulation duration and frequency. Stimulation of isolated rat soleus muscle for 1 or 10 s at 60 Hz increased $^{22}Na^+$ efflux over the next 5-min by 22 and 57%, respectively (Everts *et al.* 1994). Stimulation for 10 s at 60, 90 or 120 Hz increased $^{22}Na^+$ efflux in the 30 s immediately after stimulation by 1000, 1800, or 2200% (Nielsen *et al.* 1997), the latter being not significantly different from the theoretical maximum Na^+ transport capacity of the Na^+,K^+-ATPase.

Evidence of excitation-induced activation of the Na^+,K^+-ATPase in human skeletal muscle comes from the rapid and immediate decline in venous $[K^+]$ (Hallén *et al.* 1993, Vøllestad *et al.* 1994) and reversal from a negative to positive arteriovenous $[K^+]$ difference ($[K^+]_{a-v}$) across active muscle beds (Juel *et al.* 1990, Lindinger *et al.*

1990, Lindinger *et al.* 1992, Sostaric *et al.* 2006) following the cessation of exercise (Figure 2.4). It was assumed that at the end of exercise K^+ efflux ceases suddenly and that the rate of change in interstitial volume was zero, thus the rate of change of venous $[K^+]$ ($[K^+]_v$) was assumed to reflect K^+ reuptake by the Na^+,K^+-ATPase (Hallén *et al.* 1993, Sejersted *et al.* 2000, Vøllestad *et al.* 1994). Based on these assumptions, the Na^+,K^+-ATPase-mediated K^+ uptake at the end of exercise has been calculated at ~5 - 30 $\mu mol.s^{-1}.(kg\ wet\ weight)^{-1}$ (Hallén *et al.* 1993, Vøllestad *et al.* 1994). This equates to ~7 – 45% of the predicted maximum uptake rate of 70 $\mu mol.s^{-1}.(kg\ wet\ weight)^{-1}$ (Nørgaard *et al.* 1984a). The $Na^+,K^+,2Cl^-$ cotransporter (NKCC) has been shown to contribute to 35% of total ouabain-sensitive resting K^+ influx under isotonic conditions (Lindinger *et al.* 2002, Lindinger *et al.* 2001). Therefore, the above calculations may overestimate Na^+,K^+-ATPase-mediated K^+ uptake. Thus, during muscle contraction in humans, the Na^+,K^+-ATPase does not appear to be fully activated.

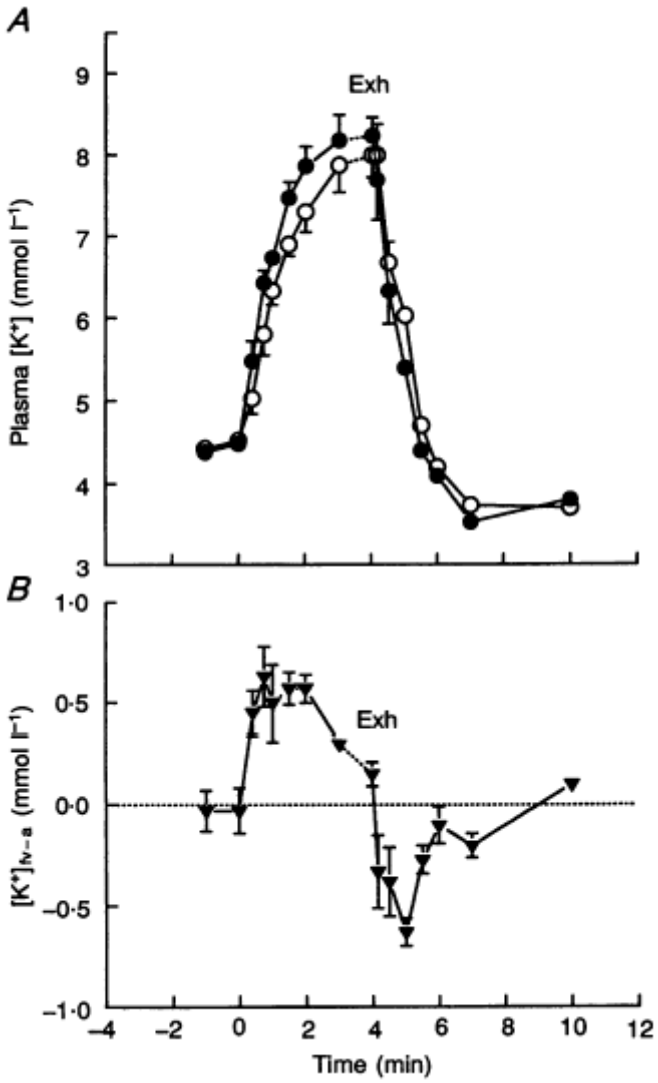

Figure 2.4. Arterial, venous and veno-arterial difference in plasma K^+ after exhaustive intensive exercise.

(A), changes in arterial (○) and femoral venous (●) plasma [K^+] during and after exhaustive bicycle exercise at 110% $\dot{V}O_{2max}$. B, veno-arterial [K^+] differences. From (Vøllestad *et al.* 1994).

2.2.6.1.2 Contraction-induced translocation

Aside from increased Na^+,K^+-ATPase turn-over rate, muscle contractions have also been proposed to increase Na^+,K^+-ATPase activity by increasing sarcolemmal

Na^+,K^+-ATPase content via translocation of Na^+,K^+-ATPase from an intracellular pool. It was reported that ~ 5 min of fatiguing knee-extensor exercise in humans markedly increased the Na^+,K^+-ATPase α_2 (70%) and β_1 (26%) abundance in skeletal muscle sarcolemmal giant vesicles (Juel *et al.* 2000a). Similar findings have been reported in rat muscle, with 1 h of treadmill running increasing α_1 and α_2 protein abundance in a plasma membrane fraction in red (64 and 43%, respectively) and white (55 and 94%, respectively) muscle fibres (Tsakiridis *et al.* 1996). The authors suggested that the isoforms may have been translocated from a separate intracellular pool (*ibid*). However, no corresponding decrease was observed for these isoforms in the measured intracellular membrane fraction (*ibid*). Furthermore, studies have so far failed to identify and quantify the intracellular pool, which may be partly located in the t-tubules (Clausen 2003). In another rat study, low-intensity treadmill running caused a 29% increase in [^{3}H]ouabain binding site content and a respective 13 – 32% increase in the α_1, α_2, and β_2 isoforms in sarcolemmal giant vesicles from oxidative muscle fibres, which was almost completely absent after 30 min recovery (Juel *et al.* 2001). Thus, there is some inconsistency between findings of increased α_1 and α_2 in the plasma membrane fraction in some studies (Juel *et al.* 2001, Tsakiridis *et al.* 1996) and only increased α_2 in others (Juel *et al.* 2000a). These translocation studies all relied on membrane isolation techniques, which have been criticised due to their low Na^+,K^+-ATPase recovery, which is in the range of 0.2 - 8.9% and may therefore not be representative of the entire Na^+,K^+-ATPase pool (Hansen *et al.* 1996). To date, no studies have detected translocation during muscle contraction in non-fractionated tissue. Similarly, no change in [^{3}H]ouabain binding site content was observed in isolated rat soleus and EDL muscles stimulated at various frequencies and durations (McKenna *et al.* 2003b). Furthermore, [^{3}H]ouabain binding site content was not

different when performed on intact and cut muscles specimens (Clausen *et al.* 1974, McKenna *et al.* 2003b), suggesting no intracellular Na^+,K^+-ATPase pool.

Studies of Na^+,K^+-ATPase content in human muscle biopsy samples have found no increase in [^{3}H]ouabain binding immediately after intense incremental or prolonged exercise (Aughey *et al.* 2006, Aughey *et al.* 2005, Leppik *et al.* 2004, Murphy *et al.* 2006b) or within 4 h post-isometric contractions (Fowles *et al.* 2002b). However, increased [^{3}H]ouabain binding site content was observed following ultra-prolonged running (~10 h) (Overgaard *et al.* 2002). Thus, whether acute muscle contractions increase skeletal muscle Na^+,K^+-ATPase content is equivocal.

2.2.6.1.3 Contraction-induced inhibition

Muscle contractions induce a marked increase in Na^+,K^+-ATPase activity, however recent evidence also suggests that the maximal activity of the Na^+,K^+-ATPase in human skeletal muscle is depressed during or immediately after exercise. Maximal in-vitro skeletal muscle Na^+,K^+-ATPase activity measured by 3-O-MFPase activity was reduced by 14% after a bout of single-leg kicking (Fraser *et al.* 2002). Since then, maximal in-vitro 3-O-MFPase activity was also found to be reduced by 12 – 38% after sprint-, incremental-, and prolonged-cycling (Aughey *et al.* 2006, Aughey *et al.* 2005, Leppik *et al.* 2004, McKenna *et al.* 2006a, Murphy *et al.* 2006b, Sandiford *et al.* 2004) and following repeated isometric contractions (Fowles *et al.* 2002b). As Na^+,K^+-ATPase activity contributes to membrane excitability (Nielsen *et al.* 2000), depressed muscle maximal Na^+,K^+-ATPase activity may contribute to fatigue in exercising humans. If so, an early post-fatigue recovery in Na^+,K^+-ATPase activity would be expected, but the recovery time course remains uncertain. After 30 min of intermittent isometric contractions, Na^+,K^+-ATPase activity was 35% lower in an exercised compared to a non-exercised leg, with no difference between legs at 1 h

post-exercise (Fowles *et al.* 2002b). Whilst this suggested that exercise transiently impaired muscle maximal in-vitro Na^+,K^+-ATPase activity, they also reported no difference between rest and post-exercise when sampled from different legs. Furthermore, they found no differences within the exercised leg between 0, 1, or 4 h after exercise. Together these internally inconsistent findings suggest either that activity was not depressed, or did not recover following exercise. The recovery time course is important, as it has implications for understanding whether Na^+,K^+-ATPase impairment with exercise is part of a fatigue-, damage- and/or some other regulatory process. This was therefore investigated in this thesis (Chapter 3).

2.2.6.2 Ionic regulation

Muscle contractions increase $[Na^+]_i$ and $[K^+]_I$ (section 2.1.1). Of these, only increased $[Na^+]_i$ is likely to be a major regulator of Na^+,K^+-ATPase activity, with increased $[Na^+]_i$ shown to activate Na^+,K^+-ATPase (Everts *et al.* 1992, Sejersted *et al.* 1988). A correlation between intracellular Na^+ content and ouabain-suppressible K^+ uptake in rat soleus and EDL muscles has also been reported (Everts *et al.* 1994). Increasing $[Na^+]_i$ in isolated rat soleus muscles by incubation in K^+-free buffer or veratridine, an activator of the voltage-gated Na^+ channels, increased ouabain-suppressible K^+ uptake by 117 to 152% in rat soleus, and by 126 to 194% in rat EDL muscle (Everts *et al.* 1992).

2.2.6.3 Hormonal regulation

Numerous hormones regulate skeletal muscle Na^+,K^+-ATPase activity, including adrenaline and noradrenaline, insulin, insulin-like growth factor, calcitonin gene-related peptide, and amylin. These are very briefly reviewed.

2.2.6.3.1 Catecholamines

Exercise increases sympathetic nervous system activity, stimulating the release of adrenaline and noradrenaline (Kjaer 1992). In isolated rat soleus muscle, elevated levels of adrenaline increased the rate of ouabain binding (Clausen *et al.* 1977), ouabain-suppressible K^+ uptake (Clausen *et al.* 1987b), and caused a hyperpolarisation of 9 – 22 mV (*ibid*). The stimulatory effects of catecholamines were not additive to those of contractile activity (Everts *et al.* 1988b), however, blocking the β_1- and β_2-adrenoreceptors with propranolol increased the exercise induced hyperkalaemia (Hallén *et al.* 1994, McKelvie *et al.* 1991). Blockade of β_1-adrenoreceptors alone did not alter the rise in plasma $[K^+]$ during exercise (Unsworth *et al.* 1998). Measurement of arterio-venous $[K^+]$ difference ($[K^+]_{a-v}$) showed that the higher plasma $[K^+]$ during exercise with β_1- and β_2-blockade was not due to greater muscle K^+ loss but rather from reduced K^+ redistribution to inactive tissues and an increased lag time of Na^+,K^+-ATPase activation (Hallén *et al.* 1994). This was supported by a study showing that β_1- and β_2-blockade did not effect the rise in plasma $[K^+]$ during exercise, but slowed K^+ reuptake after exercise (Unsworth *et al.* 1998).

2.2.6.3.2 Insulin and IGF-I

During exercise, insulin secretion is reduced (Kjaer *et al.* 1987), however muscle insulin delivery is increased due to increased muscle blood flow. In humans, insulin infusion decreased plasma $[K^+]$ and increased skeletal muscle K^+ uptake (Alvestrand *et al.* 1984). Thus, it is possible that both muscle excitation and increased insulin delivery work in concert to stimulate Na^+,K^+-ATPase activity during exercise. A recent study found that the decrease in maximal Na^+,K^+-ATPase activity typically observed following exercise (see Section 2.2.6.1.3) was attenuated in glucose

supplemented subjects in whom serum insulin was maintained during exercise (Green *et al.* 2007). However, this was not attributed to the elevated insulin levels (*ibid*). In human forearm muscle, insulin induced a 31-fold increase in ouabain-suppressible K^+ uptake (Ferrannini *et al.* 1988), indicating that the stimulatory effect of insulin for K^+ uptake was via Na^+,K^+-ATPase activation. Studies on animals have provided further evidence of the stimulating effect of insulin on the Na^+,K^+-ATPase. In isolated rat soleus muscle, insulin increased ouabain-sensitive K^+ uptake and Na^+ efflux by 25 and 45%, respectively (Clausen *et al.* 1987b). Insulin also decreased $[Na^+]_i$ by 22% and increased $[K^+]_i$ by 10% and induced a 4.3 mV hyperpolarisation (Flatman *et al.* 1979). Insulin may also cause translocation of additional Na^+,K^+-ATPase from intracellular stores to the sarcolemma. Insulin-induced translocation was first reported in frog sartorius muscle, in which insulin increased [^{3}H]ouabain binding by 65% (Grinstein *et al.* 1974). Since then, insulin-induced Na^+,K^+-ATPase translocation has been reported in rat muscle (Al-Khalili *et al.* 2003, Hundal *et al.* 1992, Lavoie *et al.* 1996, Marette *et al.* 1993) and human skeletal muscle cell cultures (Al-Khalili *et al.* 2003). However, insulin incubation increased ^{86}Rb uptake and decreased $[Na^+]_i$, but did not affect [^{3}H]ouabain binding site content in rat soleus (McKenna *et al.* 2003b). Insulin has also failed to cause translocation in other tissues (Clausen *et al.* 1977, Feraille *et al.* 1994, Longo *et al.* 2001, Sargeant *et al.* 1995).

Insulin-like growth factor-I (IGF-I) is structurally similar to proinsulin and exerts similar effects on the Na^+,K^+-ATPase as insulin (Dørup *et al.* 1995). In human subjects, IGF-I induced hypokalaemia, which was attributed to enhanced K^+ uptake in extrarenal tissues (Giordano *et al.* 1995). In isolated rat soleus muscle, IGF-I increased ouabain-sensitive $^{86}Rb^+$ and $^{42}K^+$ uptake by 30 -54% and 56%, respectively and also stimulated $^{22}Na^+$ efflux (Dørup *et al.* 1995). The effects of IGF-I and insulin

were not additive, indicating that they stimulate the Na^+,K^+-ATPase via the same pathway (*ibid*).

2.2.7 Chronic regulation

Chronic regulation of the Na^+,K^+-ATPase is primarily achieved through changes in the content of the enzyme. Numerous factors have been identified that influence Na^+,K^+-ATPase content in human skeletal muscle, including physical activity, hormonal regulation, and K^+ intake, whereas other factors, such as ageing, require further investigation.

2.2.7.1 Training and inactivity

2.2.7.1.1 Training

Several studies have shown that physical training increases skeletal muscle Na^+,K^+-ATPase content, as measured by [^{3}H]ouabain binding site content. Increased [^{3}H]ouabain binding site content has been observed following each of endurance, sprint, and resistance training (Table 2.3). Interestingly, the training-induced upregulation of Na^+,K^+-ATPase content of 14 – 22% (median = 15%), was irrespective of the initial fitness level of the subjects. This suggests that already highly trained individuals are capable of further increasing their Na^+,K^+-ATPase content and thus the potential for training-induced Na^+,K^+-ATPase upregulation may be larger than suggested by these studies. This is supported by the 30-40% greater skeletal muscle Na^+,K^+-ATPase content in chronically active versus sedentary aged subjects (Klitgaard *et al.* 1989).

2.2.7.1.2 Inactivity

The effect of physical inactivity on skeletal muscle Na^+,K^+-ATPase content has not been thoroughly investigated in humans. Immobilisation of the deltoid muscle due to shoulder impingement syndrome reduced [^{3}H]ouabain binding site content by 27%

compared to a control group (Leivseth *et al.* 1994). This was similar to reductions observed in immobilisation studies of animals, where [^{3}H]ouabain binding site content was reduced by 20 - 30% after just one week in rat soleus, immobilised by denervation, plaster cast, or tenotomy (Kjeldsen *et al.* 1986b).

Table 2.3. Effects of physical training on skeletal muscle Na^+,K^+-ATPase content in healthy humans.

Reference	**Training type**	**Duration**	**% change in [^{3}H]ouabain binding site content**
(Klitgaard *et al.* 1989)	Aged active vs. aged sedentary (x-s)		↑30-40%
(Kjeldsen *et al.* 1990a)	Army training	10 wk	n.c.
(Green *et al.* 1993)	Submaximal cycling	6 days	↑14%
(McKenna *et al.* 1993)	Sprint cycling	7 wk	↑16%
(Madsen *et al.* 1994)	Intensified running	6 wk	↑15%
(Evertsen *et al.* 1997)	X-country skiing	5 mths	↑17% females ↑14% males
(Green *et al.* 1999a)	Cycling	11 wk	↑22%
	Resistance training	12 wk	↑16%
(Green *et al.* 1999b)	Submaximal cycling	8 wk	↑14%
(Medbø *et al.* 2001)	Resistance training	3 mths	↑15%
(Fraser *et al.* 2002)	Endurance trained vs. untrained (x-s)		↑18%
(Harmer *et al.* 2006)	Sprint cycling	7 wk	↑8%
(Murphy *et al.* 2007)	Endurance trained vs. recreationally active (x-s)		↑16%

n.c., no change; ↑, increased; x-s, cross-sectional

2.2.7.2 Hormonal regulation

2.2.7.2.1 Thyroid hormone

Thyroid hormones have the single largest hormonal influence on Na^+,K^+-ATPase content in skeletal muscle. Hyperthyroidism dramatically increases and hypothyroidism decreases skeletal muscle Na^+,K^+-ATPase content, in both humans and rats (Azuma *et al.* 1993, Biron *et al.* 1979, Everts *et al.* 1988a, Harrison *et al.* 1998, Kjeldsen *et al.* 1986a, Kjeldsen *et al.* 1984b). The number of [^{3}H]ouabain binding sites of biopsies taken from the vastus lateralis of hyper- and hypothyroid patients was 68% higher and 50% lower, respectively when compared to euthyroid subjects (Kjeldsen *et al.* 1984b). Following treatment to normalise thyroid status, the [^{3}H]ouabain binding site content also returned to normal and a significant correlation was also found between the number of [^{3}H]ouabain binding sites and the level of free thyroxine (*ibid*). The regulation of skeletal muscle Na^+,K^+-ATPase by thyroid hormones is isoform specific. In thyroid hormone treated rats α_2 and β_2 subunit mRNA expression and protein abundance increased while no change was detected in α_1 or β_1 mRNA expression or protein abundance (Azuma *et al.* 1993).

2.2.7.2.2 Glucocorticoids

Endogenous glucocorticoids do not appear to be of major importance in the regulation of Na^+,K^+-ATPase content in skeletal muscle, as [^{3}H]ouabain binding site content was not changed following adrenalectomy in rats (Dørup *et al.* 1997). However, large doses of glucocorticoids are often used to treat various inflammatory diseases and for immune suppression following organ transplant (Lundberg *et al.* 2004) and have been linked with upregulation of Na^+,K^+-ATPase content in skeletal muscle (Clausen 2003). In humans treated with the glucocorticoid dexamethasone (~0.05 $mg.kg^{-1}.day^{-1}$) for 5 days, vastus lateralis [^{3}H]ouabain binding site content increased by 18 – 42%

(Djurhuus *et al.* 2002, Nordsborg *et al.* 2005a) and maximal in-vitro Na^+,K^+-ATPase activity increased by 14% (Nordsborg *et al.* 2005a). In chronic obstructive lung disease patients treated with the glucocorticoid prednisone, skeletal muscle [^{3}H]ouabain binding was 61% higher than controls and a correlation was found between prednisone dose and [^{3}H]ouabain binding site content (Ravn *et al.* 1997). Dexamethasone treatment increased the α_2 and β_1 protein abundances in rat soleus, EDL, gastrocnemius and diaphragm by 53 – 78% and increased α_1 protein abundance by 65% in diaphragm only (Thompson *et al.* 2001). Thus exogenous glucocorticoids appear to be potent regulators of Na^+,K^+-ATPase content in skeletal muscle, which is important when interpreting results from renal transplantation recipients (Chapter 5).

2.2.7.3 K^+ depletion and supplementation

2.2.7.3.1 K^+ depletion

Hypokalaemia, induced by treatment with diuretics, caused a 17% decrease in [^{3}H]ouabain binding sites in humans, (Dørup *et al.* 1988, Dørup *et al.* 1993) and a correlation was found between muscle [K^+] and [^{3}H]ouabain binding sites (Dørup *et al.* 1988). Following correction of muscle K^+ content, [^{3}H]ouabain binding sites were restored to normal levels (Dørup *et al.* 1993).

2.2.7.3.2 K^+ supplementation

Rats fed a high K^+ diet had lower plasma [K^+] following an acute K^+ load (Alexander *et al.* 1968). It was later shown that that the adaptation was due to increased K^+ uptake by skeletal muscle, as $^{86}Rb^+$ uptake was 87% greater in skeletal muscle of K^+ supplemented rats than controls, whereas the $^{86}Rb^+$ uptake in spleen, liver and heart was unchanged (Blachley *et al.* 1986). More recently, K^+ supplementation has been shown to increase [^{3}H]ouabain binding site content in rat gastrocnemius by 68% and in EDL and soleus by 48 and 26%, respectively (Bundgaard *et al.* 1997b). This effect

of K^+ levels on Na^+,K^+-ATPase is important for renal disease patients treated by haemodialysis (Chapters 4 and 5), as they are typically hyperkalaemic.

2.2.7.4 Ageing

In adult humans, skeletal muscle Na^+,K^+-ATPase content was not different between adults ranging from 25 – 80 years (Nørgaard *et al.* 1984a). Na^+,K^+-ATPase content was 14% lower in a group of six aged (68 ± 0.6 yr) vs. six young (28 ± 0.2 yr) subjects, however this was not statistically significantly, possibly due to the low sample size (Klitgaard *et al.* 1989). [^{3}H]ouabain binding site content decreased by 35% in rats from 12 – 86 weeks of age (Kjeldsen *et al.* 1984a). Thus, the effect of ageing on skeletal muscle Na^+,K^+-ATPase content is equivocal and requires further investigation.

2.2.8 Digoxin

Cardiac glycosides such as digoxin and ouabain are specific and potent inhibitors of the Na^+,K^+-ATPase. Digoxin is used to treat patients with severe heart failure (Rose *et al.* 1994) and has also been used experimentally, to block Na^+,K^+-ATPase in animals and humans (Bruce *et al.* 1968, Cumberbatch *et al.* 1981, Wai Ching Li *et al.* 1993). Digoxin is derived from the leaves of the foxglove plant (Digitalis purpurea) (Figure 2.5) and its medicinal use for the treatment of congestive heart failure (CHF) was described by William Withering over 200 years ago (Whitfield 1985).

2.2.8.1 Digoxin and skeletal muscle Na^+,K^+-ATPase

Digoxin and other cardiac glycosides bind to a specific receptor on the third extracellular loop (between transmembrane segments 5 and 6) of the α subunit (Horisberger 2004), locking it in the E2-P conformation (see Figure 2.2) and making it resistant to dephosphorylation by K^+, thus inactivating the enzyme (Sen *et al.* 1969). Digoxin and ouabain appear to have similar inhibitory effects on Na^+,K^+-ATPase, as

the IC_{50} in human red blood cells was 2.2×10^{-6} and 0.85×10^{-6} M, respectively (Senn *et al.* 1988).

When used medicinally, the target organ of digoxin is the left ventricular myocardium, in which it has a positive inotropic effect (Fozzard *et al.* 1985). However, only 3-4% of a given digoxin dose binds to the heart (Steiness 1978), with the largest proportion binding to skeletal muscle (~50%) and the remainder distributed among plasma and other tissues, or excreted by the kidney (Doherty *et al.* 1967, Steiness 1978). Thus, skeletal muscle is important in regulating plasma and myocardial concentrations of digoxin ([digoxin]).

In skeletal muscle of congestive heart failure (CHF) patients, a typical maintenance digoxin dose of 2-4 $\mu g.kg^{-1}.d^{-1}$ resulted in occupancy of 9-35% of Na^+,K^+-ATPase (Green *et al.* 2001, Schmidt *et al.* 1995, Schmidt *et al.* 1993b). This could have potentially detrimental effects on muscle function, as the skeletal muscle Na^+,K^+-ATPase is vitally important for maintaining muscle membrane excitability and contractility (section 2.1.3). Compared to healthy controls, $\dot{V}O_{2peak}$ was 24% lower in a group of CHF patients in whom digoxin occupied 35% of skeletal muscle Na^+,K^+-ATPase (Green *et al.* 2001), however, 9% occupancy of skeletal muscle Na^+,K^+-ATPase did not affect exercise performance (Schmidt *et al.* 1995). The degree of occupancy needed to impair exercise performance in healthy subjects has not been investigated. Whether digoxin similarly occupies Na^+,K^+-ATPase in skeletal muscle from healthy subjects, and the subsequent performance effects, are not known and were investigated in Chapter 6.

2.2.8.1.1 Digoxin-induced compensatory upregulation of Na^+,K^+-ATPase

It has been hypothesised that chronic digoxin administration may cause an upregulation of Na^+,K^+-ATPase activity or content to compensate for Na^+,K^+-pumps

inhibited by digoxin (Ford *et al.* 1979). However, whether compensatory upregulation of Na^+,K^+-ATPase occurs during digitalisation is uncertain. Several studies have reported compensatory upregulation of Na^+,K^+-ATPase content or activity following chronic digoxin administration in erythrocytes from humans and pigs (Ford *et al.* 1979, Whittaker *et al.* 1983), and guinea-pig myocardium (Bonn *et al.* 1978). Conversely, others have found no evidence of compensatory upregulation in myocardium (Schmidt *et al.* 1993a, Schmidt *et al.* 1991a) or skeletal muscle (Schmidt *et al.* 1993b) from CHF patients. However, these studies in CHF patients were cross-sectional and lacked a pre-digitalis sample, making the results equivocal. Thus, whether chronic digoxin administration causes compensatory upregulation in skeletal muscle remains unclear and was investigated in Chapter 6.

2.2.8.2 Endogenous digitalis-like factors

As the Na^+,K^+-ATPase α subunit has a digitalis binding site, this has led to the hypothesis that an endogenous digitalis-like factor (EDLF) may exist (Hamlyn *et al.* 1991). Hamlyn *et al.* (Hamlyn *et al.* 1991) purified a factor from human plasma that was identical to ouabain, could bind to the Na^+,K^+-ATPase digitalis binding site, and inhibited ouabain-sensitive $^{86}Rb^+$ uptake. Many substances resembling ouabain have been isolated from numerous mammalian tissues (Schoner 2002), however growing evidence suggests that the adrenal gland is the major site of synthesis/storage (Hamlyn *et al.* 1998, Li *et al.* 1998). The EDLF is believed to play a role in regulating blood pressure, as the concentration of a factor isolated from plasma was found to inhibit the Na^+,K^+-ATPase and to be correlated with the blood pressure of the donors (Hamlyn *et al.* 1982). Physical stress may elevate plasma EDLF concentration as increased plasma $[Na^+]$ (Hasegawa *et al.* 1987), hypoxia (De Angelis *et al.* 1998), and exercise (Bauer *et al.* 2005) have all been shown to increase the plasma concentration

of EDLF. Despite these findings, controversy still exists surrounding the existence and physiological significance of EDLF (Forni *et al.* 2005, Hansen 2003, Hilton *et al.* 2005). Some studies have been unable to detect EDLF (Lewis *et al.* 1994). Furthermore, several researchers have reported plasma EDLF concentrations or adrenal gland secretion rates that were either so low (Boulanger *et al.* 1993) that they are unlikely to be of physiological significance (Hansen 1994, Hansen 2003), or were so high (Bauer *et al.* 2005) that they would inhibit the majority of Na^+,K^+-ATPase in proximity to the plasma with potentially lethal effects (Hilton *et al.* 2005).

Figure 2.5. Foxglove plant (Digitalis purpurea).

From (Whitfield 1985)

2.2.9 Conclusions

The skeletal muscle Na^+,K^+-ATPase maintains trans-sarcolemmal [Na^+] and [K^+] gradients and membrane excitability and is thus vitally important for the maintenance of muscle contractility and exercise performance. Numerous factors are involved in the acute and chronic regulation of the Na^+,K^+-ATPase, including contractile activity, ionic changes, hormones, K^+ intake, ageing and digoxin. Exercise depresses skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity, which may contribute to fatigue. However, whether inhibition of skeletal muscle Na^+,K^+-ATPase activity expedites the onset of fatigue in healthy humans is unknown. Furthermore, the recovery time-course of the exercise-induced depression in Na^+,K^+-ATPase activity also remains uncertain. Chronic exercise upregulates skeletal muscle Na^+,K^+-ATPase content, however the effects of an acute exercise bout on Na^+,K^+-ATPase content are equivocal. Thyroid hormones, insulin, and exogenous, but not endogenous, glucocorticoids all regulate Na^+,K^+-ATPase content. Insulin and catecholamines are regulators of Na^+,K^+-ATPase activity. The effects of ageing on muscle Na^+,K^+-ATPase are equivocal. Digoxin acutely inhibits Na^+,K^+-ATPase activity and chronic digoxin exposure may cause upregulation of Na^+,K^+-ATPase content and activity, but this is yet to be investigated in healthy humans.

SECTION II: SKELETAL MUSCLE Na^+,K^+-ATPase, K^+ REGULATION, AND EXERCISE PERFORMANCE IN RENAL DISEASE

Exercise performance is markedly impaired in patients with renal disease, and this has been linked with their impaired plasma K^+ regulation during exercise. As the skeletal muscle Na^+,K^+-ATPase regulates plasma $[K^+]$, this suggests that the skeletal muscle Na^+,K^+-ATPase may also be impaired in renal disease. In healthy humans, exercise training increases Na^+,K^+-ATPase content and improves plasma K^+ regulation and muscle performance during exercise. Exercise training also improves exercise performance in renal disease, however whether improved K^+ regulation contributes to this is unknown. Therefore, this section reviews the impaired exercise performance in renal disease. There is particular focus on haemodialysis patients and renal transplant recipients as these are investigated in Chapters 4 and 5. It also outlines the possible causes of their poor exercise performance, with emphasis on the possible role of impaired K^+ regulation and skeletal muscle Na^+,K^+-ATPase. Finally, it discusses the effects of exercise training on exercise performance in renal disease, and whether improved K^+ regulation after training may contribute to their improved performance.

2.3 EXERCISE PERFORMANCE IN HD AND RTX

Patients with renal disease undergoing haemodialysis therapy (HD) or following renal transplantation (RTx) have limited physical functioning which impacts on their quality of life. They display markedly impaired exercise performance compared to healthy control subjects (CON), with significant reductions in both cardiorespiratory fitness (as measured by peak oxygen consumption, $\dot{V}O_{2peak}$), and absolute muscle strength. Measurement of cardiorespiratory fitness and muscle strength and fatiguability can provide valuable information regarding an individual's ability to

perform daily living tasks. Therefore, section 2.3 reviews the current literature on $\dot{V}O_{2peak}$, muscle strength and fatiguability in HD and RTx.

2.3.1 Cardiorespiratory fitness

Cardiorespiratory fitness, as measured by peak oxygen consumption ($\dot{V}O_{2peak}$), has been well documented in HD patients, with the majority of studies finding values of ~50 – 70% (median 58%) of matched CON or predicted values (Table 2.4). Only one study has reported normal $\dot{V}O_{2peak}$ in HD (Clark *et al.* 1996), however the control subjects in that study had below-predicted $\dot{V}O_{2peak}$ (Wasserman *et al.* 1994) and were older than the HD patients. It is important to note that most of these studies have stringent subject exclusion criteria, and thus only the healthy and better functioning patients are tested. Therefore, it is likely that the $\dot{V}O_{2peak}$ of the HD population is even lower than these studies suggest.

Table 2.4. $\dot{V}O_{2peak}$ in HD patients.

Reference	***n***	**sex (M:F)**	**Age (years)**	**[Hb] ($g.dl^{-1}$)**	**Hct (%)**	**$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)**	**Comparison (% control group)**
(McMahon *et al.* 1999)	14		43	10.0		1.61 ($l.min^{-1}$)	70%[c]
(Hsieh *et al.* 2006)	27	19:8	61			11.5 ± 2.9	77%
(Storer *et al.* 2005)	12	7:5	44	12.0		14.8 ± 4.2	57%
(Robertson *et al.* 1990)	19	11:8	47		21	15.3 ± 5.0	40%[d]
(Shalom *et al.* 1984)	14	7:7	45		26	15.4 ± 4.3	
(Moore *et al.* 1993b)	9	8:1				15.8 ± 1.0	
(Mayer *et al.* 1988)	8	4:4	36	5.9		16.0 ± 3.4	
(McMahon *et al.* 1992)	10	5:5	48	6.4		17.4 ± 2.6	65-70%[a]
(Diesel *et al.* 1990)	10	2:8	38	8.1		17.7 ± 3.6	
(Kouidi *et al.* 1998)	7	5:2	44		31	17.7 ± 5.0	
(Carney *et al.* 1983)	4	3:1	39			17.9 ± 4.3	46%[b]
(Akiba *et al.* 1995)	20	9:11	40	6.5		18.7 ± 3.5	
(Lewis *et al.* 1993)	9	5:4	36	7.0		18.7 ± 4.2	
(Molsted *et al.* 2004)	9					18.8	
(Painter *et al.* 1986a)	18	11:7	45		24	19.1 ± 5.8	64%[b]
(Painter *et al.* 2002a)	48	27:21	46		32	19.5 ± 7.0	62%[b]

(Painter *et al.* 1986b)	20	12:8	42		24	19.6 ± 4.1	
(Barany *et al.* 1993)	21	11:10	39	7.3		20.0 ± 5.0	59%
(van den Ham *et al.* 2005)	16	10:6	49	12.1		21.4 ± 8.0	81%
(Latos *et al.* 1987)	9	6:3	40		25	21.8 ± 3.3	60%
(Kettner Melsheimer *et al.* 1987)	35	28:7	31	9.1		22.3 ± 2.7	
(Baraldi *et al.* 1990)	7	4:3	14	6.3		24.1 ± 7.1	54%
(Sangkabutra *et al.* 2003)	8	4:4	26	7.8	25	24.5 ± 4.6	56%
(Levendoglu *et al.* 2004)	14	8:6	33	12.2	36	24.8 ± 7.0	
(Bonzel *et al.* 1991)	12	7:5	14	7.2		25.1	57%
(Violan *et al.* 2002)	9	7:2	27			25.4 ± 5.7	
(Lundin 1981)	10	9:1	33		32	28.6 ± 5.6	
(Clark *et al.* 1996)	8	6:2	37		32	26.9 ± 9.0	90% (n.s.)

CON, healthy control subjects; BM, body mass; [Hb], haemoglobin concentration; Hct, haematocrit; n.s., not significantly different; a, age-predicted; b, age- and sex-predicted; c, age-, sex- and body mass-predicted; d, age-, sex-, and height-predicted.

Following successful renal transplantation, $\dot{V}O_{2peak}$ is still reduced compared to CON (Table 2.5) however whether $\dot{V}O_{2peak}$ is improved compared to pre-renal transplantation is equivocal. $\dot{V}O_{2peak}$ increased by 31 and 38%, respectively at 6 and 15 weeks post- renal transplantation (Gallagher-Lepak 1991) and by 25% at 7.5 weeks post-renal transplantation (Painter *et al.* 1987) compared to pre-renal transplantation. However, other studies found no difference in $\dot{V}O_{2peak}$ or maximum work rate achieved during an incremental exercise test between HD and RTx (Bullock *et al.* 1984, van den Ham *et al.* 2005) or in maximum work rate pre- and post-renal transplantation (Nyberg *et al.* 1995). Anaemia contributes to reduced exercise performance in renal disease patients prior to renal transplantation (McMahon *et al.* 1999, Morris *et al.* 1993, Painter *et al.* 2002a), thus the ~10% increase in haematocrit (Hct) reported post-renal transplantation (Gallagher-Lepak 1991, Painter *et al.* 1987) may partially account for any gain in $\dot{V}O_{2peak}$. Yet, no studies have compared $\dot{V}O_{2peak}$ in HD and RTx with similar [Hb], therefore this was investigated in Chapter 4.

2.3.2 Muscle strength

Absolute muscle strength as measured by maximal voluntary contraction (MVC), 1RM, or isokinetic peak torque is reduced by 20 - 64% in HD patients compared to CON (DePaul *et al.* 2002, Fahal *et al.* 1997, Johansen *et al.* 2005, Johansen *et al.* 2003, Kemp *et al.* 2004, Kettner Melsheimer *et al.* 1987, Rogerson *et al.* 1989, van den Ham *et al.* 2005). In RTx, absolute muscle strength is also significantly impaired, being 12, 24, 46, and 64% less than in age- and sex-matched CON (Horber *et al.* 1987, Kettner Melsheimer *et al.* 1987, Painter 2003, van den Ham *et al.* 2005). These results suggest that muscle strength is not different between HD and RTx. This is supported by cross-sectional studies showing no difference in muscle strength between age-, sex- and body mass-matched RTx and HD (Kettner Melsheimer *et al.*

1987, van den Ham *et al.* 2005). Furthermore, unchanged handgrip strength 1 year after RTx suggests that muscle strength is not improved post-RTx (Nyberg *et al.* 1995).

The deficit in absolute muscle strength in HD may be due to reduced muscle mass rather than impaired contractile properties of the muscle, as several studies have reported normal muscle strength in HD after accounting for differences in body mass (Fahal *et al.* 1997), muscle cross-sectional area (CSA) (Kemp *et al.* 2004) or muscle contractile-tissue CSA (Johansen *et al.* 2003). Similarly, the lack of improvement in muscle strength following RTx may be due to the muscle wasting effects of glucocorticoids, as thigh muscle CSA and peak torque in RTx recipients treated with prednisone were reduced by 20 – 45% and 20 – 30%, respectively, compared to a group of controls (Horber *et al.* 1985). Only one study has investigated whether reduced muscle strength in RTx is due to muscle atrophy, reporting reduced knee-extensor isokinetic torque after correction for lean limb mass compared to CON (van den Ham *et al.* 2005). Likewise, some studies have shown that muscle strength is still decreased in HD when normalised to limb CSA (Johansen *et al.* 2005) or lean limb mass (van den Ham *et al.* 2005). Therefore, whether muscle atrophy is the primary cause of poor muscle strength in HD and RTx is equivocal and is important for the design of appropriate therapies. Therefore, muscle strength was measured in HD and RTx in this thesis (Chapter 5) and was normalised to both body mass and to limb muscle CSA.

Table 2.5. $\dot{V}O_{2peak}$ in RTx recipients.

Reference	***n***	**sex (M:F)**	**Age (years)**	**[Hb] ($g.dl^{-1}$)**	**Hct (%)**	**$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)**	**Comparison (% control group)**
(van den Ham *et al.* 2005)	35	18:17	52	13.8		21.3 ± 6.2	81%
(Painter *et al.* 2003)	14	11:3	47		32	23.7 ± 8.6	83%
(Painter 2003)	1	1:0	65		43	24.3	72%
(Painter *et al.* 2002b)	43	30:13	44	11.6	35	24.7 ± 6.7	71%[a]
(Kettner Melsheimer *et al.* 1987)	18	14:4	36	13.7		24.8 ± 4.5	
(Violan *et al.* 2002)	12	11:1	35			28.0 ± 7.9	
(Gallagher-Lepak 1991)	9	3:6	37		37	28.7 ± 9.1	
(Kempeneers *et al.* 1990)	16	9:7	33			29.0 ± 7.8	61%
(Painter *et al.* 1987)	20	14:6	29		36	29.3 ± 4.6	
(Painter *et al.* 1997)	76	45:31	45			31.6 ± 12.6	98%[a]
(Painter *et al.* 1986a)	20	13:7	34		45	31.7 ± 7.0	93%[a]
(Horber *et al.* 1987)	9	5:4	34			32.8 ± 5.9	82%
(Bonzel *et al.* 1991)	11		17	14.6		35.9	81%
(Feber *et al.* 1997)	26	12:14	14			40.9 ± 14	

CON, healthy control subjects; BM, body mass; [Hb], haemoglobin concentration; Hct, haematocrit; n.s., not significantly different; a, age- and sex-predicted.

2.3.3 Muscle fatigue resistance

Many studies investigating $\dot{V}O_{2peak}$ have reported early leg fatigue as a primary cause of incremental exercise termination in HD patients (Diesel *et al.* 1990, McMahon *et al.* 1999, Sangkabutra *et al.* 2003). This suggests that muscle fatigue resistance may be impaired, and may be a limiting factor in $\dot{V}O_{2peak}$, in these patients. However, there has been surprisingly little research into muscle fatigue resistance during repeated contractions in HD. The decline in knee-extensor torque following 15 maximal knee-extensions at $30^{o}.s^{-1}$ was ~23% (Robertson *et al.* 1990), however no comparison was made with a healthy control group. The time to fatigue during repeated isometric plantarflexion exercise at 50% MVC was reduced by 30% in HD compared to CON (Kemp *et al.* 2004). Fatigue resistance during isometric contractions of the dorsiflexors was three-fold higher in HD than in CON (Johansen *et al.* 2005). However, ischaemic isometric contractions do not reflect movement patterns during typical daily tasks. Thus, it is important to determine muscle fatigue resistance during dynamic contractions. However, whether muscle fatigue resistance during repeated dynamic contractions is altered in HD has not been determined. Furthermore, there has been no research to date investigating muscle fatigue resistance in RTx. Thus, whether muscle fatigue resistance is impaired and might limit $\dot{V}O_{2peak}$ in RTx has also not been determined. Therefore, this thesis also measured muscle fatigue resistance during repeated dynamic contractions in HD (Chapters 4 and 5) and RTx (Chapter 5).

In summary, $\dot{V}O_{2peak}$, and muscle strength are markedly impaired in HD and RTx, which may potentially limit their physical functioning and reduce their quality of life. Whether their poor strength is due to muscle atrophy or some other impairment remains uncertain. Muscle fatigue resistance may also be impaired in HD and RTx,

with likely adverse effects on physical functioning, however this is yet to be determined.

2.3.4 Factors contributing to impaired exercise performance in HD and RTx

It is essential to understand the underlying mechanisms of impaired exercise performance in renal disease in order to develop effective therapies. Renal disease results in numerous abnormalities of the endocrine, biochemical, cardiovascular, pulmonary and neuromuscular systems (Morrison 1985), any of which may impair exercise performance. This review will focus on possible limiting factors within skeletal muscle.

2.3.4.1 Factors contributing to impaired exercise performance in HD

2.3.4.1.1 Oxygen delivery limitations

The poor exercise performance seen in HD patients in part must be consequent to the marked anaemia that develops with ESRD, as $\dot{V}O_{2peak}$ is improved following treatment with erythropoietin (EPO) (Marrades *et al.* 1996b, McMahon *et al.* 1992, Painter *et al.* 1994). However, correction of anaemia does not fully restore muscle function and $\dot{V}O_{2peak}$. For example, after EPO treatment, the proportional increase in $\dot{V}O_{2peak}$ was substantially less in comparison to the rise in [Hb] (Marrades *et al.* 1996b). Furthermore, normalisation of [Hb] to 140 $g.l^{-1}$ with EPO in HD patients did not result in attainment of normal $\dot{V}O_{2peak}$ (McMahon *et al.* 1999). Similarly, [Hb] and Hct are typically normalised after renal transplantation, however exercise performance is still subnormal (Kettner Melsheimer *et al.* 1987, van den Ham *et al.* 2005). Thus, whilst correction of anaemia is essential for improvement in exercise tolerance in renal disease, deficits remain and thus other factors must also be involved. Peak single-leg O_2 delivery during single-leg extensions was 0.64 $l.min^{-1}$ in mildly anaemic HD patients, which was lower than CON (0.79 $l.min^{-1}$) (Sala *et al.*

2001). There is controversy whether leg blood flow is reduced (Bradley *et al.* 1990) or unchanged (Marrades *et al.* 1996a, Sala *et al.* 2001), in HD. Therefore the depressed single-leg O_2 delivery appeared to be primarily due to a lower arterial O_2 content (C_aO_2). Resting leg blood flow was not different between RTx and CON (Boden *et al.* 1993), however blood flow during exercise was not measured. Increasing C_aO_2 in HD patients, by correction of anaemia with EPO, reduced leg blood flow during exercise and thus did not increase single-leg O_2 delivery (Marrades *et al.* 1996a, Marrades *et al.* 1996b). Yet, single-leg O_2 delivery and leg $\dot{V}O_2$ were increased by ~25% in HD when C_aO_2 was increased by breathing hyperoxic air (100% O_2) (Sala *et al.* 2001). Low muscle capillary to mitochondria O_2 conductance has been reported in HD (Marrades *et al.* 1996b, Sala *et al.* 2001) which would be expected to impede mitochondrial O_2 uptake. This may be due to capillary to muscle fibre dissociation and reduced capillary density (Bradley *et al.* 1990, Diesel *et al.* 1993, Moore *et al.* 1993b). Skeletal muscle capillary density was reduced by 31% in RTx compared to CON and this coincided with an 18% lower $\dot{V}O_{2peak}$ (Horber *et al.* 1987). However, due to a lack of quantitative data in HD, no comparison can be made. Thus, there is substantial evidence for an O_2 supply limitation to $\dot{V}O_{2peak}$ in HD, however other factors also appear to be involved. Further research is required to determine if skeletal muscle oxygen delivery is a limiting factor of exercise performance in RTx.

2.3.4.1.2 Abnormal energy metabolism

Oxidative energy metabolism is impaired in HD. ^{31}P-magnetic resonance spectroscopy (MRS) has become a popular tool for the non-invasive measurement of energy metabolism during exercise in HD patients and there has been a lot of focus on this area of research. Changes in muscle pH, inorganic phosphate concentration ($[P_i]$), or phosphocreatine concentration ([PCr]) are used as indicators of anaerobic ATP

production (Moore *et al.* 1993a). Thus, greater fluctuations in muscle pH, [P_i], or [PCr] during contraction in HD, indicate a greater reliance on anaerobic metabolism and therefore, reduced relative contribution from oxidative metabolism. All studies to date have reported larger fluctuations in pH, [P_i], or [PCr] during dynamic muscle contractions in HD compared to CON (Durozard *et al.* 1993, Johansen *et al.* 2005, Kemp *et al.* 2004, Moore *et al.* 1993a, Sala *et al.* 2001, Thompson *et al.* 1993, Thompson *et al.* 1996b). Still, while there is consensus regarding the occurrence of impaired oxidative metabolism in HD, controversy exists surrounding the mechanism(s) involved. As discussed above (section 2.3.4.1.1), mitochondrial O_2 supply is impaired in HD. When O_2 supply and O_2 consumption were matched between HD and CON, there was no difference in oxidative metabolism, implying an O_2 supply limitation to oxidative metabolism in HD (Sala *et al.* 2001). Conversely, impaired O_2 supply was indicated not to be the cause of defective oxidative metabolism in HD, which occurred despite adequate cellular O_2 supply, as determined by near-infrared spectroscopy (Kemp *et al.* 2004). Mitochondrial abnormalities, decreased mitochondrial content, or reduced activity of key oxidative enzymes such as succinate dehydrogenase, isocitrate synthase, or cytochrome oxidase may also cause impaired oxidative metabolism in renal disease. Mitochondrial abnormalities were observed by electron microscopy in vastus lateralis muscle biopsies from HD patients (Diesel *et al.* 1993, Kouidi *et al.* 1998). However, isolated mitochondria had normal morphology and function (Miro *et al.* 2002). Also, ATP production in isolated mitochondria from HD before EPO treatment was 35% higher than CON (Barany *et al.* 1991). After 1 yr of EPO treatment, ATP production was not different between HD and CON (*ibid*). The elevated mitochondrial function prior to EPO treatment may be due to increased activity of Krebs cycle enzymes, as elevated NADH dehydrogenase

activity was detected in muscle biopsies from HD patients, and was suggested to be an adaptation to chronic hypoxia caused by impaired O_2 supply (Bradley *et al.* 1990). Conversely, in predialysis patients, the enzymes citrate synthase and β-hydroxyacyl-CoA dehydrogenase were reduced by ~ 35%, however whether this also occurs in HD has not been determined.

There is conflicting evidence as to whether oxidative metabolism is impaired in RTx and whether it is improved compared to HD. The changes in $[P_i]$ and [PCr] during handgrip contractions were elevated in HD but were the same in RTx and CON (Moore *et al.* 1993a), indicating normalised oxidative metabolism in RTx. In contrast, muscle oxidative capacity of vastus lateralis biopsy specimens from RTx was 1278 ± 358 μL $O_2.g^{-1}.h^{-1}$ (Kempeneers *et al.* 1990), which is similar to that of HD (1170 ± 342 μL $O_2.g^{-1}.h^{-1}$) (Diesel *et al.* 1993), but lower than values from healthy moderately trained subjects (2004 ± 288 μL $O_2.g^{-1}.h^{-1}$) (Kempeneers *et al.* 1990). However the difference between the renal disease and healthy subjects may be due to differences in training status. Unfortunately, no comparison was made with sedentary control subjects. Also, no difference was detected in mitochondrial volume between RTx and CON (Horber *et al.* 1987).

HD patients tend to have a predominance of type II muscle fibres, and this could also contribute to their impaired oxidative metabolism, but the findings on muscle fibre-type in RTx are less clear (section 2.3.4.1.4).

Whether anaerobic glycolysis is also impaired in HD is equivocal. Serum $[Lac^-]$ was reduced by 33% following ischemic forearm exercise in HD compared to CON, attributed to an inhibition of glycolysis (Nakao *et al.* 1982). Conversely, calculated muscle Lac^- accumulation was greater in HD than CON (Thompson *et al.* 1993). Finally, similar muscle intracellular pH, $[P_i]$, and [PCr] in HD and CON following

static maximal handgrip contractions, which are likely to be completely ischemic, suggests normal anaerobic metabolism in HD (Moore *et al.* 1993a).

Anaerobic metabolism appears to be normal in RTx. During static (ischemic) handgrip contractions the changes in [P_i] and [PCr] were the same in RTx and CON (Moore *et al.* 1993a). Furthermore, serum [Lac^-] after ischemic forearm contractions was not different from CON (Nakao *et al.* 1982).

In summary, oxidative energy metabolism is impaired in HD, which is likely to play a role in their poor exercise performance. Controversy surrounds the mechanisms involved, but mitochondrial abnormalities, decreased mitochondrial content and oxidative enzyme activity, and altered muscle fibre-type have all been implicated. Anaerobic energy metabolism may also be impaired, however this is also controversial. It is uncertain whether oxidative metabolism is improved in RTx compared to HD, although anaerobic metabolism appears to be normalised.

2.3.4.1.3 Muscle atrophy

It is well established that patients with renal disease undergoing haemodialysis therapy suffer from muscle atrophy. The contractile tissue cross-sectional area (CSA) of the dorsiflexor muscles was 7.2 cm^2 in HD compared to 9.1 cm^2 in CON (Johansen *et al.* 2003). Type II fibres may be affected more than type I (Ahonen 1980, Bradley *et al.* 1990, Diesel *et al.* 1993, Fahal *et al.* 1997, Floyd *et al.* 1974, Kouidi *et al.* 1998, Sakkas *et al.* 2004, Shah *et al.* 1983). All muscle fibre types appear to be affected with the mean muscle fibre area reduced by 33% (Kouidi *et al.* 1998). Numerous mechanisms play a role in causing muscle atrophy in HD. Protein degradation is increased in HD (Garibotto *et al.* 1994), and is due to metabolic acidosis (Bailey *et al.* 1996, Garibotto *et al.* 1996), inflammation (Adams *et al.* 2006), and even haemodialysis itself (Ikizler *et al.* 2002). Protein synthesis was also reduced in HD,

and is caused by insulin and growth hormone resistance, and physical inactivity (Adams *et al.* 2006).

The muscle atrophy detected in uraemic patients appears to persist following renal transplantation. Total thigh CSA was the same between RTx and CON but thigh muscle CSA was 21% lower in RTx (Horber *et al.* 1987). Both type I and II muscle fibres CSA appeared to be reduced (Horber *et al.* 1986, Painter 2003). Altered muscle fibre morphology, as evidenced by disoriented myofibrils and wide interfibrillar spaces, has also been reported (Topp *et al.* 2003). These may be due to the muscle wasting effects of immunosuppressive steroids (section 2.3.4.1.5).

2.3.4.1.4 Muscle fibre-type

It is unclear whether altered muscle fibre-type contributes to impaired exercise performance in HD. Muscle fibre type composition influences $\dot{V}O_{2peak}$ and maximal work capacity in healthy subjects, which are positively related to vastus lateralis type I fibre percentage (Bergh *et al.* 1978). Muscle fatiguability and peak torque are also influenced by muscle fibre composition in healthy subjects, being positively correlated to the percentage of fast twitch fibres (Thorstensson 1976, Thorstensson *et al.* 1976). Vastus lateralis type I fibre percentage was correlated with $\dot{V}O_{2peak}$ in predialytic uraemic subjects (Clyne *et al.* 1993). Several studies have found reduced type I fibre percentage in lower limb skeletal muscle biopsies from HD patients (Ahonen 1980, Diesel *et al.* 1993, Fahal *et al.* 1997, Nakao *et al.* 1982). In contrast, results from other studies do not support this (Davenport *et al.* 1993, Sakkas *et al.* 2004, Sakkas *et al.* 2003).

There is also conflicting evidence as to whether muscle fibre type composition is abnormal in RTx. Normal muscle fibre type distribution has been reported by several researchers (Ahonen 1980, Painter 2003, Topp *et al.* 2003), whereas type II

predominance (67%) has been reported by others (Kempeneers *et al.* 1990). Therefore, whether altered muscle fibre type composition contributes to the reduced exercise performance in HD and RTx is equivocal and requires further investigation.

2.3.4.1.5 Immunosuppressive steroids

The immunosuppressive steroids used to prevent organ rejection after renal transplant may contribute to their poor exercise performance. An early study found that renal transplant recipients taking prednisone also exhibited atrophy of muscle fibre I, IIa, and IIb types (Horber *et al.* 1987), although whether this was due to the drug was not conclusively shown. Painter *et al.* compared two groups of renal transplant recipients at 3 and 12 months post-transplant; one group took the standard therapy of immunosuppressants, while the other group had prednisone removed from their therapy (Painter *et al.* 2003). At 12 months, knee-extensor peak torque and $\dot{V}O_{2peak}$ had increased in the prednisone withdrawal group but peak torque had not changed and $\dot{V}O_{2peak}$ had decreased in the standard therapy group (*ibid*). In a companion study, the prednisone withdrawal group had higher type I muscle fibre percentage (45.5 %) than the standard therapy group (26.8 %) (Topp *et al.* 2003). Cyclosporine is another commonly prescribed immunosuppressant for RTx and it has been linked with skeletal muscle abnormalities, such as atrophy, accumulation of mitochondria and lipid vacuoles in humans (Goy *et al.* 1989). In rats, cyclosporine reduced skeletal muscle capillary density, succinate dehydrogenase activity, and mitochondrial respiration (Biring *et al.* 1998, Mercier *et al.* 1995).

2.3.4.1.6 Impaired muscle membrane excitability

Few studies have investigated muscle membrane excitability in renal disease. Substantial membrane depolarisation was recorded in a group of severely ill patients with uraemia or chronic renal disease with resting muscle membrane potential of only

-64 mV (Cunningham *et al.* 1971). This might be expected to reduce action potential amplitude and muscle contractility. Conversely, in adequately dialysed haemodialysis patients, E_m (-91 ± 0.9 mV) was the same as healthy controls (-91 ± 0.9 mV) (Cotton *et al.* 1979). More recently, it was found that the muscle compound action potential (which reflects the excitability of the neuromuscular junction and muscle membrane) was depressed in HD compared to CON by 32% at rest (Johansen *et al.* 2005). This coincided with increased fatiguability during repeated isometric contractions in the HD patients (*ibid*), suggesting that impaired excitability of the neuromuscular junction or muscle membrane may have contributed to their greater fatiguability. Consistent with the latter, HD exhibit an exaggerated rise in arterial $[K^+]$ during incremental exercise (Figure 2.6), that was inversely correlated with $\dot{V}O_{2peak}$ (Sangkabutra *et al.* 2003). This suggests a link between impaired K^+ regulation and exercise performance in HD. Together, these suggest possible underlying abnormalities in muscle K^+ regulation, which impair muscle membrane excitability and may contribute to their earlier onset of fatigue. The proposed mechanism was impaired skeletal muscle Na^+,K^+-ATPase activity (*ibid*), but this has not been investigated.

In renal transplanted children and adolescents, motor unit action potential amplitude was reduced in the extensor digitorum brevis and abductor pollicis brevis muscles (El-Husseini *et al.* 2005). This suggests a possible impairment of muscle membrane excitability in RTx. The causes of this impairment may be abnormal skeletal muscle K^+ regulation and Na^+,K^+-ATPase activity, but this has not yet been investigated. Thus, evidence suggests that muscle membrane excitability is depressed in HD and RTx and this may therefore contribute to their impaired exercise performance. The mechanism(s) of this impairment have not been investigated but may be abnormal skeletal muscle K^+ regulation and Na^+,K^+-ATPase activity. Therefore, whether

skeletal muscle Na^+,K^+-ATPase activity is impaired in HD and RTx, and is related to their poor exercise performance was determined in Chapter 5.

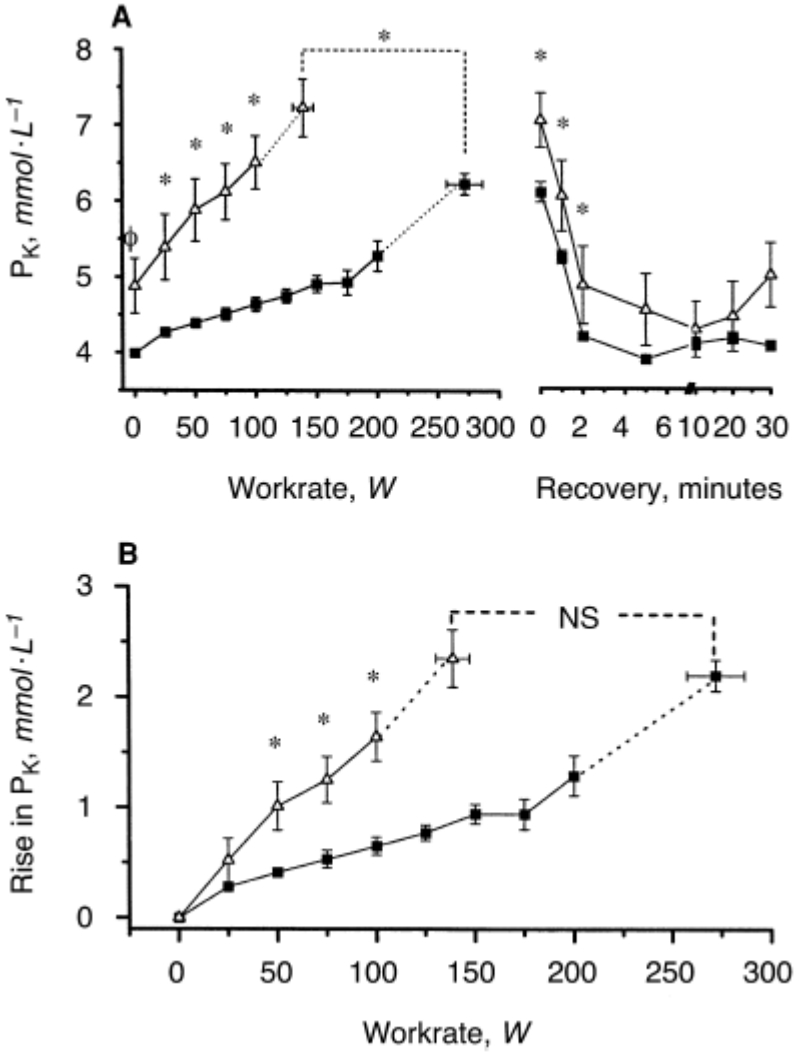

Figure 2.6. Exaggerated rise in arterial plasma [K^+] during incremental exercise in HD.

Arterial plasma [K^+] (P_K) at rest, during and following incremental cycle exercise (A) and the rise in P_K during exercise (B), for patients with end-stage renal failure (△) and healthy controls (■). From (Sangkabutra *et al.* 2003).

2.4 SKELETAL MUSCLE Na^+,K^+-ATPase IN RENAL DISEASE

There is evidence to suggest that skeletal muscle Na^+,K^+-ATPase activity might be impaired in renal disease (section 2.3.4.1.6). However, the effects of renal disease on skeletal muscle Na^+,K^+-ATPase in humans have not been investigated. This section will review the effects of renal disease on Na^+,K^+-ATPase in other human tissues and in skeletal muscle of uraemic rats.

2.4.1 Na^+,K^+-ATPase activity in renal disease

The first report of altered Na^+,K^+-ATPase in renal disease was in 1964, with decreased Na^+,K^+-ATPase activity and elevated $[Na^+]_i$ reported in erythrocytes from uraemic patients (Welt 1964). Since then, numerous authors have reported altered Na^+,K^+-ATPase activity, content and isoform abundance in a variety of tissues from patients with renal disease or uraemic rat models. Renal disease depresses Na^+,K^+-ATPase activity in erythrocytes and leucocytes from humans (Table 2.6). Reduced Na^+,K^+-ATPase activity of 14 – 45% was reported in almost all studies on CRF, Pre-D, CAPD and HD patients compared to healthy control samples or samples not exposed to uraemia. Renal transplantation appears to improve Na^+,K^+-ATPase activity, with only one study reporting depressed activity and two reporting substantial elevations. Whether Na^+,K^+-ATPase activity is depressed in other tissues in humans with renal disease is unknown. Also, whether RTx improves Na^+,K^+-ATPase activity in other tissues is not known.

Studies on nephrectomised rats suggest that renal disease substantially depresses Na^+,K^+-ATPase activity in skeletal (30 – 45%) but not cardiac muscle (Table 2.7). Whether skeletal muscle Na^+,K^+-ATPase activity is also depressed in humans with renal disease is unknown but is of particular importance, as skeletal muscle contains the largest single pool of Na^+,K^+-ATPase in the body (Kjeldsen *et al.* 1984a), and is

vitally important for extrarenal K^+ regulation and skeletal muscle function (2.1.3). Therefore, the effects of renal disease on skeletal muscle Na^+,K^+-ATPase were investigated in Chapter 5.

2.4.2 Na^+,K^+-ATPase content

Unlike Na^+,K^+-ATPase activity, there is no clear effect of renal disease on Na^+,K^+-ATPase content. Several studies have shown reduced [^{3}H]ouabain binding site content in erythrocytes from chronic renal failure patients (CRF) and HD, whereas others have found normal content in HD, CAPD, and RTx (Table 2.8). Studies on nephrectomised rats also give conflicting results with either severely depressed or unchanged Na^+,K^+-ATPase content in cardiac tissue. Only one study to date has investigated the effect of uraemia on skeletal muscle Na^+,K^+-ATPase content, reporting no change compared to sham operated rats (Table 2.8).

Table 2.6. Effects of renal disease on Na^+,K^+-ATPase activity in humans.

Reference	***n***	**Renal disease classification**	**Tissue**	**Effect on Na^+, K^+-ATPase (compared to control)**
(De_Franceschi *et al.* 1995)	10	Pre-D	RBC	↓ 45%
(De_Franceschi *et al.* 1995)	10	HD	RBC	↓ 45%
(De_Franceschi *et al.* 1995)	10	CAPD	RBC	↓ 45%
(Edmondson *et al.* 1975)	16	CRF	Leucocytes	↓ 40%
(Aparicio *et al.* 1991)	20	CRF	Leucocytes	↓ 39%
(Cole 1973)	20	CRF	RBC	↓ 38%
(Zannad *et al.* 1982)	11	HD	RBC	↓ 34%
(Vasarhelyi *et al.* 1996)	10	HD	RBC	↓ 32%
(Brod *et al.* 1984)	11	CRF	RBC	↓ 30%
(Fervenza *et al.* 1989)	13	HD	RBC	↓ 30%
(Izumo *et al.* 1984)	13	HD	RBC	↓ 24%
(Swaminathan *et al.* 1982)	15	CRF	RBC	↓ 24% (n.s.)
(Boero *et al.* 1988)	26	HD and CAPD	RBC	↓ 23%
(Fervenza *et al.* 1989)	11	Pre-D	RBC	↓ 21%
(Huang *et al.* 1996)	16	RTx	RBC	↓ 19%
(Kramer *et al.* 1976)	16	CRF	RBC	↓ 18% (n.s.)

(Zannad *et al.* 1985)	30	HD	RBC	↓ 17%
(Edmondson *et al.* 1975)	16	HD	Leucocytes	↓ 16%
(Fervenza *et al.* 1989)	13	CAPD	RBC	↓ 15%
(Kovacic *et al.* 1997)	35	HD	RBC	↓ 15%
(Welt 1964)		CRF	RBC	↓ ~15%
(Villamil *et al.* 1968)	37	CRF	RBC	↓ 14%
(Kramer *et al.* 1972)	15	CRF	RBC	n.c.
(Cheng *et al.* 1984)	45	HD	RBC	n.c.
(Gallice *et al.* 1992)	27	HD	RBC	n.c.
(Huang *et al.* 1996)	23	HD	RBC	n.c.
(Fervenza *et al.* 1989)	15	RTx	RBC	n.c.
(Zannad *et al.* 1982)	13	RTx	RBC	n.c.
(Bosch *et al.* 1991)	10	RTx	RBC	↑ 77%
(Cole *et al.* 1975)	20	RTx	RBC	↑ 101%

[Cr], serum creatinine concentration; CRF, patients with non-defined chronic renal failure; Pre-D, undialysed renal disease patients; HD, renal disease patients treated with haemodialysis; CAPD, renal disease patients treated with continuous ambulatory peritoneal dialysis; RTx, renal disease patients treated with renal transplantation; RBC, red blood cells; ↓, decreased compared to control, ↑, increased compared to control; n.c., no change compared to control; n.s., not significant.

Table 2.7. Effects of uraemia on Na^+,K^+-ATPase activity in skeletal and cardiac muscle from nephrectomised rats.

Reference	***n***	**Tissue**	**Effect on Na^+, K^+-ATPase (compared to control)**
(Bofill *et al.* 1994)	5	Soleus	↓ 45%
(Goecke *et al.* 1991)	3	Soleus	↓ 50%
(Druml 1988)	8	Epitrochlearis	↓ 30%
(Penpargkul *et al.* 1976)	5	Myocardium	n.c.
(Druml *et al.* 1990)	8	Myocardium	n.c.

↓, decreased compared to control; n.c., no change compared to control.

Table 2.8. Effects of renal disease and uraemia on Na^+,K^+-ATPase content.

Reference	*n*	Species	Renal disease classification	Tissue	Effect on Na^+, K^+-ATPase (compared to control)
(Swaminathan *et al.* 1982)	15	Human	CRF	RBC	↓ 17%
(Fervenza *et al.* 1989)	11	Human	Pre-D	RBC	↓ 21%
(Fervenza *et al.* 1989)	13	Human	HD	RBC	n.c.
(Izumo *et al.* 1984)	13	Human	HD	RBC	n.c.
(Cheng *et al.* 1984)	45	Human	HD	RBC	↓ 26%
(Fervenza *et al.* 1989)	13	Human	CAPD	RBC	n.c.
(Fervenza *et al.* 1989)	15	Human	RTx	RBC	n.c.
(Druml 1988)	8	Rat	Nephrectomised	Epitrochlearis	n.c.
(da Silva *et al.* 1994)	9	Rat	Nephrectomised	Myocardium	n.c.
(Druml *et al.* 1990)	8	Rat	Nephrectomised	Myocardium	↓ 46%
(Wald *et al.* 1995)	26	Rat	Nephrectomised	Cardiac sarcolemma	↓ 57%

CRF, patients with non-defined chronic renal failure; Pre-D, undialysed renal disease patients; HD, renal disease patients treated with haemodialysis; CAPD, renal disease patients treated with continuous ambulatory peritoneal dialysis; RTx, renal disease patients treated with renal transplantation; RBC, red blood cells; ↓, decreased compared to control; n.c., no change compared to control.

2.4.3 Na^+,K^+-ATPase isoforms

No studies to date have investigated the effects of renal disease on Na^+,K^+-ATPase isoforms in humans, and studies on nephrectomised rat skeletal and cardiac muscle give conflicting results.

2.4.3.1 Isoform mRNA expression

There is no clear effect of uraemia on Na^+,K^+-ATPase isoform mRNA expression in skeletal muscle of rats. Studies on uraemic rat myocardium tend to suggest a decrease in the α_2 and β_2 isoforms (Table 2.9).

2.4.3.2 Isoform protein abundance

Only one study has measured Na^+,K^+-ATPase isoform protein abundance in skeletal muscle, finding no effect of uraemia, however this may be partly attributable to the small sample size (Greiber *et al.* 1994). Furthermore, these analyses was performed on membrane fractions and thus the results may not be representative of the entire Na^+,K^+-ATPase pool (Hansen *et al.* 1996). Studies on rat myocardial tissue have reported mixed results (Table 2.10).

In summary, a substantial body of research suggests that renal disease causes a reduction in Na^+, K^+-ATPase activity that is prevalent in many tissues. Whether the depressed activity is due to altered content or isoform expression is uncertain. Further research is required to verify this, particularly in humans, in whom research has been limited to erythrocytes and leucocytes. Erythrocytes provide an easily accessible tissue source with minimal discomfort to the subject, however they are anucleic cells, which cannot synthesize Na^+, K^+-ATPase units, and therefore may not be representative of other cells. As skeletal muscle contains one of the largest pools of Na^+,K^+-ATPase in the body, and is vitally important for extrarenal K^+ regulation and skeletal muscle function, this represents an area of particular importance. No studies

have determined the effects of renal disease on Na^+,K^+-ATPase activity, content or isoform abundance in human skeletal muscle, therefore this was investigated in Chapter 4.

Table 2.9. Effects of uraemia on Na^+,K^+-ATPase isoform mRNA expression in skeletal and cardiac muscle from nephrectomised rats.

Reference	***n***	**Species**	**Tissue**	**Effect on Na^+, K^+-ATPase (compared to control)**				
				α_1	**α_2**	**α_3**	**β_1**	**β_2**
(Bofill *et al.* 1994)	5	Rat	Soleus	↓ ~55%				
(Bonilla *et al.* 1991)	5	Rat	Soleus	↓ ~54%	↑ ~100%			
(Greiber *et al.* 1994)	6	Rat	Soleus	n.c.	n.c.	n.c.	n.c.	n.c.
(Greiber *et al.* 1994)	6	Rat	EDL	n.c.	n.c.	n.c.	n.c.	n.c.
(da Silva *et al.* 1994)	10	Rat	Gluteus	n.c.	n.c.	n.c.	n.c.	n.c.
(Kennedy *et al.* 2003)	10	Rat	Myocardium	n.c.	↓ 26%			
(da Silva *et al.* 1994)	10	Rat	Myocardium		↓ 52%			↓ 26%

EDL, extensor digitorum longus; ↓, decreased compared to control, ↑, increased compared to control; n.c., no change compared to control.

Table 2.10. Effects of uraemia on Na^+,K^+-ATPase isoform protein abundance in skeletal and cardiac muscle from nephrectomised rats.

Reference	***n***	**Species**	**Tissue**	**Effect on Na^+, K^+-ATPase (compared to control)**				
				α_1	**α_2**	**α_3**	**β_1**	**β_2**
(Greiber *et al.* 1994)	4	Rat	Soleus	n.c.	n.c.		n.c.	n.c.
(Greiber *et al.* 1994)	4	Rat	EDL	n.c.	n.c.		n.c.	n.c.
(Kennedy *et al.* 2003)	10	Rat	Myocardium	↓ ~45%	↓ ~35%			
(da Silva *et al.* 1994)	10	Rat	Myocardium	n.c.	n.c.		n.c.	

EDL, extensor digitorum longus; ↓, decreased compared to control; n.c., no change compared to control.

2.4.4 Possible causes of Na^+,K^+-ATPase defects in renal disease

2.4.4.1 Altered substrate affinity

There have been several reports of altered substrate affinity of the Na^+,K^+-ATPase in renal disease. Kinetic studies of the Na^+, K^+-ATPase in erythrocytes from uraemic patients revealed an almost 2-fold increase in the K_m for ATP (Kramer *et al.* 1972, 1976) The significance of this finding is unclear however, since erythrocyte intracellular [ATP] was also elevated in CRF (Kramer *et al.* 1972, 1976, Welt 1964). Conversely, Welt *et al.* found no difference in the K_m for Na^+, K^+, ATP, or ouabain in erythrocytes from uraemic patients and controls (Welt 1967). It is possible that altered substrate affinities in renal disease may alter the activity of the Na^+,K^+-ATPase but the research to date suggests that this is unlikely.

2.4.4.2 Endogenous digitalis-like factors

Numerous researchers have hypothesised that an endogenously produced digoxin-like factor may inhibit the Na^+,K^+-ATPase (section 2.2.8.2). Several studies have identified such substances in plasma from uraemic patients that inhibited Na^+,K^+-ATPase activity (Stokes *et al.* 1986, Vasarhelyi *et al.* 1996). Incubation of normal cells in uraemic sera has also been shown to cause a reduction in Na^+,K^+-ATPase activity (Cole *et al.* 1968, Druml 1988, Izumo *et al.* 1984, Kramer *et al.* 1976, Minkoff *et al.* 1972), when compared to normal cells incubated in normal sera and this has been attributed to the existence of an EDLF. This concept is supported by studies showing an improvement in Na^+,K^+-ATPase activity following dialysis treatment or renal transplantation (Edmondson *et al.* 1975, Izumo *et al.* 1984, Sigstrom 1981, Vasarhelyi *et al.* 1996, Welt 1967, Zannad *et al.* 1985). However, other factors could also explain the improved Na^+,K^+-ATPase activity (section 2.4.4.3).

2.4.4.3 Uraemic toxins

Many of the studies that support the existence of endogenous digitalis-like factors (EDLF) are also consistent with the theory that the Na^+,K^+-ATPase may be inhibited by the accumulation of certain uraemic toxins. This theory is consistent with the findings of an 8 – 40% improvement in Na^+,K^+-ATPase activity following dialysis (Edmondson *et al.* 1975, Izumo *et al.* 1984, Sigstrom 1981, Welt 1967, Zannad *et al.* 1985) and a 23 – 50% decrease in Na^+,K^+-ATPase activity of normal cells incubated in uraemic sera (Cole *et al.* 1968, Druml 1988, Izumo *et al.* 1984, Kramer *et al.* 1976, Minkoff *et al.* 1972). Methylguanidine and urea are by-products of protein catabolism that are modulated by dietary protein intake. Normally, they are excreted by the kidney, however in renal disease their concentrations may be increased more than 20-fold. Ouabain-sensitive ^{86}Rb uptake of erythrocytes in the presence of urea concentrations that are commonly found in CRF (50 mM) was inhibited 15% compared to erythrocytes in the absence of urea (Kaji *et al.* 1998). Also, normal rat brain incubated in normal rat serum with the addition of methylguanidine had a 25% lower Na^+,K^+-ATPase activity than rat brain incubated in normal rat serum alone (Minkoff *et al.* 1972). Leucocyte ouabain sensitive ^{86}Rb uptake in uraemic subjects was significantly improved following three months on a low protein diet and was the same as control subjects after six months (Aparicio *et al.* 1991). This suggests that accumulation of protein metabolites in renal disease may inhibit the Na^+,K^+-ATPase.

2.4.4.4 Altered hormonal regulation

Numerous hormones regulate the activity and content of the Na^+,K^+-ATPase in normal subjects (Sections 2.2.6.3 and 2.2.7.2). Renal disease may alter the production and/or action of a number of these hormones, including insulin and thyroid hormones (Morrison 1985), and thereby may alter the activity or content of the Na^+,K^+-ATPase

in renal disease. Goecke *et al.* reported enhanced insulin sensitivity of the Na^+,K^+-ATPase in the soleus of uraemic rats, as the addition of insulin caused a 203% increase in ^{86}Rb uptake in the uraemic rats compared to a 77% increase in the control rats (Goecke *et al.* 1991). Conversely, a number of other studies have failed to find any difference in the effect of insulin on Na^+,K^+-ATPase activity in skeletal and cardiac muscle, and adipocytes from uraemic rats (Druml *et al.* 1990, Druml 1988), and skeletal muscle from uraemic humans (Alvestrand *et al.* 1984). Thyroid hormone secretion and metabolism are altered in renal disease, with decreased T_3 but normal T_4 (Morrison 1985). This would be expected to reduce Na^+,K^+-ATPase content (Kjeldsen *et al.* 1984b), however this has not been investigated in renal disease. Further studies are needed to determine the effects of renal disease on the sensitivity of the Na^+,K^+-ATPase to various other hormones, such as catecholamines and thyroid hormones.

2.4.4.5 Altered membrane lipid composition

Changes in the lipid composition of the membrane in which the Na^+,K^+-pump is embedded can also alter Na^+,K^+-ATPase activity (Wu *et al.* 2004). Abnormal membrane lipid composition has been found in erythrocytes from HD (Koorts *et al.* 2002, Vasarhelyi *et al.* 1996) and RTx (Phair *et al.* 1989) and also in kidney, liver and testes microsomal membranes from rats following RTx compared to sham operated rats (Lausada *et al.* 2005, Lausada *et al.* 2002). Therefore, altered membrane lipid composition in ESRD may contribute to their impaired Na^+,K^+-ATPase activity. However, Vasarhelyi *et al.* reported altered membrane lipid composition and depressed Na^+,K^+-ATPase activity in erythrocytes from HD adolescents, but found no correlation between the two (Vasarhelyi *et al.* 1996). Thus, whether altered membrane

lipid composition contributes to Na^+,K^+-ATPase inhibition in ESRD remains equivocal.

2.4.4.6 Immunosuppressive steroids

Patients with renal disease treated by RTx are given large doses of immunosuppressive steroids to prevent organ rejection. The most commonly used immunosuppressants are prednisone/prednisolone, cyclosporine, azathioprine and tacrolimus. These steroids have been shown to alter Na^+,K^+-ATPase activity and content (Table 2.11). Prednisone is a glucocorticoid and, as discussed in section 2.2.7.2.2, these have been shown to have potent upregulatory effects on Na^+,K^+-ATPase content and activity. Skeletal muscle Na^+,K^+-ATPase content was increased by 61% following 30 days of high dose (6 $mg.kg^{-1}.d^{-1}$) prednisone administration. The effects appear to be dose dependant, as 0.1 - 0.2 $mg.kg^{-1}.d^{-1}$ did not increase Na^+,K^+-ATPase activity in human erythrocytes, whereas 0.2 – 0.5 $mg.kg^{-1}.d^{-1}$ did (Table 2.11). Cyclosporine and tacrolimus appear to have inhibitory effects, whereas azathioprine caused no change. As it is common for RTx patients to be prescribed more than one immunosuppressant, the compound effect on the Na^+,K^+-ATPase is thus very difficult to determine. For instance, erythrocyte Na^+,K^+-ATPase activity in patients taking cyclosporine and prednisone was reduced (Borca *et al.* 1992, Huang *et al.* 1996), in those taking prednisone and azathioprine it tended to increase (Borca *et al.* 1992, Cole *et al.* 1975), and in those taking prednisone, cyclosporine, and azathioprine it was also increased (Bosch *et al.* 1991).

2.4.5 Significance of impaired skeletal muscle Na^+,K^+-ATPase in renal disease

The skeletal muscle Na^+,K^+-ATPase is critical in maintaining transmembrane $[Na^+]$ and $[K^+]$ gradients and thus, muscle membrane excitability and muscle contraction (section 2.1.3). Therefore, a reduction in skeletal muscle Na^+,K^+-ATPase activity in

renal disease could impair muscle Na^+ and K^+ regulation and membrane excitability and expedite the onset of muscle fatigue. Both arterial K^+ regulation and muscle membrane excitability may be impaired in HD, and these were linked with reduced exercise performance (section 2.3.4.1.6). However, whether these are related to depressed skeletal Na^+,K^+-ATPase activity remains to be determined. Furthermore, whether skeletal muscle K^+ regulation and Na^+,K^+-ATPase activity are impaired and related to the poor exercise performance in RTx has also not been investigated. Therefore, this thesis investigated whether skeletal muscle Na^+,K^+-ATPase was impaired in HD and RTx and whether this was related to their impaired K^+ regulation and poor exercise performance (Chapter 5).

Table 2.11. Effects of immunosuppressive steroids on Na^+,K^+-ATPase activity in muscle and red blood cells.

Reference	Dose ($mg.kg^{-1}.d^{-1}$) + additional drug	Duration	Species	Condition	Tissue	Effect on Na^+,K^+-ATPase activity
Prednisone						
(Cole *et al.* 1975)	~0.2-0.5 + Aza ~1.5		Human	RTx	RBC	↑ 101%
(Borca *et al.* 1992)	+ Aza		Human	RTx	RBC	↑ 32%
(Zannad *et al.* 1982)	~0.1-0.2 + Aza ~1-2		Human	RTx	RBC	n.c.
(Bosch *et al.* 1991)	+ Cys + Aza		Human	RTx	RBC	↑ 77%
Cyclosporine						
(Rabini *et al.* 1990)	5	7 days	Human	RA	RBC	↑ 35%
(Huang *et al.* 1996)	+ Pred		Human	RTx	RBC	↓ 19%
(Borca *et al.* 1992)	+ Pred		Human	RTx	RBC	↓ 6%

(Bosch *et al.* 1991)	+ Aza		Human	RTx	RBC	n.c.
(Mardini *et al.* 2001)	5	7 days	Rabbit		Myocytes	n.c
(Mardini *et al.* 2001)	10	7 days	Rabbit		Myocytes	↓ 43%
Tacrolimus						
(Mardini *et al.* 2001)	1	7 days	Rabbit		Myocytes	n.c
(Mardini *et al.* 2001)	5	7 days	Rabbit		Myocytes	↓ 40%
Azathioprine						
(Rabini *et al.* 1990)	2	7 days	Human	RA	RBC	n.c

Pred, prednisone; Cys, cyclosporine; Aza, azathioprine; Tac, tacrolimus; RBC, red blood cells; RA, rheumatoid arthritis; RTx, renal disease patients treated with renal transplantation; ↓, decreased compared to control, ↑, increased compared to control; n.c., no change compared to control.

2.5 EXERCISE TRAINING IN HD PATIENTS

An increasing number of studies have reported substantial benefits of exercise training in HD patients. This section will focus on exercise training induced improvements in exercise performance and quality of life (QOL) and the possible underlying mechanisms.

2.5.1 Training and exercise performance in HD patients

2.5.1.1 Training effects on $\dot{V}O_{2peak}$ in HD

Improvements in $\dot{V}O_{2peak}$ following exercise training in HD range from 13 – 70%, and appears to depend on the mode, but not the duration of training (Table 2.12). The largest increases in $\dot{V}O_{2peak}$ have resulted from a combination of aerobic and resistance training (Deligiannis *et al.* 1999a, Deligiannis *et al.* 1999b, Konstantinidou *et al.* 2002, Kouidi *et al.* 1998, Kouidi *et al.* 2004, Kouidi *et al.* 1997). This is in keeping with the finding that muscle strength is a strong predictor of $\dot{V}O_{2peak}$ in HD (Diesel *et al.* 1990). However, numerous studies have also found no improvement in $\dot{V}O_{2peak}$ following training (Akiba *et al.* 1995, Goldberg *et al.* 1980b, Goldberg *et al.* 1979, Moore *et al.* 1993b, Violan *et al.* 2002). This might be explained in some studies by low statistical power (n = 5 - 6) (Goldberg *et al.* 1980b, Goldberg *et al.* 1979), but not in others (Akiba *et al.* 1995, Moore *et al.* 1993b, Violan *et al.* 2002). Interestingly, several studies have reported that 17 - 36% of HD patients did not improve $\dot{V}O_{2peak}$ after training (Goldberg *et al.* 1986, Goldberg *et al.* 1980a, Goldberg *et al.* 1980b, Goldberg *et al.* 1979, Moore *et al.* 1993b, Suh *et al.* 2002). Thus, it is possible that there is a subset of HD patients in whom $\dot{V}O_{2peak}$ does not improve after training, although the reasons for this are unclear.

2.5.1.2 Training effects on muscle strength in HD

Resistance and endurance training also increase muscle strength in HD. A 13% increase in leg-extensor isokinetic peak torque was reported following 12 weeks of resistance training (Headley *et al.* 2002). Other studies have also reported increases of 37 – 82% in lower body 1, 3, or 5 repetition maximum (RM) or hand-grip strength following resistance training (DePaul *et al.* 2002, Johansen *et al.* 2006, Kouidi *et al.* 1998, Oh-Park *et al.* 2002, Ota 1996). Stationary endurance-based cycle training has also been reported to improve leg press 5RM by 16% (Storer *et al.* 2005).

2.5.1.3 Mechanisms of improved exercise performance following training in HD

Mechanistic exercise training studies have identified numerous training adaptations in HD patients that may contribute to their improved exercise performance. This section reviews the adaptations that have been reported in skeletal muscle, which include increased O_2 delivery, muscle CSA, and oxidative muscle fibre percentage, and improved energy metabolism.

Table 2.12. Exercise training effects on $\dot{V}O_{2peak}$ in HD.

Reference	**Training protocol**					**% increase in $\dot{V}O_{2peak}$**
	Mode	**Time ($min.d^{-1}$)**	**Frequency ($d.wk^{-1}$)**	**Duration (weeks)**	**Intensity[a]**	
(Kouidi *et al.* 2004)	cycle/calisthenics/ resistance	60	3	208	50-70% $\dot{V}O_{2peak}$	70%
	cycle/resistance	60-90	3	208	Borg RPE = 13	50%
(Kouidi *et al.* 1998)	cycle/calisthenics/ resistance	90	3	26		48%
(Deligiannis *et al.* 1999a)	calisthenics, swimming or ball games/resistance	90	3	26	60-70% HR_{max}	43%
(Konstantinidou *et al.* 2002)	cycle/resistance	60	3	26	60-70% $\dot{V}O_{2peak}$	43%
(Shalom *et al.* 1984)	cycle/jog/calisthenics	45	5	12	75-80% HR_{max}	42%
(Deligiannis *et al.* 1999b)	calisthenics, swimming or ball games/resistance	90	3-4	26	60-70% HR_{max}	41%
(Kouidi *et al.* 1997)	cycle/jog/calisthenics/ swimming or ball games	90	3	26	50-60% $\dot{V}O_{2peak}$	38%
(Painter *et al.* 1986b)	cycle	30	3	26	60-85% $\dot{V}O_{2peak}$	28%
(Storer *et al.* 2005)	cycle	20-40	3	8.5	50% workload max	22%

(Zabetakis *et al.* 1982)	walk/jog	25-45	3	10	100% AT	21%
(Levendoglu *et al.* 2004)	cycle or walk/jog	60	3	12	40-60% HR_{max}	20%
(Goldberg *et al.* 1983)	cycle/walk	45-60	3-5	52	40-80% $\dot{V}O_{2peak}$	19%
(Goldberg *et al.* 1986)	cycle/walk	45-60	3-5	52	40-80% $\dot{V}O_{2peak}$	18%
(Hagberg *et al.* 1983)	cycle/walk/calisthenics	30	3-5	60	50-85% $\dot{V}O_{2peak}$	17%
(Koufaki *et al.* 2002)	cycle	40	3	13	90% VT	17%
(Painter *et al.* 2002a)	cycle	30	3	24		16%
(Suh *et al.* 2002)	cycle, walk	60	3	12	40-60% HR_{max}	13%
(Goldberg *et al.* 1979)	cycle/jog/calisthenics	45-60	3-5	30	40-80% $\dot{V}O_{2peak}$	n.c.
(Goldberg *et al.* 1980a)	cycle/jog/calisthenics	45-60	3-5	30	40-80% $\dot{V}O_{2peak}$	n.c.
(Violan *et al.* 2002)	jog/aerobics/ball games	50	3	26	≥60% HR_{max}	n.c.
(Akiba *et al.* 1995)	cycle	10-20	3	12	RPE ≥ 12	n.c.
(Moore *et al.* 1993b)	cycle	30	3	12	70% HR_{max}	n.c.

HR_{max}, maximum heart rate; VT, ventilatory threshold; AT, anaerobic threshold; n.c., no change; a, initial – final exercise intensity expressed relative to pre-training test scores.

2.5.1.3.1 Improved oxygen delivery

Several studies have reported elevations in [Hb] of 7 – 37% following aerobic exercise training in HD (Fitts *et al.* 1999, Goldberg *et al.* 1986, Goldberg *et al.* 1983, Goldberg *et al.* 1980a, Goldberg *et al.* 1980b, Hagberg *et al.* 1983). Following aerobic or combined aerobic and resistance training, increases were observed in left ventricular mass, left ventricular end-diastolic volume, ejection fraction, stroke volume, cardiac output and oxygen pulse (Deligiannis *et al.* 1999a, Shalom *et al.* 1984). These indicate improved left ventricular systolic function and cardiac performance with training in HD. Furthermore, exercise training resulted in increased muscle capillarisation and capillary contact per muscle fibre (Kouidi *et al.* 1998, Sakkas *et al.* 2003). Thus, exercise training in HD improves oxygen transport capacity of the blood, whole body blood flow, and possibly also muscle perfusion. These changes would be expected to improve $\dot{V}O_{2peak}$ in HD, as O_2 delivery appears to be a limiting factor (section 2.3.4.1.1).

2.5.1.3.2 Energy metabolism

Six months of combined aerobic and resistance training caused qualitative improvements in mitochondria structure and number in HD (Kouidi *et al.* 1998). Phosphofructokinase activity increased by 45% (Moore *et al.* 1993b), and there was a tendency for increased succinate dehydrogenase (*ibid*) and cytochrome c oxidase activities (Sakkas *et al.* 2003) following 12 and 26 weeks of cycle training during dialysis, respectively. These findings suggest that the capacity for both glycolytic and oxidative metabolism is enhanced after exercise training in HD, and may contribute to their improved $\dot{V}O_{2peak}$.

2.5.1.3.3 Muscle CSA

Thigh muscle CSA increased by 2.5% after 12 weeks of resistance training in HD (Johansen *et al.* 2006). Type I and II muscle fibre area increased by 26 and 24%, respectively, following 6 months of combined aerobic and resistance training (Kouidi *et al.* 1998). The gastrocnemius muscle mean fibre area increased by 46% after aerobic cycle training for 6 months (Sakkas *et al.* 2003). On the contrary, rectus femoris type I and II fibre areas were unchanged after 12 weeks of aerobic cycle training (Moore *et al.* 1993b). As muscle CSA was related to $\dot{V}O_{2peak}$ in HD (Diesel *et al.* 1990, Kettner Melsheimer *et al.* 1987), and is important for muscle strength, these changes would be expected to improve exercise performance and physical functioning in HD.

2.5.1.3.4 Muscle fibre-type

The percentage of oxidative fibres of the vastus lateralis from HD increased from 33% pre-training to 53% after 6 months combined aerobic and resistance training (Kouidi *et al.* 1998). Conversely, rectus femoris muscle fibre composition was unchanged in HD after 6 months stationary aerobic cycle training (Sakkas *et al.* 2003).

2.5.1.3.5 Muscle membrane excitability

Impaired muscle membrane excitability, caused by abnormal skeletal muscle Na^+,K^+-ATPase activity and K^+ regulation, may be a potential cause of poor exercise performance in HD (section 2.3.4.1.6). In healthy subjects exercise training increases skeletal muscle Na^+,K^+-ATPase content (Table 2.3) and ameliorates the exercise-induced rise in plasma and interstitial [K^+] during exercise (Green *et al.* 1993, McKenna 1995, McKenna *et al.* 1996, McKenna *et al.* 1997, McKenna *et al.* 1993, Nielsen *et al.* 2004a). Improved plasma K^+ regulation during exercise has also been observed after training in patients with chronic heart failure (Barlow *et al.* 1994). If

plasma and interstitial K^+ regulation during exercise are also improved in HD following training, this may enhance muscle membrane excitability and contribute to the improvements observed in exercise performance. However, no studies have determined the effects of training on K^+ regulation during exercise in HD, therefore this was investigated in Chapter 4.

2.5.2 Training and health-related quality of life in HD patients

Health-related quality of life (HRQOL) is typically low in HD and is attributed to their poor physical functioning, low employment rate, and emotional disturbances (Kouidi 2004). Furthermore, HRQOL scores predicted mortality and hospitalisation rates (DeOreo 1997). Exercise training results in improved physical functioning, and as such, also has beneficial effects on HRQOL (Kouidi 2004). HRQOL was measured in 180 HD patients before and after 16 weeks of exercise training (Painter *et al.* 2000b). Significantly improved scores in the physical, but not mental HRQOL components were reported (*ibid*). Similar reports of improved physical HRQOL component scores with exercise training have also been reported in other studies (Molsted *et al.* 2004, Oh-Park *et al.* 2002, Painter *et al.* 2002a). Conversely, several studies found no change in HRQOL following exercise training in HD (DePaul *et al.* 2002, Fitts *et al.* 1999, Parsons *et al.* 2004). Patients with low self-reported HRQOL may benefit the most from exercise training as HRQOL physical component scores improved in those with low scores after exercise training but not in those with high initial scores (Painter *et al.* 2000a). The main objective of exercise training in HD is to improve exercise performance and thus physical functioning and quality of life. Therefore, HRQOL measures are important subjective indicators of the efficacy of exercise training interventions. Thus, HRQOL was measured before and after stationary cycle training in HD patients in Chapter 4.

2.6 CONCLUSIONS

Many facets of exercise performance are reduced in renal disease. Impaired $\dot{V}O_{2peak}$ and muscle strength have been well documented in HD and to some extent in RTx. However, comparisons of exercise performance between HD and RTx are limited. For instance, it is unclear whether $\dot{V}O_{2peak}$ is improved in RTx and whether this is related to increased [Hb] compared to HD. Muscle strength is low in both HD and RTx but may be due to reduced muscle mass in each, yet this is uncertain. Muscle fatiguability during repeated dynamic contractions in HD and RTx has not been investigated, but is likely reduced.

Numerous skeletal muscle abnormalities may cause the reduced exercise performance in HD and RTx. Evidence suggests that impaired muscle membrane excitability, possibly caused by abnormal skeletal muscle Na^+,K^+-ATPase and [K^+] regulation, may be a contributing factor. However, the effects of HD and RTx on the skeletal muscle Na^+,K^+-ATPase have not been determined. Impaired plasma K^+ regulation has been observed in HD and may be related to their anaemia. Anaemia treatment improves their plasma K^+ regulation (McMahon *et al.* 1999), but whether it is still impaired compared to CON has not been investigated. Whether K^+ regulation is improved by exercise training in HD is unknown, and whether this is related to their improved exercise performance after training is also unknown.

SECTION III: AIMS AND HYPOTHESES

The skeletal muscle Na^+,K^+-ATPase enzyme regulates trans-membrane Na^+ and K^+ fluxes during muscle contractions, and therefore plays an important role in delaying muscle fatigue. Consequently, any decrease of Na^+,K^+-ATPase content or activity has the potential to adversely affect muscle fatiguability. Therefore, this thesis investigated three factors thought to impair or down-regulate the skeletal muscle Na^+,K^+-ATPase – acute exercise, renal disease and digoxin. The related effects on plasma $[K^+]$ during exercise and on muscle performance were also examined. This thesis firstly investigated the acute effects of brief intense exercise on muscle Na^+,K^+-ATPase content and maximal in-vitro activity (Study 1). Study 2 investigated the effects of end-stage renal disease on plasma $[K^+]$ regulation during exercise; the relationship between impaired $[K^+]$ regulation and muscle performance; and the effects of endurance training in these patients. Study 3 investigated the impacts of end-stage renal disease and renal transplantation on skeletal muscle Na^+,K^+-ATPase and its relationship with muscle performance. Finally, Study 4 investigated the effects of chronic digoxin administration on skeletal muscle Na^+,K^+-ATPase content and maximal in-vitro activity and on muscle performance.

2.7 AIMS

2.7.1 Study One

Acute, fatiguing exercise has been shown to reduce skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity, with potentially detrimental effects on exercise performance. Therefore, the first aim of this study was to investigate whether the depressed muscle Na^+,K^+-ATPase activity with exercise reflected a loss of Na^+,K^+-ATPase units. The second aim was to determine the time-course of it's recovery post-exercise. The third

aim was to ascertain whether this depressed activity was related to increased Na^+,K^+-ATPase isoform gene expression.

2.7.2 Study Two

Plasma K^+ regulation during exercise was impaired in haemodialysis patients (HD) with anaemia and this was related to their poor exercise performance. Both treatment of anaemia with erythropoietin (EPO) and exercise training improve exercise performance in HD. Thus, this study aimed to determine whether plasma K^+ regulation is impaired in HD that have received anaemia correction with EPO, compared to CON. It also aimed to ascertain whether improved exercise performance after training is correlated to improved plasma K^+ regulation.

2.7.3 Study Three

HD exhibit impaired K^+ regulation and this is related to their poor exercise performance, but this relationship has not been investigated in RTx. Furthermore, no studies have compared exercise performance in HD and RTx with similar [Hb]. A potential cause of impaired K^+ regulation in HD is depressed skeletal muscle Na^+,K^+-ATPase activity, but no studies have investigated this in HD or RTx. Therefore, the first aim of this study was to compare exercise performance between HD and RTx with similar [Hb]. The second aim was to determine if K^+ regulation during exercise and skeletal muscle Na^+,K^+-ATPase activity are impaired in HD and in RTx. The third aim was to investigate whether depressed skeletal muscle Na^+,K^+-ATPase activity and impaired K^+ regulation during exercise in HD contribute to their poor exercise performance.

2.7.4 Study Four

Digoxin is a specific Na^+,K^+-ATPase inhibitor and in digoxin-treated chronic heart failure patients, skeletal muscle Na^+,K^+-ATPase content and exercise performance are

each reduced. However, whether chronic digoxin administration reduces skeletal muscle Na^+,K^+-ATPase content and activity and thus impairs maximal exercise performance in healthy humans has not been investigated. Thus, this study aimed to determine if chronic digoxin administration would depress skeletal muscle Na^+,K^+-ATPase content and activity in healthy humans, and whether acute exercise would further depress activity. An additional aim was to investigate the effects of inhibition of Na^+,K^+-ATPase activity by digoxin on exercise performance in healthy subjects.

2.8 HYPOTHESES

2.8.1 Study One

The specific hypotheses tested were that:

1. Acute fatiguing exercise will only transiently depress skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity at fatigue, with recovery by 3 or 24 hours post-exercise.
2. Skeletal muscle Na^+,K^+-ATPase content would be unchanged immediately and within 24 h after acute, short-duration exercise.
3. The reduction in Na^+,K^+-ATPase activity immediately following an acute bout of exhaustive exercise will be correlated with increases in the mRNA of the Na^+,K^+-ATPase catalytic α isoforms.

2.8.2 Study Two

The specific hypotheses tested were that in HD treated by chronic EPO administration:

1. The plasma $[K^+]$ response to incremental exercise would still be impaired compared to healthy controls, as evidenced by increased plasma $[K^+]$, rate of

rise in plasma [K^+], and rise in plasma [K^+] relative to total work done ($\Delta[K^+].work^{-1}$ ratio).

2. Aerobic cycle ergometer training would improve their plasma [K^+] response to incremental exercise, together with $\dot{V}O_{2peak}$, muscle strength and fatiguability, and quality of life.

2.8.3 Study Three

The specific hypotheses tested were that:

1. $\dot{V}O_{2peak}$ would be reduced compared to CON but would not differ between HD and RTx with similar [Hb].
2. Both muscular strength and fatiguability during dynamic contractions would be worsened in HD and RTx compared to CON.
3. The plasma [K^+] response to incremental exercise, as assessed by plasma [K^+], rate of rise in plasma [K^+], and the $\Delta[K^+].work^{-1}$ ratio, would be impaired in HD but not RTx compared to CON.
4. Skeletal muscle Na^+,K^+-ATPase activity would be low in HD, but normal in RTx compared to CON, whilst muscle Na^+,K^+-ATPase content and isoform abundance would be unchanged in HD and RTx.

2.8.4 Study Four

The specific hypotheses tested were that digoxin administration for 14 days in healthy humans would:

1. Depress skeletal muscle Na^+,K^+-ATPase content and activity, with the latter being further depressed by acute exercise.
2. Reduce time to fatigue during moderate duration intermittent cycling exercise.

CHAPTER 3. DEPRESSED Na^+,K^+-ATPase ACTIVITY IN SKELETAL MUSCLE AT FATIGUE IS CORRELATED WITH INCREASED Na^+,K^+-ATPase mRNA EXPRESSION FOLLOWING INTENSE EXERCISE

3.1 INTRODUCTION

In isolated rat skeletal muscle, electrical stimulation can increase Na^+,K^+-ATPase activity by 18-22-fold above resting levels (Clausen 2003, McKenna *et al.* 2003b, Nielsen *et al.* 1997, 2000). However, several studies have found that the maximal in-vitro Na^+,K^+-ATPase activity is reduced with exercise, as determined in-vitro by K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) activity, in humans (Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Leppik *et al.* 2004, Sandiford *et al.* 2004) and in rats (Fowles *et al.* 2002a). This suggests that the maximal attainable Na^+,K^+-ATPase activity and thus capacity for Na^+/K^+ exchange is reduced with exercise. In isolated rat muscle, inhibition of Na^+,K^+-ATPase activity by ouabain accelerated fatigue and retarded the force recovery rate (Harrison *et al.* 1997, Nielsen *et al.* 1996). As Na^+,K^+-ATPase activity counters excitation-induced Na^+ influx and K^+ efflux (Hodgkin *et al.* 1959b) and contributes to membrane excitability (Clausen 2003, Nielsen *et al.* 2000), depressed muscle maximal Na^+,K^+-ATPase activity may also contribute to fatigue in exercising humans. If so, an early post-fatigue recovery in Na^+,K^+-ATPase activity would be expected, but the recovery time course remains uncertain. After 30 min of intermittent isometric contractions, Na^+,K^+-ATPase activity was 35% lower in an exercised compared to a non-exercised leg, with no difference between legs at 1 h post-exercise (Fowles *et al.* 2002b). Whilst this suggested that exercise transiently impaired muscle maximal in-vitro Na^+,K^+-ATPase activity, they also reported no difference between rest and post-exercise, when

sampled from different legs. Furthermore, they found no differences within the exercised leg between 0, 1, or 4 h after exercise. Together these internally inconsistent findings suggest either that activity was not depressed, or did not recover following exercise and clarification is required. The recovery time course is important, as it has implications for understanding whether Na^+,K^+-ATPase impairment with exercise is part of a fatigue-, damage- and/or some other regulatory process. Therefore, this study tested the first hypothesis, that exercise will only transiently depress maximal in-vitro Na^+,K^+-ATPase activity at fatigue, with recovery by 3 or 24 hours post-exercise.

Relatively few studies have investigated the effects of brief, intense exercise on muscle Na^+,K^+-ATPase content, as fully quantified by [^{3}H]ouabain binding (Nørgaard *et al.* 1984a). An upregulation is suggested by the increased muscle [^{3}H]ouabain binding with ~10 h running (Overgaard *et al.* 2002) and training (Green *et al.* 1993, McKenna *et al.* 1993). In contrast, no increase in [^{3}H]ouabain binding occurred immediately after incremental or prolonged exercise (Aughey *et al.* 2005, Leppik *et al.* 2004) or within 4 h post-isometric contractions (Fowles *et al.* 2002b). However, these studies may have missed any increase due to either the lack of a recovery biopsy (Aughey *et al.* 2005, Leppik *et al.* 2004) or an insufficient recovery period (Fowles *et al.* 2002b). Acute upregulation of muscle Na^+,K^+-ATPase content is also suggested by the finding that five min fatiguing knee-extensor exercise markedly increased the Na^+,K^+-ATPase α_2 (70%) and β_1 (26%) sarcolemmal abundance (Juel *et al.* 2000a). Since [^{3}H]ouabain binding did not differ between intact and cut muscle pieces in isolated rat muscles, suggesting no internal Na^+,K^+-ATPase stores (McKenna *et al.* 2003b), increased sarcolemmal α_2 abundance with exercise (Juel *et al.* 2000a) implies an increased sarcolemmal Na^+,K^+-ATPase content. However, [^{3}H]ouabain binding was not measured and membrane isolation has been criticized in this context due to

low Na^+,K^+-ATPase recovery (Hansen *et al.* 1988). Finally, electrical stimulation of isolated rat muscles failed to elevate [^{3}H]ouabain binding (McKenna *et al.* 2003b), suggesting that acute exercise does not elevate muscle Na^+,K^+-ATPase content. However, this could conceivably have been influenced by the absence of local hormonal or temperature effects. To resolve this issue, this study therefore tested the second hypothesis that muscle Na^+,K^+-ATPase content would be unchanged immediately and within 24 h after acute, short-duration exercise.

In addition to Na^+/K^+ exchange, Na^+,K^+-ATPase also acts as a signal transducer in cardiac muscle, (for review, see (Xie *et al.* 2002)) and in liver, where low K^+-inhibition of Na^+,K^+-ATPase activity increased Na^+,K^+-ATPase mRNA abundance (Pressley *et al.* 1988). Whether exercise-induced Na^+,K^+-ATPase inhibition also exerts a similar signalling function in skeletal muscle is unknown. Acute exercise increases the mRNA expression for each of Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2, and the β_3 isoforms (Murphy *et al.* 2004). Thus, acute inhibition of Na^+,K^+-ATPase activity with exercise might then be linked to increased gene expression (Murphy *et al.* 2004) and also increased Na^+,K^+-ATPase content with chronic training (Green *et al.* 1993, McKenna *et al.* 1993). This may occur via direct protein-protein interaction between Na^+,K^+-ATPase and it's neighbouring proteins, triggering a signalling cascade culminating in increased gene transcription (Xie *et al.* 2002). Alternately, ionic disturbances with depressed Na^+,K^+-ATPase activity may stimulate synthesis of new Na^+,K^+-ATPase enzymes to re-establish favourable Na^+ and K^+ gradients (Bundgaard *et al.* 1997a), (Wolitzky *et al.* 1986). This study therefore tested the third hypothesis that the reduction in Na^+,K^+-ATPase activity immediately following an acute bout of exhaustive exercise will be correlated with increases in the gene transcripts of the Na^+,K^+-ATPase catalytic α isoforms.

This laboratory has recently reported the muscle function, aerobic power, as well as muscle Na^+,K^+-ATPase isoform gene and protein expression before and after exercise in these subjects (Murphy *et al.* 2004). Here this study reports new data on the acute effects of exercise on muscle Na^+,K^+-ATPase content and maximal in-vitro activity, on the recovery time-course of Na^+,K^+-ATPase maximal in-vitro activity, and novel correlations between depressed muscle maximal in-vitro Na^+,K^+-ATPase activity and gene transcription changes.

3.2 METHODS

3.2.1 Subjects and overview of exercise tests

Fifteen healthy volunteers (8 male, 7 female) participated in the study. Sample size was calculated using Power and Precision software based on the first specific hypothesis. Comparing by ANOVA the expected Na^+,K^+-ATPase activity at the 4 biopsy times (rest, fatigue, +3hr, +24 hr), expected means 1221, 1058, 1221, 1300 $pmol.min^{-1}.mg\ protein^{-1}$; within cell SD of 150 $pmol.min^{-1}.mg\ protein^{-1}$ (Fraser *et al.* 2002), α=0.05, a sample size of n=12 yielded a Power = 97%. Subject characteristics were (mean ± SD) age 24.7 ± 6.7 yr, height 174.5 ± 6.8 cm, body mass 73.2 ± 11.4 kg, and $\dot{V}O_{2\ peak}$ 50.5 ± 2.8 $ml.kg^{-1}.min^{-1}$. Each subject refrained from vigorous exercise, alcohol and caffeine for 24 h before each exercise test. Subjects consumed standardised meals for 24 h before and after the invasive trial and were informed of all possible risks before giving written consent. This study was approved by the Human Research Ethics Committee at Victoria University of Technology.

3.2.2 Knee extensor muscle fatigue test

The knee extensor strength and fatigue tests were performed on an isokinetic dynamometer (Cybex Norm 770, Henley HealthCare, Massachusetts, USA) and have been described in detail elsewhere (Murphy *et al.* 2004). In brief, subjects performed

an isokinetic knee extensor muscle strength test, which involved three consecutive maximal contractions at $180°.s^{-1}$. The muscle fatigue test involved continuous one-legged knee extensions at $180°.s^{-1}$, repeated every 1.5 s, until fatigue. The work rate was ~40% of the total work produced during the maximal isokinetic knee extensor strength test. Fatigue was defined as an inability to maintain more than 90% of their target work rate for three successive contractions. This exercise model was chosen as it is similar to one that showed an apparent increase in sarcolemmal Na^+,K^+-ATPase α_2 and β_1 subunits following knee-extension exercise (Juel *et al.* 2000a).

3.2.3 Blood sampling and processing

A 20-gauge catheter was inserted into a dorsal hand vein, covered by a waterproof patch (Tegaderm) and the hand sheathed in a plastic bag. The hand was heated in a 45ºC water bath throughout the duration of the sampling period to enable arterialised samples to be drawn at rest, during exercise, at fatigue, and at 1, 2, 5 and 10 min post-exercise. The catheter was kept patent by periodic infusions of heparinised isotonic saline. The blood sample was transferred to a tube containing lithium heparin; 1 ml of blood was analysed in duplicate for [Hb] and Hct (Sysmex, K-800, Kobe, Japan) and another 1.5 ml was centrifuged at 4,500 rpm for 2 min with the plasma removed and frozen until later duplicate analysis of plasma $[K^+]$ using an ion selective electrode (Ciba Corning 865pH/Blood Gas Analyzer, Bayer, MA, USA).

3.2.4 Muscle biopsies

A muscle needle biopsy was taken from the middle third of the vastus lateralis muscle at rest, fatigue, 3 h and 24 h post exercise. After injection of a local anaesthetic into the skin and fascia (1% Xylocaine), a small incision was made and a muscle sample taken (~120mg) using a Stille biopsy needle. A small portion of muscle was homogenised and then frozen in liquid N_2 for later analysis of maximal in-vitro

Na^+,K^+-ATPase activity (3-O-methylfluorescein phosphatase activity). The remaining sample was immediately frozen in liquid N_2 until later analysis of Na^+,K^+-ATPase content ([^{3}H]ouabain binding site content), and gene expression (Real-Time RT-PCR).

3.2.5 3-O-MFPase assay

Maximal in-vitro Na^+,K^+-ATPase activity was measured using the K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay (Fraser *et al.* 2002, Fraser *et al.* 1998). Approximately 20 mg of muscle was blotted on filter paper, weighed, then homogenised on ice for 2 x 20 s at 20,000 rpm (Omni 1000, Omni International) in a homogenate buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris (pH 7.4). Muscle homogenates were then rapidly frozen and stored in liquid nitrogen until later analysis. Before analysis, homogenates were thawed, diluted one-fifth with ice-cold homogenate buffer, and then freeze-thawed a further 3 times. Thirty µl of the diluted, freeze-thawed homogenate was incubated in 2.5 ml of assay medium containing 100 mM Tris, 5 mM $MgCl_2$, 1.25 mM EDTA and 80 nM 3-O-methylfluorescein (pH 7.4) for 5 min at 37°C, before adding 40 µl of 10 mM 3-O-MFP (final concentration 156 µM) to start the reaction. The reaction was measured for 80 s before 10 µl of 2.58 M KCl (final concentration 10 mM) was added to stimulate K^+-dependent phosphatase activity. All assays were performed at 37°C, with continuous stirring, in a spectrofluorometer (Aminco Bowman AB2 SLM, Urbana, IL). Excitation wavelength was 475 nm, and emission wavelength was 515 nm, with 4 nm slit widths. The K^+-stimulated 3-O-MFPase activity was calculated by subtracting the initial activity from the activity obtained after addition of 10 mM KCl.

3.2.6 [^{3}H]ouabain binding site content

Skeletal muscle total Na^+,K^+-ATPase content was determined by vanadate-facilitated [^{3}H]ouabain binding site content analysis (Nørgaard *et al.* 1984a, Nørgaard *et al.* 1983). Muscle samples were cut into 2-5 mg pieces and washed for 2 x 10 min in 37°C vanadate buffer containing 250 mM sucrose, 10 mM Tris, 3 mM $MgSO_4$, and 1 mM $NaVO_4$ (pH 7.2-7.4). Muscle samples were then incubated for 120 min at 37°C in the above buffer with the addition of [^{3}H]ouabain (10^{-6} M, 2.0 μCi.ml^{-1}). After incubation, muscle samples were washed for 4 x 30 min in ice-cold vanadate buffer to remove any unbound [^{3}H]ouabain, blotted on filter paper and weighed before being soaked overnight in vials containing 0.5 ml of 5% trichloroacetic acid and 0.1 mM ouabain. The following morning, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard) was added prior to liquid scintillation counting of the [^{3}H]activity. The content of [^{3}H]ouabain binding sites was calculated on the basis of the sample wet weight and the specific activity of the incubation medium and samples and expressed as pmol.(g wet wt)$^{-1}$. The final [^{3}H]ouabain binding site concentration was then calculated by subtracting the non-specific [^{3}H]ouabain uptake (2.5 %) (Nørgaard *et al.* 1984a) and multiplying by a correction factor of 1.13 to allow for impurity of the [^{3}H]ouabain (1.05) (measured by supplier; Amersham Pharmacia Biotech - Castle Hill, Australia), loss of specifically bound [^{3}H]ouabain during washout (1.05) (Nørgaard *et al.* 1984a), and incomplete saturation (1.025) (T Clausen, personal communication).

3.2.7 Real-time RT-PCR measurement of mRNA

Total RNA was extracted from 5-10 mg of muscle using the FastRNA reagents (BIO 101, Vista, CA, USA) using methods previously employed in this laboratory (Murphy *et al.* 2003). The resulting RNA pellet was dissolved in EDTA-treated water and total RNA concentration was determined spectrophotometrically at 260 nm. RNA (1 μg)

was transcribed into cDNA using the Promega AMV Reverse Transcription Kit (Promega, Madison, Wisconsin, USA), and the resulting cDNA was stored at –20°C for subsequent analysis.

Real-Time PCR (GeneAmp 5700 Sequence Detection System) was run for 1 cycle (50°C for 2 min, 95°C for 10 min) and 50 cycles (95°C for 15 s, 60°C for 60s). Primer sequences were designed for the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 genes as described elsewhere (Murphy *et al.* 2004). All samples were run in triplicate and measurements included a no-template control as well as a human skeletal muscle sample endogenous control. Cyclophilin (CYC) mRNA expression was unchanged with exercise (data not shown), and was therefore used as a control (housekeeping gene) to account for any variations in the amount of input RNA and the efficiency of reverse transcription. Gene expression was quantified using a cycle threshold (C_T) method, whereby the relative expression of the genes compared with resting samples was made using the expression, $2^{-\Delta\Delta CT}$, in which the expression of each gene was normalised for input cDNA using the housekeeping gene CYC. Muscle mRNA are presented for 14 subjects (7 males, 7 females), due to insufficient sample for one subject.

3.2.8 Calculations

Details of the time course of changes in Na^+,K^+-ATPase isoform mRNA response to exercise in these individuals have been reported elsewhere (Murphy *et al.* 2004). The mRNA in resting muscle was contrasted against mRNA at fatigue for each isoform, as well as against the average post-exercise mRNA expression (average of fatigue, 3 h, and 24 h post-exercise) for each of the Na^+,K^+-ATPase α_1–α_3 and β_1–β_3 isoforms. The average post-exercise mRNA expression of each isoform was determined to account for variability in the individual time response of mRNA upregulation post-

exercise (Murphy *et al.* 2004). Correlations were calculated between the percentage change in 3-O-MFPase activity from rest to fatigue (%Δ 3-O-MFPase$_{rest\text{-}fatigue}$) and the percentage changes in both mRNA at fatigue, and the average post-exercise mRNA (Murphy *et al.* 2004).

3.2.9 Statistics

Data are presented as means ± SD. A one-way ANOVA with repeated measures was used to analyse all variables except mRNA, with Post-hoc analyses using Fisher's Least Significant Difference test. Correlations were determined by least squares linear regression. Single comparisons (e.g. rest vs. fatigue mRNA) were tested using a paired *t* test. Statistical significance was accepted at $P < 0.05$.

3.3 RESULTS

3.3.1 Exercise time, plasma volume and [K^+]

Knee extensor time to fatigue was 352 ± 268 s. Plasma volume decreased by 4.3 ± 2.1 % after 1 min of exercise ($P < 0.05$), by 12.3 ± 4.5 % at fatigue ($P < 0.05$), and remained 3.3 ± 4.3 % below rest at 10 min post-exercise ($P < 0.05$). Arterialised venous plasma [K^+] increased ($P < 0.05$) after 1 min of exercise and increased further ($P < 0.05$) at fatigue (Figure 3.1). In recovery, plasma [K^+] fell rapidly by 1 min post-exercise ($P < 0.05$), dropped below pre-exercise values at 2 and 5 min post-exercise ($P < 0.05$), but had recovered by 10 min post-exercise.

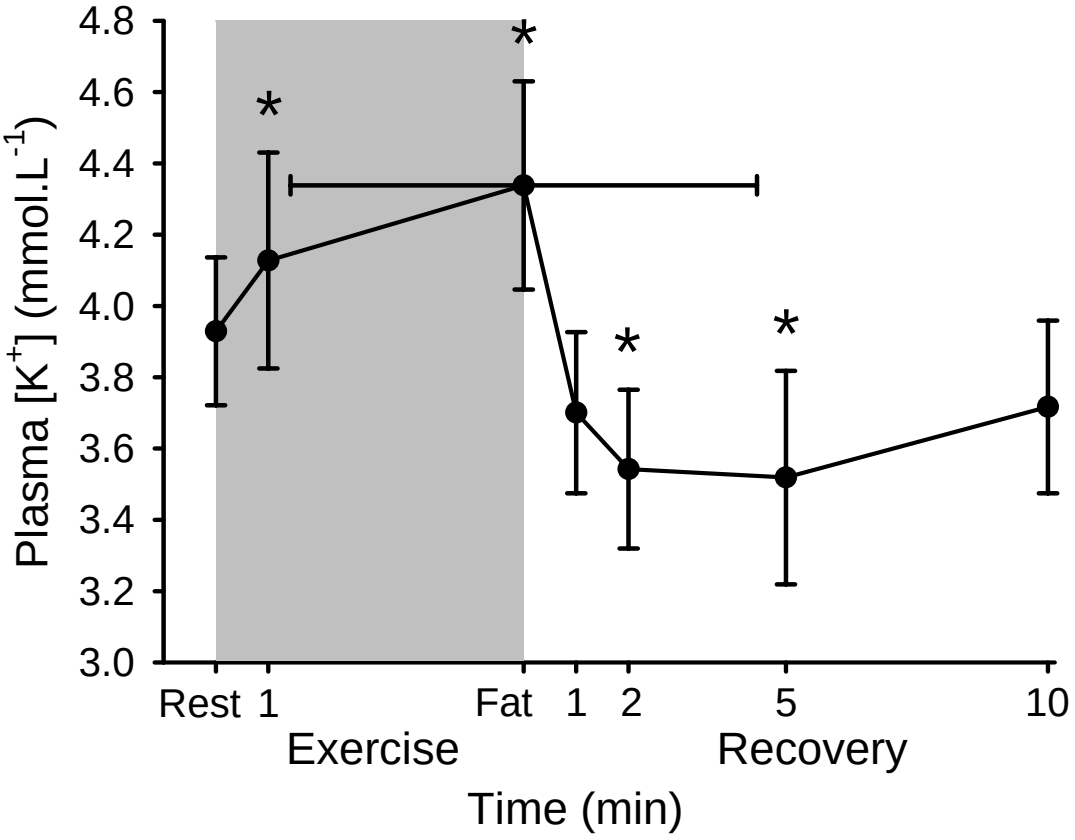

Figure 3.1. Arterialised venous plasma $[K^+]$ at rest, during single-leg knee extensions continued to fatigue (Fat), and for 10 min after exercise.

n = 15, except fatigue and 1 min where n = 14, and 10 min where n = 13. Shaded bar represents exercise bout. * Different to rest, $P < 0.05$. Values are means ± S.D.

3.3.2 Muscle Na^+,K^+-ATPase activity

Exercise decreased muscle in-vitro maximal 3-O-MFPase activity expressed relative to muscle wet weight by 10.7 ± 8.0 % ($P < 0.05$). This decline occurred in 11 out of 12 subjects. Muscle in-vitro 3-O-MFPase activity did not differ significantly from rest at either 3 h or 24 h post-exercise (Figure 3.2). Conversely, muscle in-vitro maximal 3-O-MFPase activity expressed relative to muscle protein content did not change with

exercise (rest 1566 ± 342, fatigue 1439 ± 201, +3 h 1404 ± 204, +24 h 1305 ± 214 nmol.min^{-1}.(g protein)$^{-1}$).

3.3.3 Muscle Na^+,K^+-ATPase content

Muscle [^{3}H]ouabain binding site content was not affected by the exercise bout, being unchanged from rest at fatigue, 3, and 24 h post-exercise, whether expressed relative to muscle wet weight (Figure 3.2) or muscle protein content (rest 1805 ± 504, fatigue 1893 ± 552, +3 h 1796 ± 524, + 24 h 1595 ± 398 pmol.g protein^{-1}).

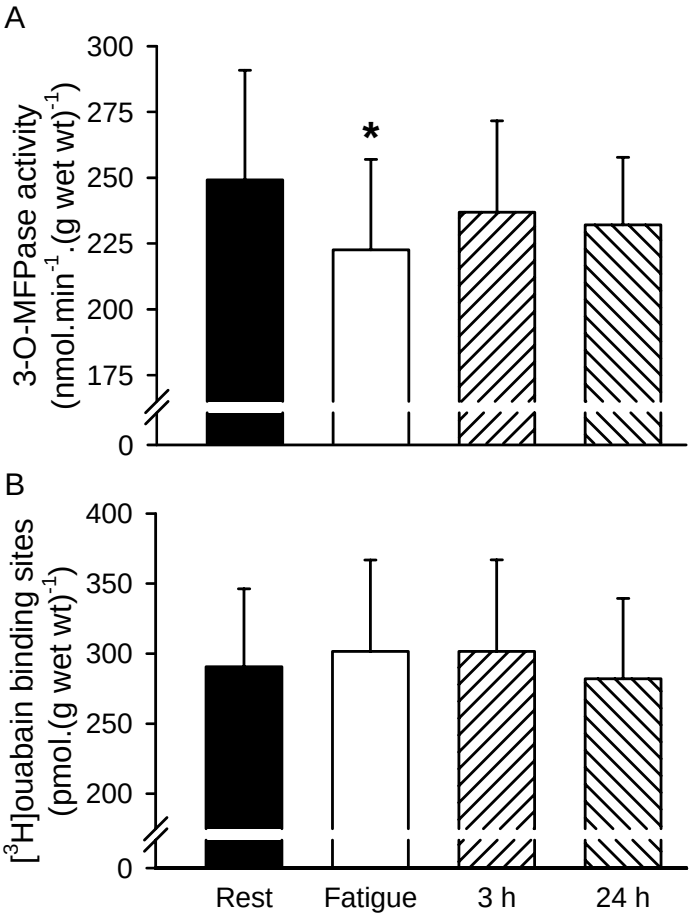

Figure 3.2. Maximal in-vitro 3-O-MFPase activity (A) and [^{3}H]ouabain binding site content (B) at rest, at fatigue induced by repeated isokinetic single-leg knee extensions, and at 3 and 24 h post-exercise.

Values are means ± S.D. 3-O-MFPase activity: n = 12. [^{3}H]ouabain binding site: n = 14. * Less than rest, $P < 0.05$.

3.3.4 Correlations between changes in muscle Na^+,K^+-ATPase mRNA and 3-O-MFPase activity

3.3.4.1 α_1 isoform

Exercise elevated α1 mRNA by 1.5-fold at fatigue ($P < 0.05$, Figure 3.3). The percent-change in α1 mRNA at fatigue was inversely correlated with the percentage change in 3-O-MFPase activity from rest to fatigue (%Δ 3-O-MFPase$_{rest\text{-}fatigue}$, -10.7 ± 8.0 %) (r = -0.60, $P < 0.05$, Figure 3.3). The average post-exercise (average of fatigue, 3 and 24 h) α1 mRNA was increased by 1.4-fold above rest ($P < 0.05$, Figure 3.3). The percentage change in average post-exercise α_1 mRNA approached a significant inverse correlation with %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ ($r = -0.56$, $P < 0.10$, Figure 3.3).

3.3.4.2 α_2 isoform

Exercise elevated α_2 mRNA at fatigue by 2.5-fold ($P < 0.05$, Figure 3.4). The percentage change in α_2 mRNA at fatigue was significantly correlated with %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ ($r = -0.60$, $P = 0.05$, Figure 3.4). The average post-exercise α_2 mRNA was increased by 2.2-fold ($P < 0.05$, Figure 3.4) and the percentage change was similarly inversely correlated with the %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ ($r = -0.68$, $P < 0.05$, Figure 3.4).

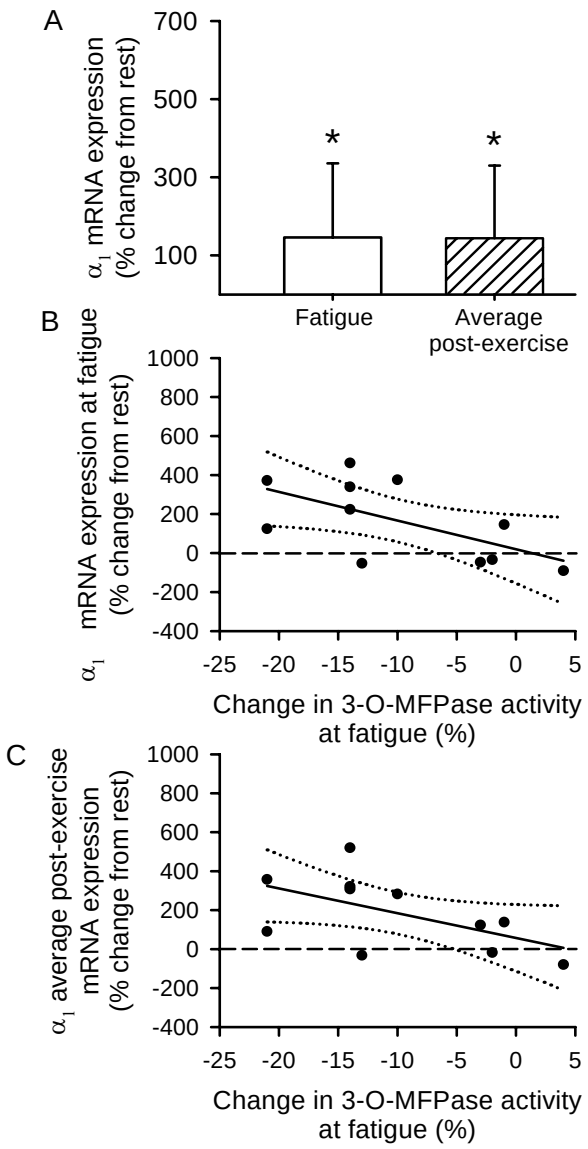

Figure 3.3. Na^+,K^+-ATPase α_1 isoform mRNA expression and relationship with 3-O-MFPase activity.

Na^+,K^+-ATPase α_1 isoform mRNA at fatigue induced by repeated isokinetic single-leg knee extensions, and the average post-exercise α_1 mRNA expression (average of fatigue, 3 h, and 24 h post-exercise) expressed as percentage change relative to rest (A). Values are means ± S.D. n = 14. * Greater than rest, $P < 0.05$. Scatter plot of the percentage change in 3-O-MFPase activity from rest to fatigue induced by repeated isokinetic single-leg knee extensions, against (B) the percentage change in α_1 mRNA expression from rest to fatigue (n = 11, r = -0.68, $P < 0.05$, y = -14.7x + 19.6) and (C) the percentage change in average post-exercise α_1 mRNA expression relative to rest (n = 11, r = -0.56, $P < 0.10$, y = -12.7x + 57.1). Dotted curves indicate 95% confidence intervals, dashed line indicates mRNA expression at rest.

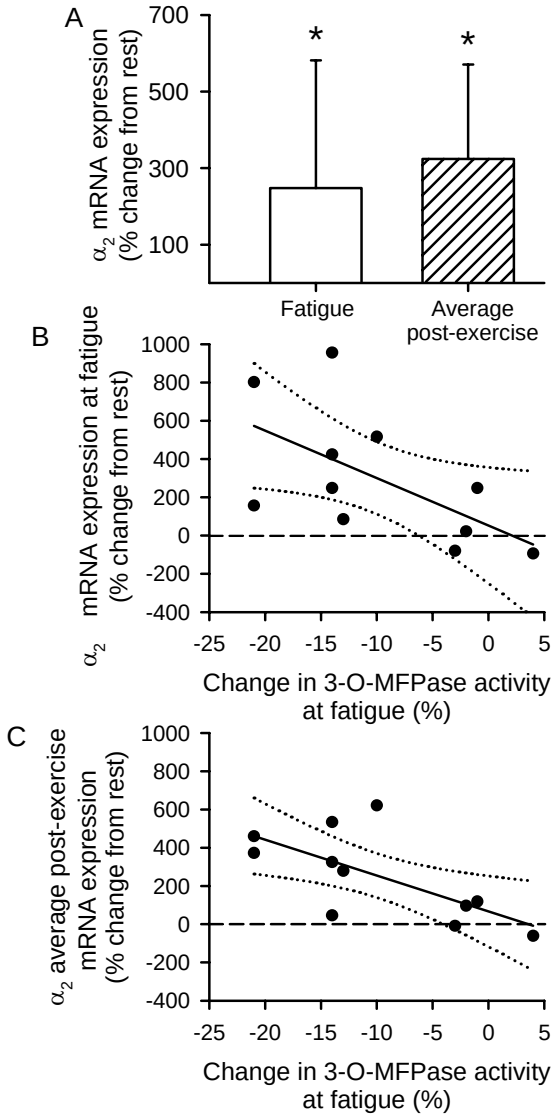

Figure 3.4. Na^+,K^+-ATPase α_2 isoform mRNA expression and relationship with 3-O-MFPase activity.

Na^+,K^+-ATPase α_2 isoform mRNA at fatigue induced by repeated isokinetic single-leg knee extensions, and the average post-exercise α_2 mRNA expression (average of fatigue, 3 h, and 24 h post-exercise) expressed as percentage-change relative to rest (A). Values are means ± S.D. n = 14. * Greater than rest, $P < 0.05$. Scatter plot of the percentage change in 3-O-MFPase activity from rest to fatigue induced by repeated isokinetic single-leg knee extensions, against (B) the percentage change in α_2 mRNA expression from rest to fatigue (n = 11, r = -0.60, P = 0.05, y = -24.8x + 52.7), and (C) the percentage change in average post-exercise α_2 mRNA expression relative to rest (n = 11, r = -0.60, $P < 0.05$, y = -18.8x + 66.8). Dotted curves indicate 95% confidence intervals; dashed line indicates mRNA expression at rest.

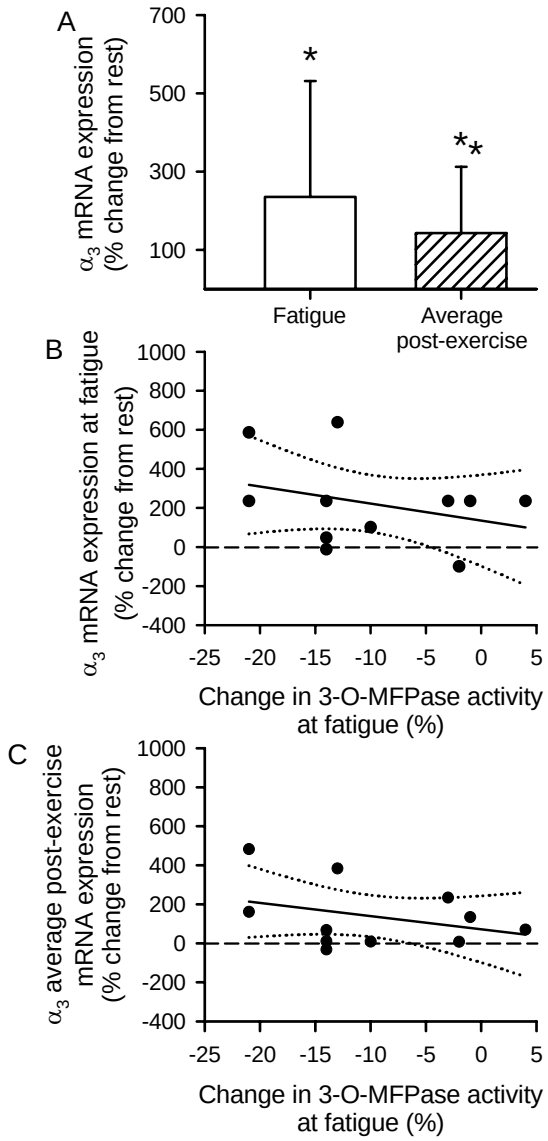

Figure 3.5. Na^+,K^+-ATPase α_3 isoform mRNA expression and relationship with 3-O-MFPase activity.

Na^+,K^+-ATPase α_3 isoform mRNA at fatigue induced by repeated isokinetic single-leg knee extensions, and the average post-exercise α_3 mRNA expression (average of fatigue, 3 h, and 24 h post-exercise) expressed as percentage-change relative to rest (A). Values are means ± S.D. $n = 14$. * Greater than rest, $P < 0.05$. Scatter plot of the percentage change in 3-O-MFPase activity from rest to fatigue induced by repeated isokinetic single-leg knee extensions, against (B) the percentage change in α_3 mRNA expression from rest to fatigue ($n = 11$, $r = -0.32$, $P < 0.40$, $y = -8.7x + 135.2$) and (C) the percentage change in average post-exercise α_3 mRNA expression relative to rest ($n = 11$, $r = -0.34$, $P < 0.40$, $y = -6.8x + 71.9$). Dotted curves indicate 95% confidence intervals, dashed line indicates mRNA expression at rest.

3.3.4.3 α_3 isoform

Exercise elevated α_3 mRNA expression at fatigue by 2.4-fold ($P < 0.05$, Figure 3.5). However, in contrast to both α_1 and α_2, the percentage change in α_3 mRNA expression at fatigue was not significantly correlated with the %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ ($r = -0.32$, $P < 0.40$, Figure 3.5). The average post-exercise α_3 mRNA was also elevated by 1.1-fold ($P < 0.05$, Figure 3.5). The percentage change in average post-exercise α_3 mRNA also showed no significant correlation with the %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ ($r = -0.34$, $P < 0.40$, Figure 3.5).

3.3.4.4 β_1, β_2, and β_3 isoforms

Exercise had no significant effect on β_1 or β_3 mRNA at fatigue, but the average post-exercise mRNA for β_1 and β_3 were elevated by 1.1- and 1.0-fold ($P < 0.05$), respectively. The β_2 isoform mRNA expression was elevated by 1.7-fold at fatigue ($P < 0.05$), and the average β_2 post-exercise mRNA was doubled ($P < 0.05$). There were no significant correlations for any of the three β subunit isoforms between the %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ and either the percentage change from rest to fatigue (β_1: $r = -0.22$, N.S.; β_2: $r = -0.08$, N.S.; β_3: $r = -0.33$, N.S.) or the percentage change in average post-exercise mRNA expression (β_1: $r = -0.44$, N.S.; β_2: $r = -0.24$, N.S.; β_3: $r = -0.38$, N.S.).

3.4 DISCUSSION

There are three major findings of this study. First, brief, exhaustive muscle contractions only transiently depressed skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity, as measured by maximal K^+-stimulated 3-O-MFPase activity, with no significant difference in 3-O-MFPase activity evident from rest after 3 h and until

24 h recovery. Second, exhaustive muscle contractions did not modify muscle Na^+,K^+-ATPase content, as measured by [^{3}H]ouabain binding site content, either immediately, or for up to 24 h after exercise. Third, and our most novel findings, significant inverse relationships were found between the percentage change in 3-O-MFPase activity from rest to fatigue (%Δ3-O-MFPase$_{rest\text{-}fatigue}$) and the percentage increase in mRNA expression at fatigue, for both the α_1 and α_2 Na^+,K^+-ATPase isoforms, and for the percentage increase in the average post-exercise α_2 mRNA expression. These findings suggest that this reversible depression in muscle 3-O-MFPase activity with fatiguing exercise, that is independent of muscle [^{3}H]ouabain binding site content, may exert a modulatory role in muscle Na^+,K^+-ATPase gene expression.

3.4.1 Reversible depression in muscle maximal in-vitro Na^+,K^+-ATPase activity with exercise.

The 3-O-MFPase activity measurements were performed on crude muscle homogenates with no membrane purification, to ensure maximal Na^+,K^+-ATPase recovery (Hansen *et al.* 1988). This assay is the preferred method for measuring Na^+, K^+-ATPase activity in small muscle biopsy samples due to its high sensitivity and specificity for Na^+, K^+-ATPase (Fraser *et al.* 1998). Maximal in-vitro K^+-stimulated 3-O-MFPase activity was reduced by ~11 % immediately after fatiguing exercise, consistent with several previous studies (Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Leppik *et al.* 2004, Sandiford *et al.* 2004). We unequivocally demonstrate that this depression in human muscle was transient, with activity not significantly different from rest at 3 or 24 h after the exercise bout. Thus our work clarifies a previous finding which also suggested an early post-exercise recovery, but

which had internally inconsistent results and was thus inconclusive (Fowles *et al.* 2002b).

The transient nature of this Na^+,K^+-ATPase impairment following exercise suggests that this phenomenon reflects enzyme inactivation as part of an exercise-induced process and/or some other cellular regulatory process, rather than being due to muscle damage. That this depression in Na^+,K^+-ATPase activity is an obligatory response to exercise is further suggested, by having been demonstrated across a wide range of exercise intensities and durations (Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Leppik *et al.* 2004, Sandiford *et al.* 2004). During prolonged cycling, muscle 3-O-MFPase activity was progressively decreased with increased exercise time to fatigue (Leppik *et al.* 2004), suggesting that the decrease in 3-O-MFPase activity was due to progressive exercise-induced changes in the cellular milieu. Previous studies have speculated that increased intracellular $[Ca^{2+}]$, or free radical damage are the most likely mechanisms for the reduced maximal in-vitro 3-O-MFPase activity (Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Sandiford *et al.* 2004). Whilst the exact mechanisms remain to be determined, they appear to be rapidly reversible, evidenced by recovery of maximal in-vitro 3-O-MFPase activity within 3 h post-exercise.

It has been suggested that depressed maximal in-vitro Na^+,K^+-ATPase activity may contribute to fatigue by contributing to reduced transcellular Na^+ and K^+ gradients and membrane inexcitability (Aughey *et al.* 2005, Leppik *et al.* 2004).

There is considerable evidence that intense muscle contractions are accompanied by muscle K^+ loss, elevated interstitial K^+ concentration, membrane depolarisation of between 10-20 mV and reduced M-wave area (see reviews by Sejersted and Sjøgaard (Sejersted *et al.* 2000), Clausen (Clausen 2003) and Nielsen (Nielsen *et al.* 2000)).

However, at this time there is as yet no direct evidence that depressed maximal Na^+,K^+-ATPase activity exacerbates these ionic changes.

Data on the effects of decreased Na^+ and K^+ gradients on muscle excitability in humans is limited. Calculations of membrane potential in exercising humans suggest a depolarisation of 14 mV at fatigue (Sjøgaard *et al.* 1985), whereas animal studies have shown both hyperpolarisation (Hicks *et al.* 1989) and depolarisation (Balog *et al.* 1996) following exercise. Measurements of the compound muscle action potential (M-wave) in humans have shown either decreased (Behm *et al.* 1997, Fowles *et al.* 2002b, Fuglevand *et al.* 1993, Lepers *et al.* 2002), unchanged (Millet *et al.* 2002, Sandiford *et al.* 2004), or increased (Behm *et al.* 1997) area or amplitude following exercise. Therefore, whether membrane inexcitability occurs during exercise in humans is equivocal, and it is not yet possible to determine whether a decrease in 3-O-MFPase activity during exercise affects excitability.

These in-vitro measures of depressed maximal Na^+,K^+-ATPase activity most likely do not reflect in-vivo changes. Firstly, in-vivo Na^+,K^+-ATPase activity is highly likely to be substantially elevated with exercise, based on the 18-22 fold increase in Na^+,K^+-ATPase activity that occurs with electrical stimulation in isolated rat muscles (Clausen 2003, McKenna *et al.* 2003b, Nielsen *et al.* 1997, 2000); the rapid post-exercise reversal of arterio-venous K^+ differences across exercising muscles; and the rapid post-exercise decline in $[K^+]$ observed here. These all provide evidence of increased in-vivo Na^+,K^+-ATPase activity in human muscles. Second, these findings indicate that the maximal attainable Na^+,K^+-ATPase activity is decreased following exercise, whilst evidence based on ion selective electrodes and arterio-venous K^+ differences suggest that the in-vivo Na^+,K^+-ATPase activity in human muscles is considerably below the levels achieved in isolated rat muscles (see Sejersted and

Sjøgaard (Sejersted *et al.* 2000) and McKenna (McKenna 1998)). The in-vivo pump activity may also be adversely affected during exercise due to any localised decline in glycogen, phosphocreatine and ATP, but this has not yet been demonstrated. Further studies with more sophisticated techniques are required to determine the exact relationship between depressed maximal in-vitro 3-O-MFPase activity, in-vivo Na^+/K^+ exchange in human muscles with exercise.

The relatively small depression in Na^+,K^+-ATPase activity found in this study (11%), may cast doubt on the functional significance of these findings. Interestingly, the percentage decline in Na^+,K^+-ATPase activity at fatigue is similar to the percentage gain in total Na^+,K^+-ATPase content ([^{3}H]ouabain binding) with intense exercise training in humans and the percentage decline with inactivity (for review see (Clausen 2003)). It is apparent that these interventions therefore produce only relatively small changes in Na^+,K^+-ATPase in human muscles. Thus the 11% decline in maximal activity with fatigue here might also be expected to have important implications for the muscle. It is also unknown whether this depression reflects a similar decline in Na^+,K^+-ATPase activity in all muscle fibres, or a more marked depression in Na^+,K^+-ATPase activity in some fibres.

It is not possible to state that phosphatase activity measured enzymatically directly reflects functional pump activity. Nonetheless, the 3-O-MFPase assay is specific for Na^+,K^+-ATPase, as shown by ouabain inhibition (Fraser *et al.* 1998) and correlates with [^{3}H]ouabain binding site content (Fraser *et al.* 2002). The 3-O-MFPase assay is the preferred method for measuring Na^+, K^+-ATPase activity in small muscle biopsy samples, due to its high specificity, sensitivity, as well as an inability to utilise more traditional assays monitoring ATP hydrolysis rate in muscle (Fraser *et al.* 1998).

3.4.2 Lack of effect of exercise on muscle [^{3}H]ouabain binding site content.

Our [^{3}H]ouabain binding measurements were conducted on cut muscle pieces in the presence of vanadate, thus enabling full quantification of muscle Na^+,K^+-ATPase content (Clausen *et al.* 1974, McKenna *et al.* 2003b). Since [^{3}H]ouabain binding measurements did not differ when measured in either muscle pieces or intact muscles, it was suggested that all functional Na^+,K^+-ATPase units in muscle are measured with this technique (Clausen *et al.* 1974, McKenna *et al.* 2003b). We show that fatiguing exercise lasting ~6 min did not affect muscle [^{3}H]ouabain binding site content, either immediately, or for up to 24 h post-exercise. The lack of change in [^{3}H]ouabain binding with fatigue confirms previous findings following acute dynamic or isometric exercise in humans (Aughey *et al.* 2005, Fowles *et al.* 2002b), or 10 s to 240 min electrical stimulation of isolated rat muscles (McKenna *et al.* 2003b). Conversely, an increase in [^{3}H]ouabain binding site content was observed following a 100 km run of ~641 min duration (Overgaard *et al.* 2002), most likely due to Na^+,K^+-ATPase synthesis during exercise. The lack of change for up to 24 h post-exercise extends earlier findings (Aughey *et al.* 2005, Fowles *et al.* 2002b, Leppik *et al.* 2004, McKenna *et al.* 2003b), as these failed to follow changes for 24 h after exercise and may therefore have missed any post-exercise upregulation. It should be sufficient to detect increased Na^+,K^+-ATPase synthesis within 24 h, as this has a half-time of 18 h (Wolitzky *et al.* 1986). Since [^{3}H]ouabain selectively binds to Na^+,K^+-ATPase in both the sarcolemma and t-tubular system (Clausen *et al.* 1974), these previous findings argue against a translocatable intracellular Na^+,K^+-ATPase pool (McKenna *et al.* 2003b). Therefore our findings of unchanged [^{3}H]ouabain binding with ~6 min exercise are inconsistent with an apparent translocation of Na^+,K^+-ATPase α_2 and β_1

isoforms from undefined intracellular stores to the sarcolemma with exercise (Juel *et al.* 2000a).

3.4.3 Correlation between depressed Na^+,K^+-ATPase activity and α isoform gene expression.

The most novel finding of this study is the significant inverse relationship found between the percentage change in maximal in-vitro 3-O-MFPase activity from rest to fatigue (%Δ 3-O-MFPase$_{rest\text{-}fatigue}$) and the percentage change from rest to fatigue for each of α_1 and α_2 mRNA expression. This finding is reinforced by the significant inverse correlation between the %Δ 3-O-MFPase$_{rest\text{-}fatigue}$ and the average post-exercise percentage-change in both α_2 and α_1 mRNA expression. This is the first time such a relationship has been demonstrated in contracting skeletal muscle. This suggests a possible modulatory role of depressed Na^+,K^+-ATPase activity in muscle Na^+,K^+-ATPase gene expression. This is consistent with findings in rat liver cells, in which reduction of Na^+,K^+-ATPase activity by incubation in low [K^+] medium for 6 h, resulted in a 60% increase in Na^+,K^+-ATPase α and β subunit mRNA expression (Pressley *et al.* 1988). These findings strongly suggest that acutely depressed maximal Na^+,K^+-ATPase activity might be one factor underlying the chronic exercise-induced upregulation of Na^+,K^+-ATPase content in skeletal muscle, as shown with exercise training (Green *et al.* 1993, McKenna *et al.* 1993). However, this study was unable to unequivocally demonstrate this link here, as no upregulation of [^{3}H]ouabain binding site content was observed within 24 h after this short exercise bout. This laboratory has previously reported that the Na^+,K^+-ATPase isoform protein expression was also unchanged (Murphy *et al.* 2004). Further studies with either very prolonged exercise (Overgaard *et al.* 2002) or chronic training (Green *et al.* 1993, McKenna *et al.* 1993) are required to verify this possibility.

Three possible mechanisms have been identified that may link decreased Na^+,K^+-ATPase activity to increased Na^+,K^+-ATPase gene transcription. The first is via ion concentration-dependent regulation, whereby decreased maximal Na^+,K^+-ATPase activity allows greater Na^+ and K^+ net fluxes across the cell membrane and, consequently, increased muscle intracellular [Na^+] and extracellular [K^+]. These ionic disturbances may stimulate synthesis of new Na^+,K^+-ATPase enzymes in order to re-establish favourable Na^+ and K^+ gradients, as evidenced by a 44% increase in rat gastrocnemius muscle [^{3}H]ouabain binding site content following seven days of diet-induced hyperkalaemia (Bundgaard *et al.* 1997a). Also, activation of voltage-sensitive Na^+ channels with veratridine increased sarcolemmal Na^+,K^+-ATPase by 60% in cultured chick skeletal muscle (Wolitzky *et al.* 1986). However, the exercise-induced elevations in muscle intracellular [Na^+] and extracellular [K^+] may be too brief to stimulate gene transcription. The second mechanism is via increased cytosolic Ca^{2+} concentrations. Tetanic and resting cytosolic [Ca^{2+}] increase during fatiguing muscle contractions (Westerblad *et al.* 1991). Incubation of mouse diaphragm in 10 mM Ca^{2+} completely and reversibly inhibits Na^+,K^+-ATPase activity (Sulova *et al.* 1998). Ca^{2+} has also been established as a regulator of gene transcription, often acting via the Ca^{2+} receptor calmodulin and Ca^{2+}/calmodulin-dependant protein kinases (Corcoran *et al.* 2001), although this signalling pathway has not yet been linked with Na^+,K^+-ATPase gene transcription. The third proposed mechanism is via decreased-Na^+,K^+-ATPase-activity-induced, direct protein-protein interaction of the Na^+,K^+-ATPase and it's neighbouring proteins, which triggers a signalling cascade, culminating in increased Na^+,K^+-ATPase gene transcription (Xie *et al.* 2002). This latter mechanism may be the most likely, as it has been shown that the gene regulation associated with Na^+,K^+-ATPase

inhibition is independent of changes in intracellular Na^+, K^+ and Ca^{2+} (Liu *et al.* 2000). It cannot be ruled out that the relationships between maximal in-vitro 3-O-MFPase activity and mRNA observed in this study were merely coincidental. It is possible that the factors responsible for depressing maximal in-vitro 3-O-MFPase activity are also involved in the regulation of Na^+,K^+-ATPase mRNA expression. Further studies are required to determine the factors responsible for depression of maximal in-vitro 3-O-MFPase activity and up-regulation of Na^+,K^+-ATPase mRNA expression with exercise.

3.4.4 Isoform-specific relationships

An important finding was that only changes in the α_1 and α_2 isoforms mRNA were related to the change in 3-O-MFPase activity with fatigue. The catalytic α subunit contains the substrate (Na^+, K^+, Mg^{2+}, and ATP) and inhibitor (ouabain) binding sites (Ewart *et al.* 1995, Hansen 1984, Lingrel 1992). The mRNA of the α_1 and α_2 isoforms are the most abundantly expressed of the α isoforms in skeletal muscle (Orlowski *et al.* 1988), consistent with the dominant protein abundance of the α_1 and α_2 isoforms (Hansen 2001, Sweadner 1989). Hence, the α_1 and α_2 isoforms probably play the most important role of the α subunit isoforms in skeletal muscle. Thus, their regulation is presumably the most tightly controlled and sensitive to possible regulatory stimuli, such as ionic concentration changes caused by decreased maximal Na^+,K^+-ATPase activity. The Na^+ affinity of the α_3 isoform in humans is 30- and 10-fold lower than α_1 and α_2, respectively (Crambert *et al.* 2000) and the rat α_3 isoform is three-times less sensitive to Na^+ activation than the α_1 and α_2 isoforms (Munzer *et al.* 1994). The lower sensitivity of α_3 to Na^+ may therefore make α_3 less sensitive to exercise-induced changes in $[Na^+]_i$ and explain the lack of a relationship between 3-O-MFPase activity and mRNA expression for this isoform. There was also no

relationship between the change in 3-O-MFPase activity and the change in mRNA expression for any of the β subunit isoforms, which are needed for the correct configuration, maturation, transport, and K^+ kinetics of the Na^+,K^+-ATPase enzyme (Ackermann *et al.* 1990, Crambert *et al.* 2000, Noguchi *et al.* 1990). This may be related to an over-abundance of β compared to α subunits (Lavoie *et al.* 1997), suggesting that there would always be adequate β subunits available to form new functional αβ complexes. Finally, several studies have found that the α_1 and α_2 isoform abundance in plasma membranes were elevated in both oxidative and glycolytic muscle fibres following treadmill running in rats (Juel *et al.* 2001, Tsakiridis *et al.* 1996).

3.5 CONCLUSIONS

This study demonstrates that acute short-duration exercise depressed maximal in-vitro 3-O-MFPase (Na^+,K^+-ATPase) activity in skeletal muscle, which recovered within 3 h post-exercise. The decrease in maximal in-vitro 3-O-MFPase activity was not due to a decline in [^{3}H]ouabain binding site (Na^+,K^+-ATPase) content, and brief exercise did not upregulate Na^+,K^+-ATPase content over the following 24 h, as [^{3}H]ouabain binding site content was not changed after exercise. There were significant inverse relationships between the decline in 3-O-MFPase activity from rest to fatigue and the increase in α_1 and α_2 mRNA expression at fatigue, as well as when averaged over 0, 3 and 24 h post-exercise. This suggests a possible signal transduction role of depressed Na^+,K^+-ATPase activity in the exercise-induced upregulation of Na^+,K^+-ATPase in skeletal muscle.

CHAPTER 4. IMPAIRED EXTRARENAL POTASSIUM REGULATION AND EXERCISE PERFORMANCE ARE IMPROVED WITH ENDURANCE TRAINING IN PATIENTS ON HAEMODIALYSIS.

4.1 INTRODUCTION

Haemodialysis patients (HD) display abnormally low exercise performance, with peak oxygen consumption ($\dot{V}O_{2peak}$) during incremental exercise and muscle strength each reduced by up to 50% compared to healthy controls (CON) (Bonzel *et al.* 1991, Kettner Melsheimer *et al.* 1987, Painter *et al.* 1986a, Sangkabutra *et al.* 2003). HD show an exaggerated rise in arterial [K^+] during incremental exercise, which was inversely correlated with $\dot{V}O_{2peak}$ (Sangkabutra *et al.* 2003). Resting muscle membrane potential was reduced in patients with uraemia or chronic renal disease (Cunningham *et al.* 1971) and a reduced muscle compound action potential was also observed in HD at rest, which persisted throughout exercise (Johansen *et al.* 2005). Together, these findings suggest that reduced muscle membrane excitability, caused by impaired muscle K^+ regulation, may contribute to the poor exercise performance in HD. Few studies have compared extrarenal K^+ regulation during exercise between HD and CON and in these studies the patients have all had severe anaemia (Clark *et al.* 1996, Huber *et al.* 1985, Sangkabutra *et al.* 2003). A relationship was identified between [Hb] and plasma K^+ regulation during exercise in HD, in whom the exercise-induced hyperkalaemia was ameliorated after treatment with erythropoietin (EPO) (McMahon *et al.* 1999). Furthermore, EPO treatment increased Na^+,K^+-ATPase activity in rat myocardium (Wald *et al.* 1995). Thus, the current clinical practice of maintaining HD at near normal [Hb] with EPO improves the impaired extrarenal K^+ regulation (McMahon *et al.* 1999) previously observed in these patients (Huber *et al.*

1985, Sangkabutra *et al.* 2003). However, whether K^+ regulation is still impaired compared to CON is unknown. This study compared exercise performance ($\dot{V}O_{2peak}$, muscle strength and fatiguability), and plasma [K^+] during incremental exercise between HD with near normal [Hb] and CON. The first hypothesis tested was that HD treated with EPO would have reduced $\dot{V}O_{2peak}$ and muscle strength, with increased muscle fatiguability and plasma [K^+] during exercise compared to CON.

Exercise training improves both exercise capacity (DePaul *et al.* 2002, Goldberg *et al.* 1986, Kouidi *et al.* 1998, Painter *et al.* 2000b) and QOL in HD (for review see (Kouidi 2004)). Improvements in QOL are mainly due to increased physical functioning scores following exercise training (Oh-Park *et al.* 2002, Painter *et al.* 2000b, Painter *et al.* 2002a). The mechanisms underlying improved exercise capacity remain unresolved but may include improvements in [Hb] (Fitts *et al.* 1999, Goldberg *et al.* 1986, Goldberg *et al.* 1980a, Painter *et al.* 2002a), muscle fibre type, capillary size and mitochondria number (Kouidi *et al.* 1998), and increased muscle fibre area (Castaneda *et al.* 2001, Kouidi *et al.* 1998) following training. Exercise training lowers the rise in plasma [K^+] during exercise in healthy subjects (Green *et al.* 1993, Harmer *et al.* 2006, McKenna *et al.* 1996, McKenna *et al.* 1997) and in patients with chronic heart failure (Barlow *et al.* 1994). If K^+ regulation is also improved in HD patients following training this may contribute to their improved exercise capacity. Therefore, this study also measured the effects of stationary cycle training on exercise performance ($\dot{V}O_{2peak}$, muscle strength and fatiguability), plasma [K^+] during incremental exercise, and quality of life in HD. It was hypothesised that these variables would be improved with training.

4.2 METHODS

4.2.1 Study design

HD patients underwent initial testing at Baseline, after six weeks of normal activity (Pre-Train), and following six weeks of stationary cycle ergometer training (Post-Train). Four tests were completed at each time point. These comprised a maximal incremental exercise test to determine $\dot{V}O_{2peak}$ and plasma [K^+] changes; knee-extensor torque-velocity and knee-extensor fatiguability tests; and a quality of life assessment. All tests were performed on a non-dialysis day and at the same time post-dialysis for each subject (Baseline 22.0 ± 8.9 h, Pre-Train 20.3 ± 9.3 h, Post-Train 20.8 ± 8.0 h, mean ± SD). The final tests were conducted within 3 days (range 1-3, 1.3 ± 0.8 d) of completing the cycle training. A group of sedentary healthy control subjects, matched for age, sex, body mass, and height (CON), also performed all Baseline tests except the quality of life assessment.

4.2.2 Subjects

Eight HD and six CON gave written informed consent and agreed to participate in the study. Sample size was calculated using Power and Precision software based on the expected plasma K^+ values. The expected means for the sub-maximal plasma K^+ during the incremental exercise test are expected to decrease 9% after the training programme (Green *et al.* 1993). Therefore, the plasma K^+ means at the same workload are expected to be 6.50, 6.50, and 5.91 ± 0.38 $mmol.L^{-1}$, at baseline, following 6 weeks normal activity, and 6 weeks cycle training respectively (Green *et al.* 1993, Sangkabutra *et al.* 2003). Comparison of the expected means by ANOVA, α =0.05, a sample size of n = 8 per group yielded a power of 86%. Six HD (five male, one female) and six CON completed the study. Two HD patients were excluded, one due to an abnormal ECG during the Baseline $\dot{V}O_{2peak}$ test and another as illness

prevented him from training. HD patients were stable, had been dialysing for at least 12 mo prior to testing (range 13 – 85, mean 53 ± 25 mo), and had [Hb] greater than 110 $g.l^{-1}$. HD were excluded if they were generally unwell, had symptomatic ischaemic heart disease, peripheral vascular disease, disabling arthritis, chronic airflow obstruction, or were pregnant. Causes of renal failure were reflux nephropathy (n = 3), IgA glomerulonephritis (n = 1), diabetic nephropathy (n = 1) and vasculitis (n = 1). Patients underwent testing and training while taking their normal medication, which included epoetin (n = 6), the β-blocker metoprolol (n = 1), angiotensin II receptor antagonists (n = 1), Ca^{2+} channel blockers (n = 2), and prednisolone (n = 2). This study was approved by the Human Research Ethics Committee at Victoria University, and the Clinical Research and Ethics Committee of the Northwest Healthcare Network, Melbourne.

4.2.3 Exercise tests

4.2.3.1 Aerobic power ($\dot{V}O_{2peak}$)

Subjects cycled on an electrically braked cycle ergometer (Lode, Groningen, Holland) at an initial power output of 15 W with progressive increments of 15 W each min. Subjects pedalled at or above 60 rpm and the test was continued until volitional fatigue, defined as an inability to maintain cadence above 60 rpm. Expired O_2 and CO_2 were continuously measured as described elsewhere (Leikis *et al.* 2006, Li *et al.* 2002).

4.2.3.2 Knee-extensor torque-velocity test

Subjects performed three maximal isokinetic contractions at each of 0, 60, 120, 180, 240, 300, and $360°.s^{-1}$, with 60 s recovery between sets on an isokinetic dynamometer (Cybex Norm 770, Henley HealthCare, Massachusetts), as previously described (Leikis *et al.* 2006). The highest value of each set of three was chosen as peak torque

(PT). Subjects first performed a familiarisation trial, then two trials to determine within-subject variability of the torque-velocity test. These tests were performed at least one week prior to the experimental trials. The within-subject coefficient of variation (CV) for the peak isometric torque during the variability trials was 2.6 ± 1.2 %.

4.2.3.3 Knee extensor fatigue test

Subjects performed 30 maximal isokinetic contractions at $180°.s^{-1}$, with ~1 s pause between repetitions (Leikis *et al.* 2006). The fatigue index was calculated as the percent decline in torque: fatigue index (%) = [(starting torque - final torque)/ starting torque] x 100, where starting torque = the average of the highest three of the first five repetitions; final torque = the average of the highest three of the last five repetitions. Subjects also performed familiarisation and variability trials of the fatigue test at least one week prior to the experimental trials (CV = 4.4 ± 2.5 %).

4.2.3.4 Blood sampling and processing

Blood was sampled before, during and for 10 min after the aerobic power test. In all HD, blood was sampled from the arterio-venous fistula via an indwelling catheter. In CON, arterialised venous blood was sampled from a dorsal hand-vein as described in Chapter 3. The blood sample was transferred to a tube containing lithium heparin. One ml of blood was analysed in duplicate for [Hb] and Hct (Sysmex, K-800, Kobe, Japan) and another 1.5 ml was centrifuged at 4,500 rpm for 2 min with the plasma removed and frozen until later analysis of plasma $[K^+]$. Plasma $[K^+]$ was analysed using an ion selective electrode (865pH/Blood Gas Analyser, Bayer, Medfield, MA, USA) and was corrected for changes in plasma volume, calculated from changes in [Hb] and Hct (Harrison 1985). The rise in plasma $[K^+]$ above rest during the aerobic power test ($\Delta[K^+]$) was expressed relative to the total work performed ($\Delta[K^+].work^{-1}$

ratio) as previously described (McKenna *et al.* 1993). The possible effect of medication on plasma [K^+] was also considered. Non-selective β-blockers increase plasma [K^+] during exercise (Hallén *et al.* 1994), however only β_1-blockers were taken by HD in this study and these do not affect plasma [K^+] during exercise (Gullestad *et al.* 1991). Prednisone increases skeletal muscle Na^+,K^+-ATPase content in humans (Ravn *et al.* 1997), which would likely lower plasma [K^+] during exercise. However, the plasma [K^+] of the patients taking prednisone was within the range of the other patients, therefore these results were not excluded.

4.2.4 Quality of life questionnaire

The Medical Outcomes Study Short Form Health Survey 36-item questionnaire (SF-36) was used to assess changes in health related quality of life in HD patients. The SF-36 includes one multi-item scale that assesses 1) limitations in physical activities because of health problems (physical functioning); 2) limitations in social activities because of physical or emotional problems (social functioning); 3) limitations in usual role activities because of physical health problems (role physical); 4) bodily pain; 5) general mental health; 6) limitations in usual role activities because of emotional problems (role emotional); 7) energy and fatigue (vitality); and 8) general health perceptions. Possible scores range from 0 – 100, with a higher score indicating a better state of health and wellbeing. Composite normalised scores are calculated from the individual scales and represent overall physical functioning (physical component scale, PCS) and overall mental functioning (mental component scale, MCS). The Baseline SF-36 scores were also compared with the Australian population norms (Australian_Bureau_of_Statistics 1997).

4.2.5 Exercise training

HD trained by pedalling a stationary cycle ergometer (Monark 868, Vansbro, Sweden) for 30 min during the first h of their haemodialysis treatment, three times per week for 6 weeks. Each training session comprised a 5 min warm-up at 25 W, 20 min of cycling at the training workrate, and a 5 min warm-down at 25 W. The training intensity started at a workrate corresponding to 50% of the Pre-Train $\dot{V}O_{2peak}$ and was increased by 10 % of the preceding value each week. Thus the training intensity was 50, 55, 60.5, 66.6, 73.2, and 80.5% of Pre-Train $\dot{V}O_{2peak}$ for weeks 1-6, respectively

This training intensity has previously been shown to increase $\dot{V}O_{2peak}$ in HD (Levendoglu *et al.* 2004, Storer *et al.* 2005).

4.2.6 Statistics

Data are presented as mean ± SD. A one-way ANOVA was used to detect differences between CON and HD at Baseline, Pre-Train, and Post-Train, except for $[K^+]$ data, for which a two-way ANOVA (group/training status, sample time) was used. The Least Significant Difference test was used for post-hoc comparisons. An unpaired t-test was used to compare Baseline QOL scores to Australian population norms and a paired t-test was used to compare initial and final training workrate. Correlations were determined by least squares linear regression**.** Statistical significance was accepted at $P < 0.05$. Effect size (ES, partial eta squared) was determined for ANOVA to determine the magnitude of effects. The coefficient of variation (CV) between Baseline and Pre-Train was calculated to determine the variability of the exercise tests.

4.3 RESULTS

4.3.1 Subject physical characteristics

Age, body mass, height, BMI and Hct did not differ between HD and CON, however [Hb] was 13-15% greater in CON (Table 4.1). HD physical characteristics did not change during the course of the study (Table 4.1).

4.3.2 Training workrate

The mean training workrate increased by 65% ($P < 0.005$) from the first (43 ± 11 W) to the last (71 ± 22 W) training session.

Table 4.1. Subject physical characteristics.

Group	Test	Age (years)	Body mass (kg)	Height (cm)	BMI	[Hb] $g.l^{-1}$	Hct (%)
CON	Baseline	40 ± 9	73.6 ± 16.8	175 ± 9	24.0± 4.4	148 ± 13	41.9 ± 3.2
HD	Baseline	42 ± 12	68.9 ± 7.1	171 ± 8	23.6 ± 2.8	129 ± 8*	37.9 ± 3.1
	Pre-Train		69.3 ± 7.1		23.3 ± 3.1	127 ± 12*	37.5 ± 4.2
	Post-Train		69.5 ± 7.3		23.4 ± 3.1	131 ± 7*	37.8 ± 1.4

Values are mean ± SD; BMI, body mass index; [Hb], haemoglobin concentration; Hct, haematocrit; $n = 6$ per group; * less than CON, ($P < 0.05$).

4.3.3 Exercise tests

4.3.3.1 Stability from Baseline to Pre-Train

The variability from Baseline to Pre-Train for HD was 9 ± 3% for $\dot{V}O_{2peak}$, 5 ± 4% for time to fatigue, and 9 ± 8% for work done during incremental exercise. The variability was 5 ± 4% for knee-extensor isometric PT and 16 ± 28% for fatiguability. The poor CV for fatiguability was due to one patient who had very high variability (72%).

When his variability data were excluded, the mean CV for fatiguability became 5 ± 5%.

4.3.3.2 Knee-extensor torque-velocity

Peak torque was depressed in HD compared to CON, at all contraction velocities, at each of Baseline, Pre-Train and Post-Train (Figure 4.1). When expressed relative to body mass, PT was still depressed in HD at each of Baseline, Pre-Train and Post-Train, at all contraction velocities except $0^{o}.s^{-1}$ (Figure 4.1). In HD, PT was not changed after training, except at $180^{o}.s^{-1}$, which was higher at Post-Train compared to Baseline and tended to be higher than Pre-Train for absolute torque ($P < 0.06$, ES = 0.57, power = 0.80) and when expressed relative to body mass ($P < 0.06$, ES = 0.54, power = 0.75).

4.3.3.3 Knee-extensor fatiguability

The fatigue index during repeated maximal contractions was 23 ± 9, 25 ± 5 and 24 ± 11 %, respectively in HD at Baseline, Pre-Train and Post-Train, which tended to be higher than in CON (13 ± 3%, $P < 0.10$, ES = 0.294, power = 0.551, main effect). The fatigue index was not changed after training in HD.

4.3.3.4 Aerobic power

Each of $\dot{V}O_{2peak}$, time to fatigue, and total work done during the aerobic power test were lower in HD at Baseline by 37, 46, and 69%, respectively, compared to CON (Table 4.2). Neither time to fatigue nor work performed during the incremental cycle test differed between Baseline and Pre-Train (Table 4.2). Training increased both time to fatigue and total work performed (Table 4.2). $\dot{V}O_{2peak}$ did not change with training.

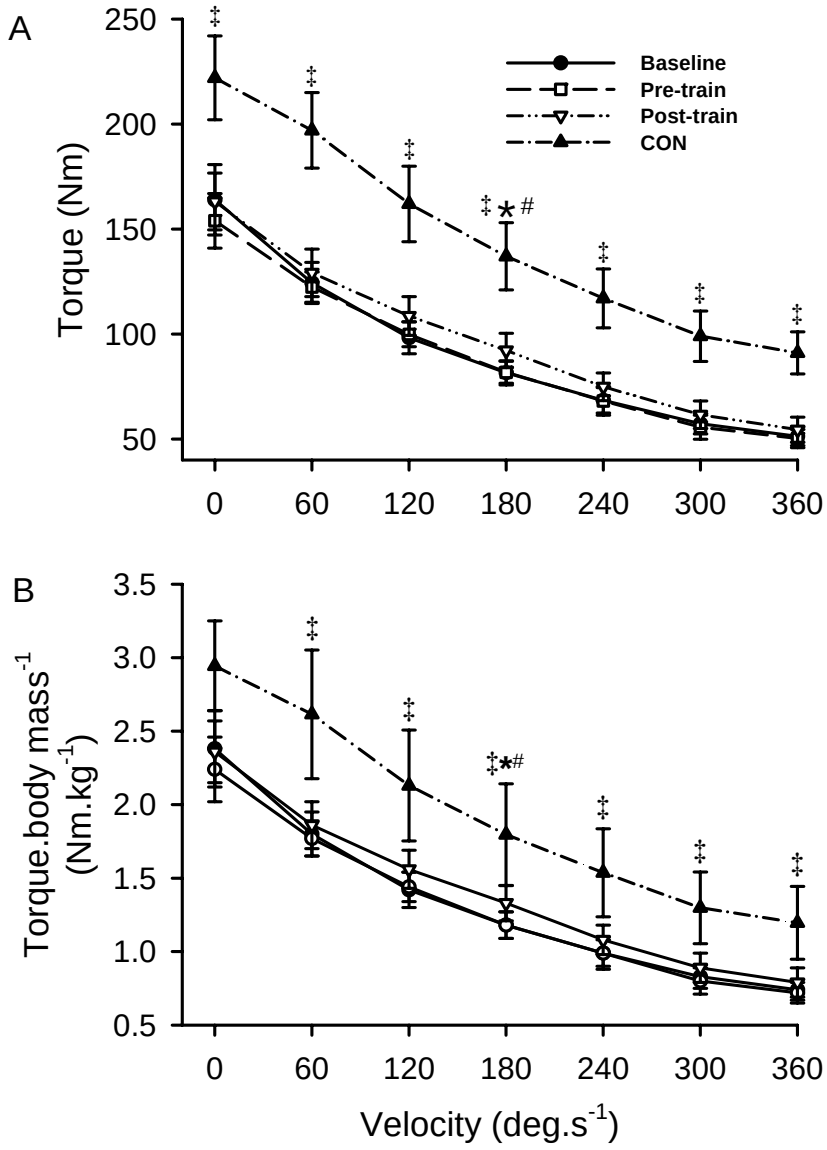

Figure 4.1. Knee-extensor peak torque in CON and before and after training in HD. Knee-extensor peak torque (A) and peak torque expressed relative to body mass (B) in CON and at Baseline, after 6 weeks of normal activity (Pre-Train) and following six weeks of stationary cycle training (Post-Train) in HD. Values are means ± SD. n = 6 per group. ‡ HD < CON at Baseline, Pre-Train and Post-Train ($P < 0.05$); * Baseline < Post-Train ($P < 0.05$); # Pre-Train < Post-Train ($P < 0.10$).

Table 4.2. $\dot{V}O_{2peak}$, time to fatigue and work done during the aerobic power test.

	CON	HD		
	Baseline	Baseline	Pre-Train	Post-Train
Time to fatigue (s)	950 ± 224	511 ± 139*	533 ± 116*	570 ± 123*†‡
Work done (kJ)	125.4 ± 51.6	38.6 ± 20.2*	40.9 ± 17.4*	46.4 ± 18.7*†‡
$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	37.4 ± 4.7	23.5 ± 7.7*	24.7 ± 5.6*	25.3 ± 6.3*

Values are mean ± SD; n = 6 per group; * less than CON, ($P < 0.05$); † > Baseline, ($P < 0.05$); ‡ > Pre-Train ($P < 0.05$).

4.3.4 Plasma [K^+]

Exercise caused a significant increase in plasma [K^+] (main effect, $P < 0.001$,Figure 4.2). Plasma [K^+] before, during and after the aerobic power test was higher in HD at Baseline and Pre-Train compared to CON (Figure 4.2). There was no effect of training on plasma [K^+] ($P < 0.20$, ES = 0.36). After training, plasma [K^+] was not different between HD and CON. The $\Delta[K^+]$ at common submaximal workrates was higher in HD at Baseline than CON, but with no difference at fatigue (Figure 4.2). During exercise, the rate of rise in plasma [K^+], calculated from rest to end of exercise, was greater in HD at Baseline and Pre-Train than CON (Figure 4.3). The $\Delta[K^+].work^{-1}$ ratio was higher in HD than CON at Baseline (2.4-fold, $P < 0.001$), Pre-Train (1.8-fold, $P < 0.005$) and Post-Train (1.3-fold, $P < 0.05$, Figure 4.3). Training decreased the $\Delta[K^+].work^{-1}$ ratio by 31%, which was lower at Post-Train compared to Baseline ($P < 0.05$) and tended to be lower than Pre-Train ($P < 0.10$, ES = 0.51).

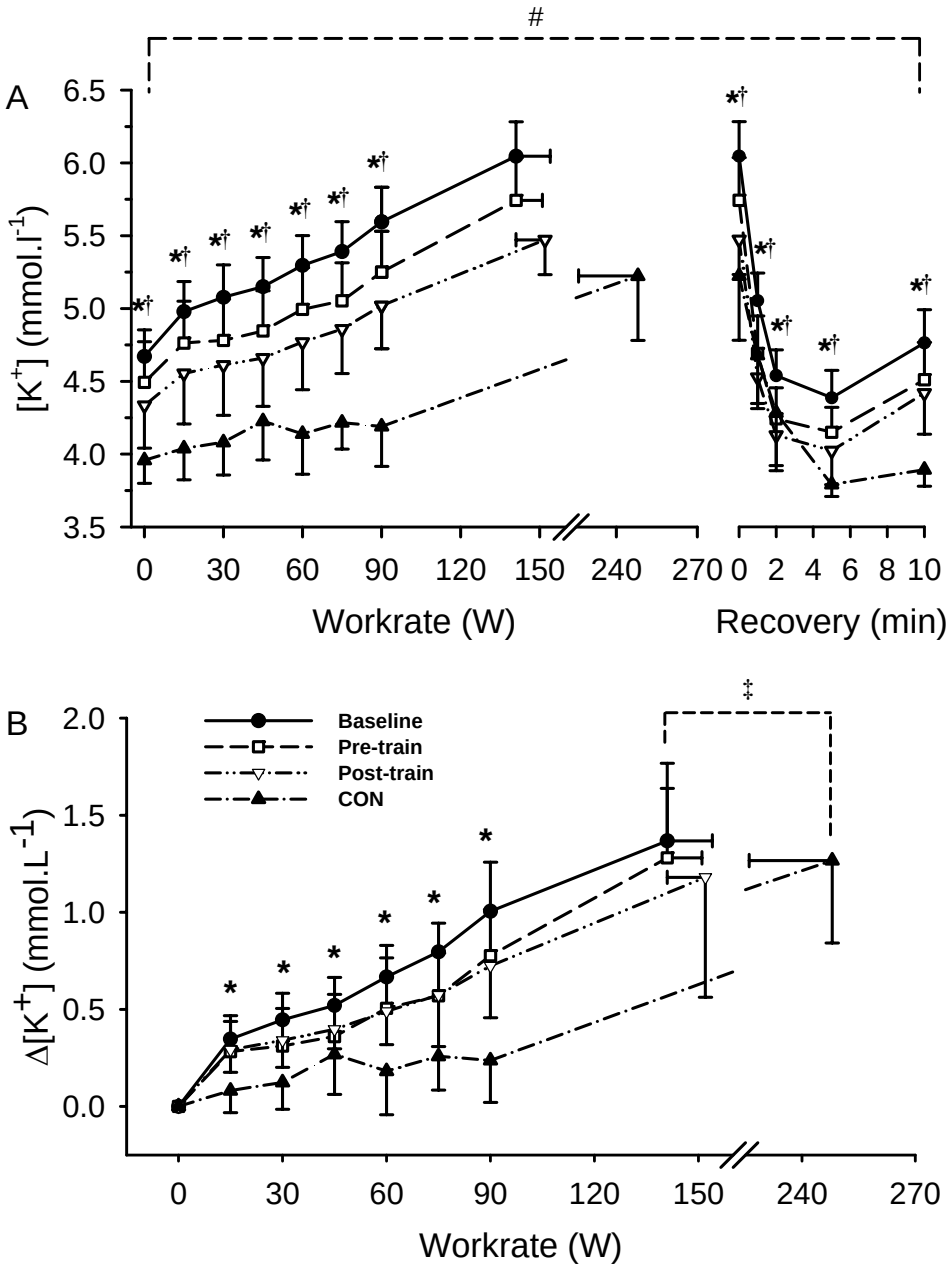

Figure 4.2. Plasma $[K^+]$ and $\Delta[K^+]$ during the aerobic power test.

Plasma $[K^+]$ before, during, and after (A), and the rise in plasma $[K^+]$ from rest during (B) an incremental cycle test to fatigue in CON and in HD at Baseline, after 6 weeks of normal activity (Pre-Train) and following six weeks of stationary cycle training (Post-Train). Values are means ± SD, n = 6, # different from rest (exercise main effect, $P < 0.0001$); * HD at Baseline > CON ($P < 0.05$); † HD at Pre-Train > CON ($P < 0.05$); ‡ peak workrate HD at Baseline, Pre-Train, and Post-Train < CON ($P < 0.05$).

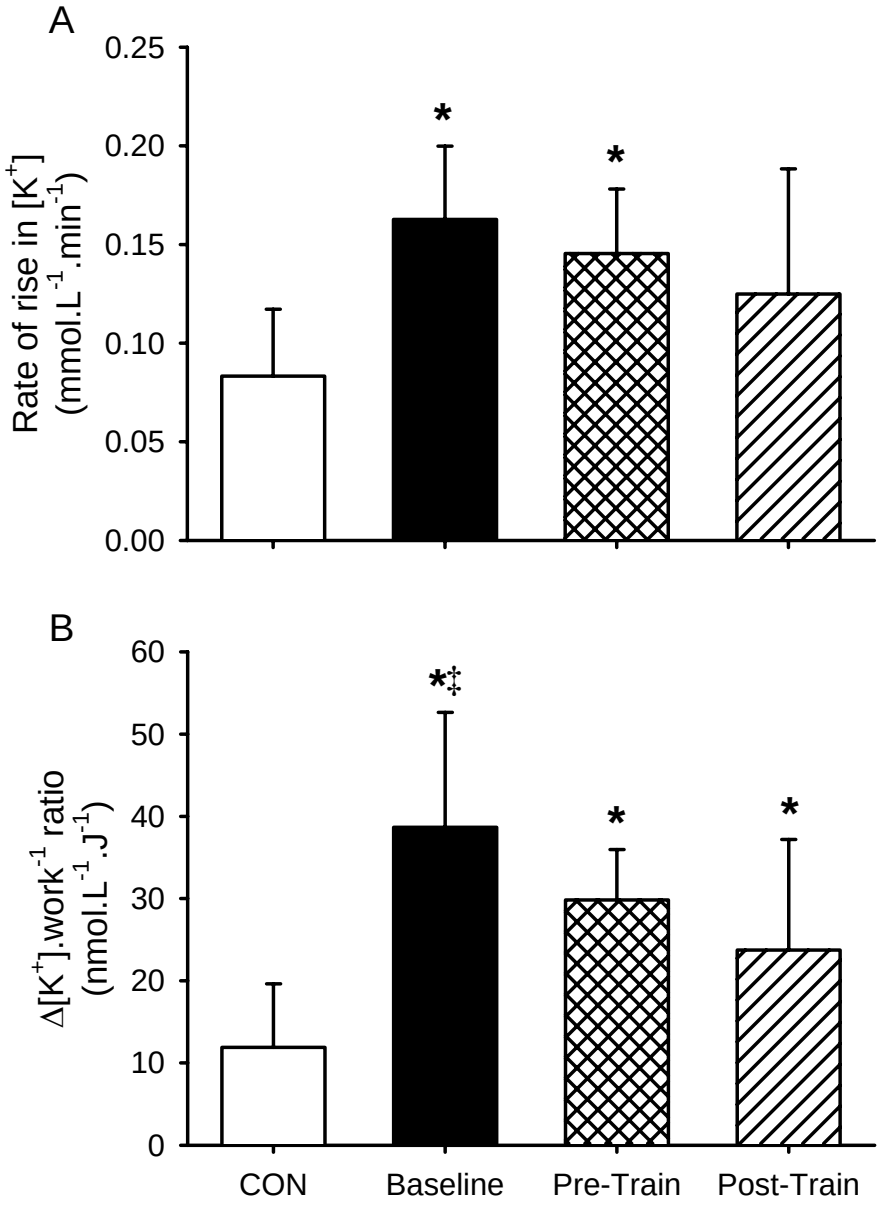

Figure 4.3. Rate of rise in $[K^+]$ and the $\Delta[K^+].work^{-1}$ ratio in HD before and after training and in CON.

The $\Delta[K^+]$ at fatigue divided by time to fatigue (A) and $\Delta[K^+]$ relative to the total work done (B) during an incremental cycle test to fatigue in healthy controls (CON) and in HD at Baseline, after 6 weeks of normal activity (Pre-Train) and following six weeks of stationary cycle training (Post-Train). Values are means ± SD, n = 6 per group, * greater than CON, ($P < 0.05$); ‡ greater than Post-Train ($P < 0.05$).

4.3.5 Quality of life

The scores for the individual and composite scales of the SF-36 in HD before and after training, and age-matched norms are shown in Table 4.3. QOL scores did not change during the study and were not different from the Australian population norms, although Physical Functioning tended to be lower in HD ($P < 0.07$).

Table 4.3. SF-36 scores and Australian norms.

		HD		
	Norms	Baseline	Pre-Train	Post-Train
Physical functioning	88 ± 26	68 ± 24	68 ± 20	76 ± 22
Role physical	85 ± 45	74 ± 23	88 ± 10	82 ± 21
Bodily pain	80 ± 32	95 ± 8	78 ± 29	88 ± 24
General health	75 ± 26	56 ± 23	50 ± 16	58 ± 21
Vitality	65 ± 26	54 ± 11	50 ± 11	54 ± 14
Social functioning	86 ± 32	71 ± 34	80 ± 15	79 ± 23
Role emotional	85 ± 45	71 ± 24	94 ± 6	86 ± 18
Mental health	75 ± 26	84 ± 6	83 ± 7	81 ± 7
Physical component scale	52 ± 13	48 ± 7	45 ± 8	49 ± 7
Mental component scale	49 ± 13	48 ± 9	53 ± 2	50 ± 5

Values are means ± SD; Norms are age-matched population norms from the Australian National Health Survey, 1995 (Australian_Bureau_of_Statistics 1997).

4.3.6 Correlations

No correlations were found between any of $\Delta[K^+]$, the rate of rise in $[K^+]$, or the $\Delta[K^+].work^{-1}$ ratio and any of the exercise variables for CON or HD at Baseline, Pre-

Train or Post-Train. When CON and HD data at Baseline were pooled, inverse correlations were found between the $\Delta[K^+].work^{-1}$ ratio and each of $\dot{V}O_{2peak}$ (Figure 4.4), peak work rate (r=-0.72, P<0.01), and time to fatigue during the aerobic power test (r=-0.70, P<0.05), and PT at $0^{o}.s^{-1}$ (r = -0.43, P < 0.05). A positive correlation was found between the $\Delta[K^+].work^{-1}$ ratio and the fatigue index (r=0.67, P<0.05).

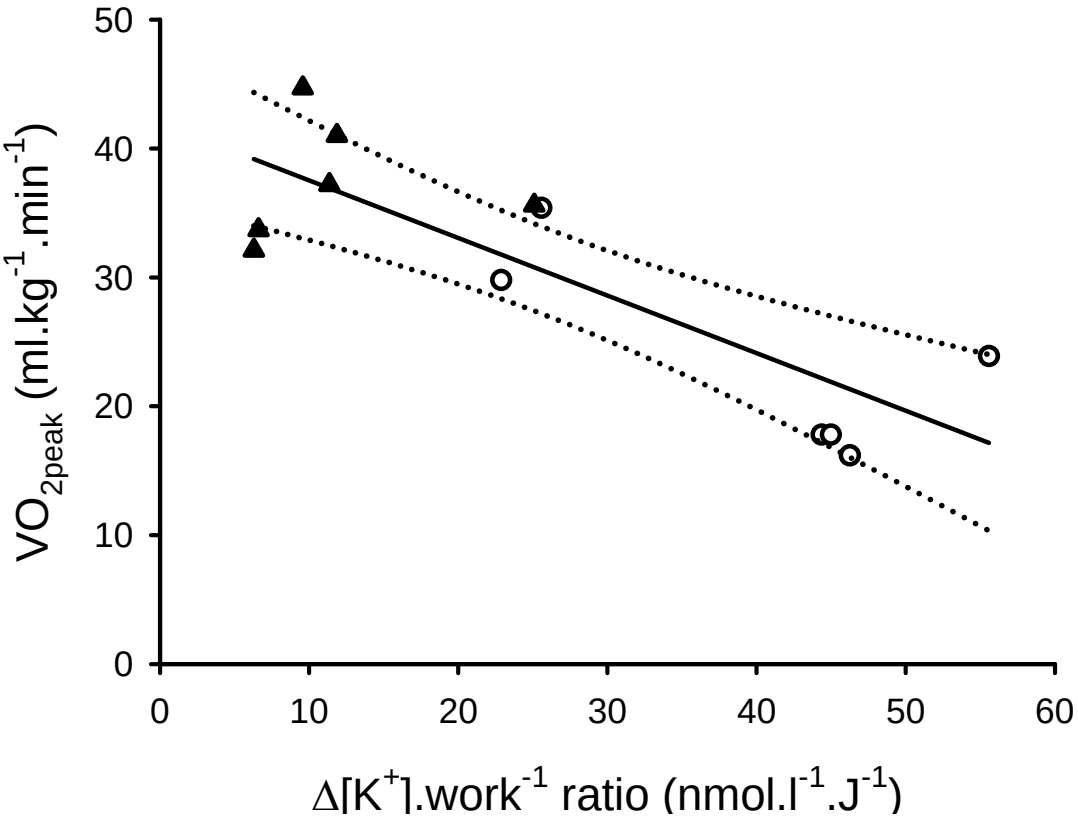

Figure 4.4. Inverse relationship between $\Delta[K^+].work^{-1}$ ratio and $\dot{V}O_{2peak}$ during the aerobic power test.

Scatter plot of $\dot{V}O_{2peak}$ against $\Delta[K^+].work^{-1}$ ratio during an incremental cycle test to fatigue (n = 12, y = -0.45x + 42, r = -0.83, P < 0.001). Solid line and dotted curves indicate regression line and 95% confidence intervals, respectively for pooled data of CON (▲), and HDP at Baseline (O).

4.4 DISCUSSION

This study demonstrated that HD with near normal [Hb] exhibited impaired $\dot{V}O_{2peak}$ and plasma [K^+] during incremental exercise compared to healthy controls. It was also found that endurance training prolonged the time to fatigue, and considerably improved extrarenal K^+ regulation, as evidenced by a 31% lower $\Delta[K^+].work^{-1}$ ratio.

4.4.1 Impaired plasma [K^+] regulation in HD taking EPO

A novel finding was that HD patients treated with EPO exhibited impaired extrarenal K^+ regulation during an incremental cycle test. Compared to CON, HD at Baseline displayed higher plasma [K^+] (18-33%), $\Delta[K^+]$ (10%), rate of rise in [K^+] (93%) and $\Delta[K^+].work^{-1}$ ratio (239%). This finding supports and extends a previous study from this laboratory in anaemic HD (Sangkabutra *et al.* 2003). All of these variables were also markedly higher in the previous study, with the $\Delta[K^+]$, rate of rise in [K^+], and $\Delta[K^+].work^{-1}$ ratio being ~50% lower in the EPO treated HD in the present study, than in the non-EPO treated HD in the previous study (*ibid*). The EPO treatment and higher [Hb] may explain the difference in K^+ regulation variables between these two studies, as the EPO treated HD in this study had ~1.7-fold higher [Hb] than the anaemic HD in the previous study (12.9 ± 0.8 vs. 7.8 ± 1.2 $g.dL^{-1}$, $P < 0.0001$). This finding is also consistent with another study from this laboratory, in which the rise in plasma [K^+] during exercise in HD was ameliorated after 4 weeks of EPO treatment (McMahon *et al.* 1999). This may be due to stimulatory effects of EPO on the skeletal muscle Na^+,K^+-ATPase, as EPO treatment increased myocardial Na^+,K^+-ATPase activity and content in rats (Wald *et al.* 1995).

Reduced Na^+,K^+-ATPase activity in uraemia has been shown in human erythrocytes, and in rat skeletal and cardiac muscle, adipocytes, brain, and intestine (Druml 1988, Fraser *et al.* 1985, Kovacic *et al.* 1997, Kramer *et al.* 1974). Therefore, reduced

Na^+,K^+-ATPase activity in skeletal muscle from uraemic humans may explain the impaired plasma K^+ regulation observed in this study, but this has not yet been investigated. Such an impairment seems likely in HD, as suggested by muscle membrane depolarisation (Cotton *et al.* 1979, Cunningham *et al.* 1971), and reduced muscle maximal compound action potential (Johansen *et al.* 2005).

Different blood sampling sites were used in HD and CON. Arterial blood was sampled from the arterio-venous fistula in HD, whereas arterialised venous blood was sampled from a dorsal hand vein in CON. Plasma [K^+] in arterialised venous blood was not different from arterial blood during low to moderate intensity incremental exercise in healthy adults, and only marginally higher (4%) during high intensity exercise (McLoughlin *et al.* 1992). Increasing the arterialised venous plasma [K^+] in CON by 4% had no effect on the differences between HD and CON (data not shown). Thus, the effect of arterial versus arterialised venous blood sampling on plasma [K^+] was negligible. However, possible differences in plasma [K^+] between the arterio-venous fistula and arterial blood in HD patients were not investigated and cannot be excluded.

4.4.2 Greater fatiguability, impaired $\dot{V}O_{2peak}$ and muscle strength in HD

This is the first report of muscle fatiguability following dynamic contractions in HD. Knee extensor fatiguability tended to be almost 2-fold higher in HD than CON, however, surprisingly, this was not significantly different. This probably reflects a type II error as effect size was moderate. The tendency to higher muscle fatiguability in HD is consistent with the 3-fold higher fatiguability following repeated isometric dorsiflexor contractions (Johansen *et al.* 2005), and 10% higher handgrip fatiguability (Moore *et al.* 1993a), respectively compared to CON.

This study shows that $\dot{V}O_{2peak}$ and knee-extensor peak torque were reduced by ~40 and by 27 - 42%, respectively, in EPO treated HD compared to CON. These deficits are similar to those previously reported in chronically EPO treated HD for $\dot{V}O_{2peak}$ (Painter *et al.* 2002a, Storer *et al.* 2005) and muscle strength (Fahal *et al.* 1997, Johansen *et al.* 2005, Johansen *et al.* 2003, Kettner Melsheimer *et al.* 1987). This strength deficit was only partially attributable to differences in body size, as PT was still reduced by 15 – 36% when expressed relative to body mass. The poor PT observed in HD may be due to reduced muscle mass, as lower-leg strength was normalised when expressed relative to muscle cross-sectional area (CSA) (Johansen *et al.* 2005, Johansen *et al.* 2003). As muscle CSA was not measured, this can not be confirmed in the present study.

Decreased physical activity levels in HD compared to CON could explain the poor exercise performance observed in HD, however physical activity levels were not measured so this cannot be determined. Due to the participant exclusion criteria to satisfy maximal exercise testing requirements, the participants in this study were relatively healthy and well motivated. Thus, the exercise performance of the general HD population is likely to be considerably worse than indicated in this study.

4.4.3 Improved plasma [K^+] regulation following training in HD

A marked improvement in acute K^+ regulation was seen with training. The $\Delta[K^+].work^{-1}$ ratio during incremental exercise was reduced by 31% after training, compared to Baseline. The lack of difference between Baseline and Post-train in plasma $[K^+]$, $\Delta[K^+]$, and rate of rise in $[K^+]$ during exercise is probably due to a type II error as effect size was moderate. For plasma $[K^+]$ the effect size was 0.36, meaning that 36% of the variability between Baseline, Pre-Train and Post-Train scores was attributable to training but the observed power for training was only 0.42, meaning

there was a 58% chance of type II error. Furthermore, the plasma $[K^+]$, $\Delta[K^+]$, and rate of rise in $[K^+]$ during exercise were all greater in HD than CON at Baseline, whereas after training they were not significantly different from CON. This strongly suggests improved extrarenal K^+ regulation with training in HD.

A link is suggested between impaired K^+ regulation and poor exercise performance in HD via the significant inverse correlations between the $\Delta[K^+].work^{-1}$ ratio and each of $\dot{V}O_{2peak}$, time to fatigue, and peak work rate during the aerobic power test, as well as PT at $0^o.s^{-1}$; and the positive correlation with knee-extensor fatiguability (when all data were pooled). This supports the previously shown inverse correlation between $\Delta[K^+].work^{-1}$ ratio and $\dot{V}O_{2peak}$ in HD not treated with EPO (Sangkabutra *et al.* 2003).

The improved $\Delta[K^+].work^{-1}$ ratio and tendency to improved plasma $[K^+]$, $\Delta[K^+]$, and rate of rise in $[K^+]$ after training, might reflect an increased skeletal muscle Na^+,K^+-ATPase content or activity. Muscle Na^+,K^+-ATPase is critical in the acute regulation of plasma $[K^+]$ (McDonough *et al.* 2002) and is elevated following training (Green *et al.* 1999a, Green *et al.* 1993, McKenna *et al.* 1993). Skeletal muscle biopsies were not taken in this study to avoid deterring potential participants, and therefore possible effects on skeletal muscle Na^+,K^+-ATPase content were not determined.

4.4.4 Improved exercise performance but not $\dot{V}O_{2peak}$ after training

Time to fatigue and total work performed during incremental cycle increased by 12 and 20%, respectively following training compared to Baseline, and by 7 and 13% compared to Pre-Train. HD patients display large functional deficits, with severely reduced $\dot{V}O_{2peak}$ and muscle strength (Bonzel *et al.* 1991, Kettner Melsheimer *et al.* 1987, Painter *et al.* 1986a, Sangkabutra *et al.* 2003) and as such, should be highly responsive to exercise training. Notably, the increased time to fatigue and total work

occurred without a concomitant improvement in $\dot{V}O_{2peak}$. A similar finding has been reported previously in five HD patients who increased their maximum achievable workrate but not $\dot{V}O_{2peak}$ following 12 weeks of stationary cycle training (Moore *et al.* 1993b). The lack of improvement in $\dot{V}O_{2peak}$ in the present study is surprising, but might reflect the relatively short training duration. However, training duration may not be the only factor as $\dot{V}O_{2peak}$ was increased in HD patients after only 9 weeks of training (Storer *et al.* 2005), but did not change following ~7 months of training (Goldberg *et al.* 1979). Thus, despite increases in the time to fatigue and work done during the aerobic power test, the lack of improvement in $\dot{V}O_{2peak}$ and knee-extensor fatiguability is perplexing. Several other studies have also reported no change in $\dot{V}O_{2peak}$ following training for 12 – 30 weeks duration in HD (Akiba *et al.* 1995, Goldberg *et al.* 1980a, Goldberg *et al.* 1979, Moore *et al.* 1993b, Violan *et al.* 2002). In the present study, 2/6 (33%) of HD failed to improve $\dot{V}O_{2peak}$. Furthermore, several studies have reported that up to 36% of HD patients did not improve $\dot{V}O_{2peak}$ after training (Goldberg *et al.* 1986, Goldberg *et al.* 1980a, Goldberg *et al.* 1980b, Goldberg *et al.* 1979, Moore *et al.* 1993b, Suh *et al.* 2002). Thus, it is possible that there is a subset of HD patients in whom $\dot{V}O_{2peak}$ does not improve following training. Further research, using a large sample of patients, is needed to verify this and determine possible mechanisms.

Knee-extensor peak torque did not increase following the training period. This is not surprising given the endurance based training programme. However, improved muscle strength following two months of cycle training has previously been observed in HD (Kouidi *et al.* 1998, Storer *et al.* 2005), probably due to their poor initial strength.

This is the first study to determine fatiguability during maximal dynamic muscle contractions before and after training in HD, finding no change. However a recent study found a 40% increase in submaximal leg-press muscular endurance following two months of cycle training in HD (Storer *et al.* 2005). Together, with the finding of increased time to fatigue after training in the present study, these results suggest that endurance training decreases fatiguability during submaximal but not maximal contractions in HD.

Due to recruitment difficulties, this study lacked a non-training HD group, however patients acted as their own controls, being tested at Baseline and Pre-Train. Thus, the improved exercise performance following training could potentially be due to a familiarisation effect. The increases in time to fatigue and work done between Pre-Train and Post-Train were larger than the CV. As the CV of the exercise performance variables from Baseline to Pre-Train represents the variability of the test and any familiarisation effect, these can only account for a portion of the increase. Physical activity levels were not monitored during this study, thus it is possible that HD increased their physical activity from Baseline to Pre-Train in anticipation of the training period. This does not appear to have occurred however, as there were no significant differences between Baseline and Pre-Train for any of the variables measured in this study.

4.4.5 No change in QOL after training

Quality of life, as measured by the SF-36 questionnaire, was not changed by physical training, which might be due to their normal QOL scores prior to the cycle training. This is consistent with a study which found improved QOL scores following exercise training in HD patients with low pre-training scores, but not in patients with high pre-training QOL scores (Painter *et al.* 2000a).

4.5 CONCLUSIONS

HD had substantially lower $\dot{V}O_{2peak}$, time to fatigue, and work done during an incremental cycle test, and lower knee-extensor PT, than CON despite being treated chronically with EPO. They also had impaired extrarenal K^+ regulation despite near normal [Hb], with higher plasma [K^+], Δ[K^+], rate of rise in [K^+], and Δ[K^+].work^{-1} ratio than CON. Further research is needed to determine the cause of the impaired extrarenal plasma K^+ regulation in HD. The rise in plasma K^+ relative to work done was related to $\dot{V}O_{2peak}$, time to fatigue, peak work rate, peak isometric torque, and fatiguability. Endurance training improved time to fatigue and plasma K^+ regulation during an incremental cycle test. Thus, impaired extrarenal K^+ regulation may contribute to the poor exercise performance in HD and the improvement in exercise performance with training may be partly due to better K^+ regulation.

CHAPTER 5. REDUCED EXERCISE PERFORMANCE IS RELATED TO IMPAIRED K^+ REGULATION AND SKELETAL MUSCLE Na^+,K^+-ATPase ACTIVITY IN HAEMODIALYSIS PATIENTS AND RENAL TRANSPLANTATION RECIPIENTS

5.1 INTRODUCTION

Haemodialysis patients (HD) display abnormally low exercise performance, with peak oxygen consumption ($\dot{V}O_{2peak}$) reduced by up to 50% compared to healthy controls (CON) (Chapter 4)(Kettner Melsheimer *et al.* 1987, Sangkabutra *et al.* 2003, van den Ham *et al.* 2005). Whether exercise performance is improved in renal transplantation patients (RTx) is equivocal, with improved $\dot{V}O_{2peak}$ reported following successful RTx (Gallagher-Lepak 1991, Kettner Melsheimer *et al.* 1987, Painter *et al.* 1987, Painter *et al.* 1986a), contrasting no difference in $\dot{V}O_{2peak}$ or maximum work capacity between HD and RTx (Bullock *et al.* 1984, van den Ham *et al.* 2005) or in maximum work rate pre- and post-RTx (Nyberg *et al.* 1995). Anaemia contributes to reduced exercise performance in HD (McMahon *et al.* 1999, Morris *et al.* 1993, Painter *et al.* 2002a), thus any increase in [Hb] and hematocrit (Hct) after transplantation would be expected to partially account for any gain in $\dot{V}O_{2peak}$. No studies have compared $\dot{V}O_{2peak}$ in HD and RTx with similar [Hb] to properly compare these patient groups, and this was therefore the first aim of the present study.

Both muscle strength and fatiguability are important determinants of exercise capability (Diesel *et al.* 1990, Kempeneers *et al.* 1990). Muscle strength is impaired in HD (Kettner Melsheimer *et al.* 1987, van den Ham *et al.* 2005), which may be due to reduced muscle mass (Kemp *et al.* 2004). Renal transplantation does not improve muscle strength (Kettner Melsheimer *et al.* 1987, Nyberg *et al.* 1995, van den Ham *et*

al. 2005), possibly due to the muscle wasting effects of glucocorticoid therapy (Horber *et al.* 1985). Muscle fatiguability during repeated maximal isometric hand-grip contractions was higher, and Hct lower in non EPO-treated HD than RTx (Moore *et al.* 1993a). Importantly, no studies have examined muscle fatiguability during repeated dynamic contractions in RTx, or between RTx and EPO-treated HD with similar [Hb]. This study therefore tested the hypotheses that $\dot{V}O_{2peak}$ would be reduced compared to CON but would not differ between RTx and EPO-treated HD with similar [Hb], and that both muscular strength and fatiguability during dynamic contractions would be worsened in EPO-treated HD and RTx compared to CON.

A possible mechanism of fatigue in HD patients is impaired muscle membrane excitability, caused by elevations in interstitial and t-tubular $[K^+]$. Consistent with this, HD show an exaggerated rise in plasma K^+ concentration during incremental exercise, which was inversely correlated with $\dot{V}O_{2peak}$ (Sangkabutra *et al.* 2003)(Chapter 4). A reduced muscle compound action potential was also observed in HD at rest, and persisted throughout exercise (Johansen *et al.* 2005). This suggests a link between impaired K^+ regulation and exercise performance in HD, where underlying muscle abnormalities in K^+ regulation impair muscle membrane excitability and enhance fatigue. Whether K^+ regulation during exercise is impaired in RTx is unknown, and this study therefore also tested the hypothesis that the rise in plasma $[K^+]$ during incremental exercise would be impaired in HD but not in RTx. Skeletal muscle contains the single largest pool of Na^+,K^+-ATPase in the body (Kjeldsen *et al.* 1984a), and is vitally important in extrarenal K^+ regulation (McDonough *et al.* 2002). Impaired skeletal muscle Na^+,K^+-ATPase activity could underlie the abnormal plasma K^+ responses in HD (Huber *et al.* 1985, Sangkabutra *et al.* 2003) as Na^+,K^+-ATPase activity was reduced in erythrocytes and leucocytes from

uraemic patients (Aparicio *et al.* 1991, Cole 1973) and in skeletal muscle from uraemic rats (Bofill *et al.* 1994, Druml 1988). Abnormalities in skeletal muscle Na^+,K^+-ATPase might include altered maximal activity, content or isoform abundance, but none of these have been investigated in either HD or RTx. However, both content and isoform abundance were normal in skeletal muscle from uraemic rats (Druml 1988, Greiber *et al.* 1994). Erythrocytic Na^+,K^+-ATPase activity was improved following RTx (Sigstrom 1981), but whether RTx also improves Na^+,K^+-ATPase activity in skeletal muscle is not known. Therefore, this study also tested the hypotheses that skeletal muscle Na^+,K^+-ATPase activity would be depressed in HD but not in RTx, whilst muscle Na^+,K^+-ATPase content and isoform abundance would be unchanged in HD and RTx. Also, depressed skeletal muscle Na^+,K^+-ATPase activity in HD would be related to their poor exercise performance.

5.2 METHODS

5.2.1 Subjects

Ten haemodialysis patients (HD), nine renal transplant recipients (RTx), and 10 sedentary, healthy controls (CON) gave written informed consent and participated in the study. Sample size was calculated using Power and Precision software based on the expected Na^+, K^+-ATPase activity means. Comparing by ANOVA the expected Na^+, K^+-ATPase activity means of the HD, transplant and control groups 1269, 1623 and 1977 pmol.mg $protein^{-1}.min^{-1}$ respectively, SD of 250 pmol.mg $protein^{-1}.min^{-1}$ (McKenna 2000) α =0.05, a sample size of n = 10 per group yielded a power of >99%. One HD patient underwent all tests except the muscle biopsy. Subjects were matched for sex, age, height, body mass and body mass index (BMI) (Table 5.1). HD were stable and had been dialysing for at least 6 mo prior to testing (range 7 – 71, 38

± 23 mo, mean ± SD). RTx were transplanted at least 12 mo prior to testing (range 16 – 171, 63 ± 53 mo), had a stable creatinine, and a calculated creatinine clearance (Cockcroft-Gault) of >40 $ml.min^{-1}$. All subjects had [Hb] >110 $g.l^{-1}$. Subjects were excluded if they were generally unwell, had symptomatic ischaemic heart disease, peripheral vascular disease, disabling arthritis, chronic airflow obstruction, or were pregnant. Causes of renal failure were reflux/congenital dysplasia (HD = 5, RTx = 2), glomerulonephritis (HD = 4, RTx = 4), cystic kidney disease (RTx = 2), diabetes (HD = 1) and unknown (RTx = 1). Subjects were tested while taking their normal medication (Table 5.1). Of the RTx recipients, two received kidneys from living related donors, two from living non-related donors, and five were from cadaveric donors. This study was approved by the Human Research Ethics Committees at Victoria University and at Royal Melbourne Hospital.

5.2.2 Exercise tests

5.2.2.1 Aerobic power ($\dot{V}O_{2peak}$)

Subjects cycled on an electrically braked cycle ergometer (Lode, Groningen, Holland) at an initial power output of 15 W with progressive increments of 15 W each minute. Subjects pedalled at or above 60 rpm and the test was continued until volitional fatigue, defined as an inability to maintain cadence above 60 rpm. Expired O_2 and CO_2 and ventilation were continuously measured to enable calculation of $\dot{V}O_{2peak}$ as described elsewhere (Chapter 4)(Li *et al.* 2002).

5.2.2.2 Knee-extensor torque-velocity test

Subjects performed three maximal isokinetic contractions at 0, 60, 120, 180, 240, 300, and 360°.s^{-1}, with 60 s recovery between sets, on an isokinetic dynamometer (Cybex Norm 770, Henley HealthCare, Massachusetts), as previously described (Chapter 4). The highest value of each set of three was defined as the peak torque (PT) and was

expressed relative to body mass ($Nm.kg^{-1}$) and thigh muscle cross-sectional area ($Nm.cm^{-2}$) to correct for differences in body size and muscle mass, respectively.

5.2.2.3 Knee extensor fatigue test

Subjects performed 30 maximal isokinetic contractions at $180°.s^{-1}$, with ~1 s pause between repetitions (Chapter 4). The fatigue index was calculated as the percent decline in peak torque: fatigue index (%) = [(starting PT - final PT)/ starting PT] x 100, where starting PT = the average of the highest three of the first five repetitions; final PT = the average of the highest three of the last five repetitions.

5.2.3 Blood sampling and processing

Blood was sampled before, during and after the aerobic power test. In all HD, and in four RTx subjects, blood was sampled from the arterio-venous fistula via an indwelling catheter. In all other subjects, arterialised venous blood was sampled from a dorsal hand-vein (Chapter 3) (Petersen *et al.* 2005). The blood sample was transferred to a tube containing lithium heparin. One ml of blood was analysed in duplicate for [Hb] and Hct (Sysmex, K-800, Kobe, Japan) and another 1.5 ml was centrifuged at 4,500 rpm for 2 min with the plasma removed and frozen until later duplicate analysis of plasma [K^+] using an ion selective electrode (865pH/Blood Electrolyte and Gas Analyser, Bayer, MA, USA). Calculations for the percentage decline in plasma volume from resting conditions (ΔPV), the rise in plasma [K^+] above rest ($\Delta[K^+]$) during the aerobic power test relative to the total work performed ($\Delta[K^+].work^{-1}$ ratio), and the correction of plasma [K^+] for changes in PV were as previously described (Fraser *et al.* 2002, McKenna *et al.* 1997, McKenna *et al.* 1993). The possible effect of medication on plasma [K^+] was also considered. Non-selective β-blockers increase plasma [K^+] during exercise (Hallén *et al.* 1994), however only β_1-blockers were taken by HD in this study and these do not affect plasma [K^+] during

exercise (Gullestad *et al.* 1991). Prednisone increases skeletal muscle Na^+,K^+-ATPase content in humans (Ravn *et al.* 1997), which would likely lower plasma [K^+] during exercise. However, the plasma [K^+] during exercise was not different within groups between the patients taking prednisone and those who were not (data not shown), and therefore these results were not excluded.

5.2.4 Computerised Tomography scan

A measure of thigh muscle cross-sectional area (TMCSA) of the dominant leg was made by a single slice Computerized Tomography (CT) scan, taken 20 cm above the medial femoral condyle. Muscle area was calculated as the total muscle compartment area minus the femur area (Leikis *et al.* 2006).

5.2.5 Muscle biopsy

A muscle biopsy was taken from the middle third of the left vastus lateralis. After injection of a local anaesthetic into the skin and fascia (1% Xylocaine), a small incision was made and a muscle sample taken (~120mg) using a Stille biopsy needle. The sample was immediately frozen in liquid N_2 until later analysis of Na^+,K^+-ATPase measures.

5.2.6 Muscle Na^+,K^+-ATPase analyses

5.2.6.1 Maximal in-vitro activity and total content

The maximal in-vitro Na^+,K^+-ATPase activity was measured fluorometrically in crude muscle homogenate using the K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) assay optimized for human skeletal muscle (Fraser *et al.* 1998)(Chapter 3). Skeletal muscle total Na^+,K^+-ATPase content was determined by vanadate-facilitated [^{3}H]ouabain binding site content analysis (Nørgaard *et al.* 1984a)(Chapter 3).

5.2.6.2 Isoform abundance

This laboratory has recently shown that human skeletal muscle expresses the α_1, α_2, α_3, β_1, β_2, and β_3 Na^+,K^+-pump isoforms (Murphy *et al.* 2004). Western blotting was performed for these six Na^+,K^+-ATPase isoforms as previously detailed (Murphy *et al.* 2004), with the modification that the muscle homogenate was deglycosylated prior to electrophoresis to enhance β-isoform identification. This involved incubating the homogenate for 1 h at 37°C with 0.5% (v/v) Nonidet P40 and 3 units N-Glycosidase F (Boehringer Mannheim) per 0.5 mg protein. The final blot intensity was normalised to the same human muscle standard that was run in all gels. The linearity of the blot signal versus protein loaded was confirmed for each antibody.

5.2.6.3 Antibodies

Blots were probed with antibodies specific to each isoform. These were for α_1: monoclonal α6F (developed by D. Fambrough and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA); α_2: polyclonal anti-HERED (kindly donated by T. Pressley, Texas Tech University); α_3: monoclonal MA3-915 (Affinity Bioreagents, Golden, Colorado); β_1: monoclonal MA3-930 (Affinity Bioreagents); β_2: monoclonal 610915 (Transduction Laboratories, Lexington, Kentucky); and β_3: monoclonal 610993 (Transduction Laboratories).

5.2.7 Statistics

Data are presented as mean ± SD. A one-way ANOVA was used for all measurements except when repeated measures were taken (e.g. $[K^+]$ data) for which a mixed-design two-way ANOVA was used. The Least Significant Difference test was used for post-hoc analyses because of the unequal group sizes. Correlations were determined by least squares linear regression. Statistical significance was accepted at $P < 0.05$.

5.3 RESULTS

5.3.1 Physical characteristics

No difference was observed between groups for sex, age, height, body mass, BMI, [Hb], Hct, or TMCSA (Table 5.1). However, TMCSA expressed relative to body mass was lower in HD ($P < 0.01$) and RTx ($P < 0.05$) than in CON.

5.3.2Exercise performance

5.3.2.1 Knee-extensor torque-velocity

Peak torque expressed relative to body mass was ~25% lower in HD and RTx than CON ($P < 0.05$) at all velocities except $120°.s^{-1}$, where only RTx was lower (Figure 5.1). However, peak torque expressed relative to TMCSA did not differ between groups (Figure 5.1).

5.3.2.2 Knee-extensor fatigue test

The fatigue index was 1.7- and 1.6-fold higher, respectively in HD ($P < 0.005$) and RTx ($P < 0.01$) than in CON, with no difference found between HD and RTx (Figure 5.2).

5.3.2.3 Aerobic power

$\dot{V}O_{2peak}$ was 35 and 32% less in HD ($P < 0.01$) and RTx ($P < 0.05$) than in CON, respectively, with no difference between HD and RTx (Figure 5.2). Peak workrate was similarly lower by 31 and 29%, respectively in HD ($P < 0.005$) and RTx ($P < 0.005$) than in CON (Figure 5.3). Total work done was also lower in HD ($P < 0.005$) and RTx ($P < 0.01$) than in CON (HD 56.8 ± 23, RTx 60.6 ± 41, and CON 114.8 ± 54 kJ). When data from all groups were pooled, $\dot{V}O_{2peak}$ was highly correlated with both the TMCSA ($r = 0.81$, $P < 0.001$, $n = 28$) and knee-extensor peak torque ($r = 0.83$, $P < 0.001$, $n = 29$) and inversely correlated with fatigue index ($r = -0.53$, $P < 0.005$, $n = 29$).

Table 5.1. Physical characteristics and medications in haemodialysis patients, renal transplant recipients and healthy controls.

	HD	**RTx**	**CON**
n	10	9	10
Age (years)	39.2 ± 8.6	41.3 ± 10.6	39.8 ± 8.8
Sex (F:M)	3:7	3:6	3:7
Body mass (kg)	76.8 ± 17.1	75.6 ± 15.7	72.4 ± 16.0
Height (m)	1.75 ± 0.11	1.71 ± 0.13	1.75 ± 0.09
BMI ($kg.m^{-2}$)	25.2 ± 5.0	25.8 ± 3.3	23.4 ± 3.7
[Hb] ($g.l^{-1}$)	133 ± 14	134 ± 9	145 ± 13
Hct (%)	38.3 ± 5.1	38.7 ± 2.5	41.0 ± 3.2
Creatinine clearance ($ml.min^{-1}$)	9.9 ± 3.4*§	75.5 ± 21.6*	108.5 ± 19.2
TMCSA (cm^2)	113 ± 20	118 ± 22	131 ± 24
Relative TMCSA ($cm^2.kg^{-1}$)	1.53 ± 0.22*	1.58 ± 0.23*	1.82 ± 0.16
Medications			
Epoetin (*n*)	6	1	
β_1-blockers (*n*)	3	2	
ACE inhibitors (*n*)		3	
Angiotensin II receptor antagonists (*n*)	1	3	
Ca^{2+} channel blockers (*n*)		3	
Cyclosporine (*n*)		4	
Prednisolone (*n*)		7	
Azathioprine (*n*)		3	
Tacrolimus (*n*)		5	
Mycophenolate mofetil (*n*)		5	

Values are mean ± SD; HD, haemodialysis patients; RTx, renal transplant recipients; CON, healthy controls; BMI, body mass index; TMCSA, thigh muscle cross-sectional area; ACE, angiotensin converting enzyme; * less than CON, $P < 0.05$; § HD less than RTx $P < 0.05$.

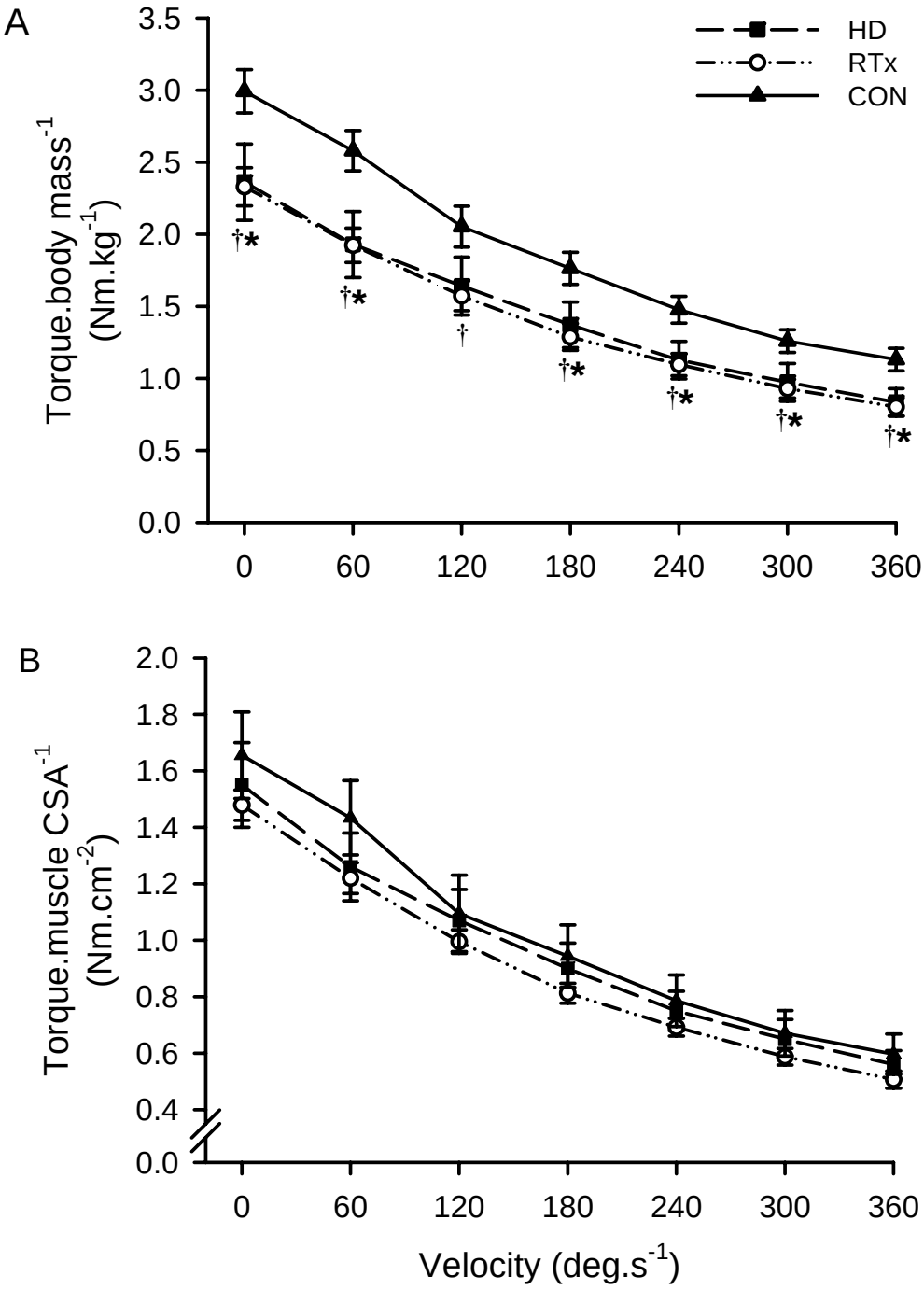

Figure 5.1. Knee extensor peak torque during isokinetic dynamometry, expressed relative to body mass (A) and to thigh muscle cross-sectional area (B).

Values are means ± SD. HD (■), haemodialysis, n = 10, RTx (○), renal transplant, n = 9, CON (▲), healthy controls, n = 10. * HD less than CON, $P < 0.05$; † RTx less than CON, $P < 0.05$.

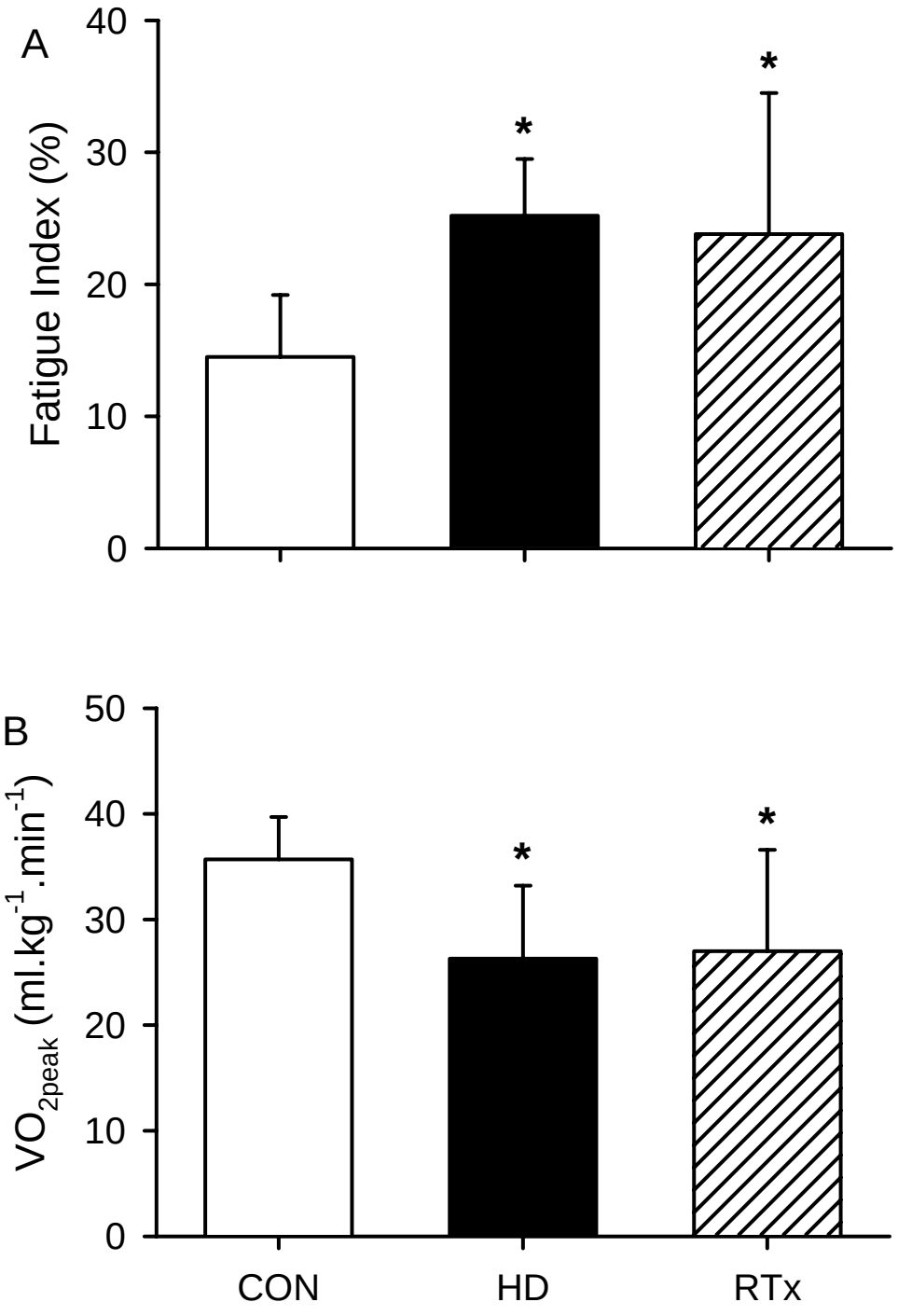

Figure 5.2. Knee-extensor fatiguability during 30 maximal isokinetic contractions (A) and $\dot{V}O_{2peak}$ during an incremental cycle test to fatigue (B).

Values are means ± SD. HD n = 10, RTx n = 9, CON n = 10. * different from CON, $P < 0.05$.

5.3.3 Plasma [K^+] and fluid shifts

5.3.3.1 Plasma [K^+]

Plasma [K^+] was higher ($P < 0.05$) in HD than in RTx and CON at rest, during exercise and at 5 and 10 min post-exercise (Figure 5.3). The $\Delta[K^+]$ did not differ between groups during common submaximal exercise workrates, but was less at fatigue in RTx ($P < 0.005$) than in CON (Figure 5.3). To correct for the longer exercise time and thus greater workrate at fatigue in CON, $\Delta[K^+]$ was expressed relative to the total work done ($\Delta[K^+].work^{-1}$ ratio). No difference was found between the groups in $\Delta[K^+].work^{-1}$ ratio (HD 36.7 ± 15.9, RTx 39.1 ± 28.4, CON 27.2 ± 17.3 $nmol.l^{-1}.J^{-1}$). When all data was pooled, $\dot{V}O_{2peak}$ was inversely correlated with the $\Delta[K^+].work^{-1}$ ratio ($r = -0.77$, $P < 0.001$, $n = 29$) and positively correlated with calculated creatinine clearance (CrCl) ($r = 0.43$, $P < 0.05$, $n = 29$).

5.3.3.2 Fluid shifts and corrected [K^+]

The change in plasma volume from rest (ΔPV), during incremental exercise to fatigue, was less in RTx than in both HD and in CON (RTx -12.7 ± 2.5, HD -15.8 ± 2.9, CON -16.2 ± 3.1 %, $P < 0.05$). Therefore, to determine whether the higher $\Delta[K^+]$ in CON than in RTx was due to greater haemoconcentration, plasma [K^+] was corrected for the ΔPV. After correction, the $\Delta[K^+]$ still did not differ between groups during common submaximal workrates, but was now less in RTx (0.80 ± 0.4, $P < 0.05$) and HD (0.91 ± 0.3, $P < 0.005$) at fatigue compared to CON (1.40 ± 0.4 $mmol.L^{-1}$). The corrected $\Delta[K^+].work^{-1}$ ratio also did not differ between groups (HD 14.9 ± 8.5, RTx 20.8 ± 15.6, CON 15.6 ± 10.4 $nmol.l^{-1}.J^{-1}$).

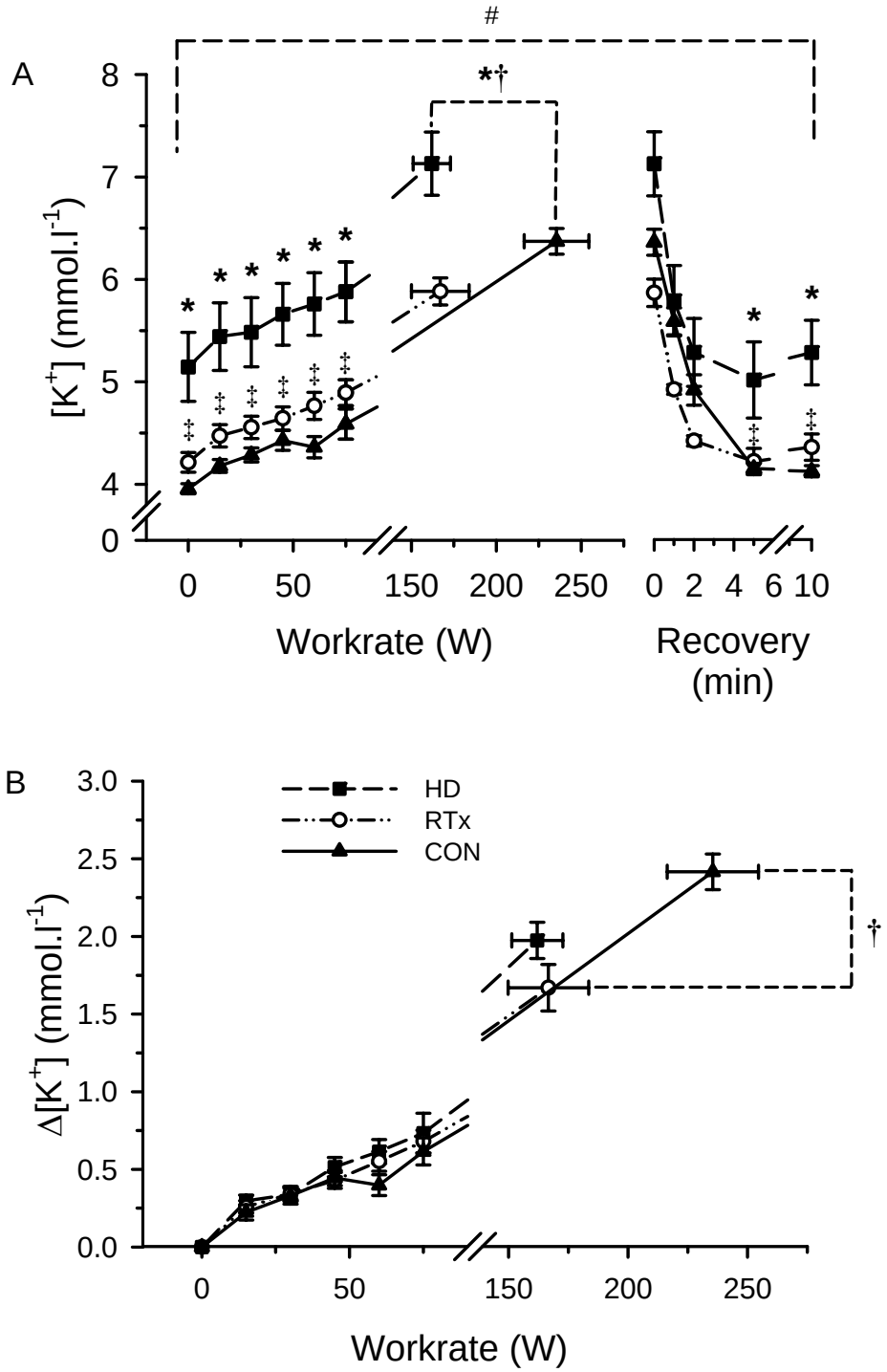

Figure 5.3. Plasma [K^+] before, during, and after (A), and the rise in plasma [K^+] from rest during (B) an incremental cycle test to fatigue.

Values are means ± SD. HD n = 10, RTx n = 9, CON n = 10. # exercise main effect ($P < 0.0001$); * [K^+] HD greater than CON, $P < 0.05$; † RTx less than CON, $P < 0.05$; ‡ HD greater than RTx, $P < 0.05$.

5.3.4 Muscle Na^+,K^+-ATPase activity, content and isoforms

5.3.4.1 Maximal in-vitro activity and content

Maximal in-vitro K^+-stimulated 3-O-MFPase activity was 31% and 28% lower in HD ($P < 0.01$) and RTx ($P < 0.05$) than CON, respectively (Figure 5.4). However, in contrast, the muscle [^{3}H]ouabain binding site content did not differ between the groups (Figure 5.4). Within HD, muscle 3-O-MFPase activity was positively correlated with $\dot{V}O_{2peak}$ ($r = 0.73$, $P < 0.05$, $n = 9$), knee-extensor isometric PT ($r = 0.84$, $P < 0.05$, $n = 9$), and time to fatigue ($r = 0.80$, $P < 0.01$, $n = 9$). No correlations were found in RTx or CON between muscle 3-O-MFPase activity and any of the exercise performance variables. For pooled data, muscle 3-O-MFPase activity was positively correlated with [^{3}H]ouabain binding site content ($r = 0.42$, $P < 0.05$, $n = 28$), CrCl ($r = 0.44$, $P < 0.05$, $n = 28$) and $\dot{V}O_{2peak}$ (Figure 5.5).

5.3.4.2 Isoform abundance

Each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2, and β_3 isoforms were expressed in muscle from all HD and RTx patients and CON. There was no significant difference between groups in protein abundance for any of these Na^+,K^+-ATPase isoforms (Figure 5.6).

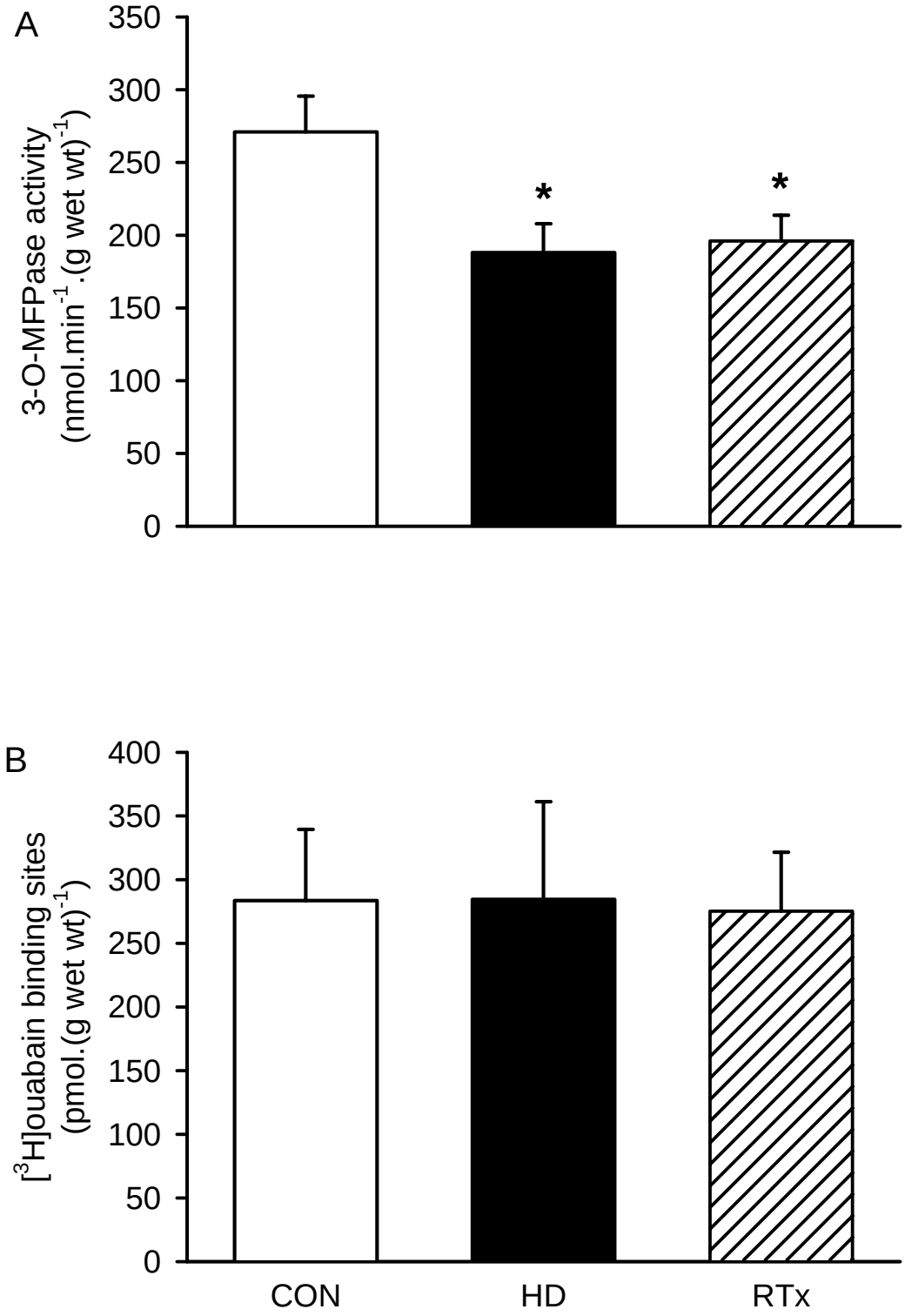

Figure 5.4. Maximal in-vitro K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) activity (A) and [^{3}H]ouabain binding site content (B).

HD, haemodialysis, n = 10, RTx, renal transplant, n = 9, CON, healthy controls, n = 10. Data expressed as mean ± SD. * less than CON, $P < 0.05$.

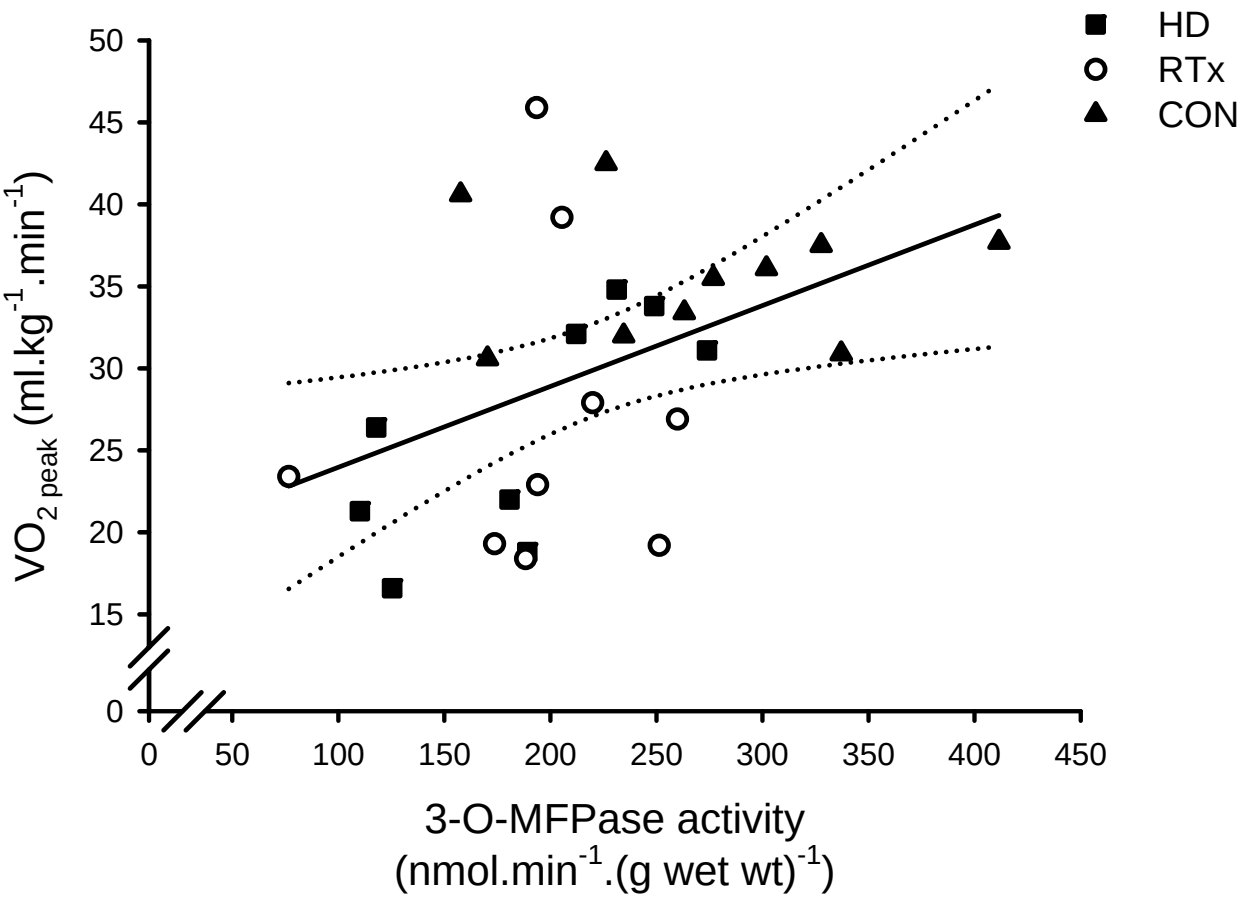

Figure 5.5. Relationship between skeletal muscle maximal in-vitro 3-O-MFPase activity and $\dot{V}O_{2peak}$.

HD (■), haemodialysis, n = 10, RTx (○), renal transplant, n = 9, CON (▲), healthy controls, n = 10. Regression line is for pooled data (solid line, n =28, r = 0.45, P < 0.05, y = 4.0x + 99.1). Dotted curves indicate 95% confidence intervals.

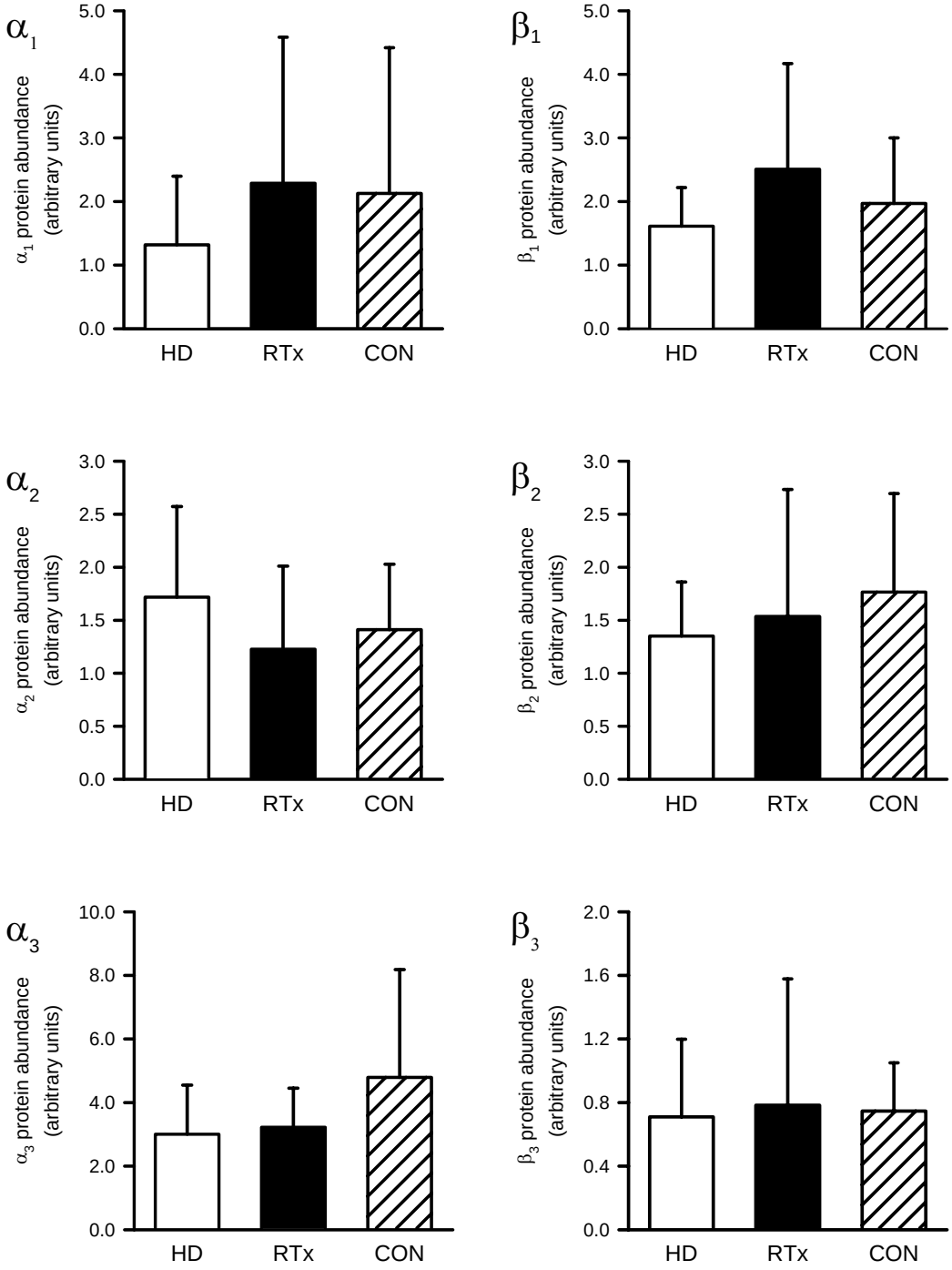

Figure 5.6. Vastus lateralis muscle Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2, and β_3 isoform expression.

HD, haemodialysis, *n* = 10, RTx, renal transplant, *n* = 9, CON, healthy controls, *n* = 10. All values are normalised to the same human muscle standard that was run in all gels. Data expressed as mean ± SD.

5.4 DISCUSSION

This is the first study to measure Na^+,K^+-ATPase activity, content or isoforms in skeletal muscle obtained from uremic patients. This study identifies an important abnormality as a possible mechanistic factor underlying impaired exercise performance in haemodialysis patients and renal transplantation recipients, showing that skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity is substantially reduced in HD and RTx compared to healthy controls. Furthermore, maximal in-vitro Na^+,K^+-ATPase activity and exercise performance did not differ between HD and RTx with normal [Hb]. Significant inter-relationships suggest that the impaired exercise performance in HD and RTx may be linked to depressed muscle maximal in-vitro Na^+,K^+-ATPase activity, which in turn was also associated with reduced renal function.

5.4.1 Reduced maximal in-vitro Na^+,K^+-ATPase activity but not abundance

The major and novel finding is that muscle maximal in-vitro Na^+,K^+-ATPase activity was markedly and similarly reduced in HD and RTx, by 31 and 28%, respectively compared to CON. This is important since skeletal muscle contains the largest single pool of Na^+,K^+-ATPase in the body (Kjeldsen *et al.* 1984a) and is therefore vital for extrarenal K^+ regulation (McDonough *et al.* 2002). These findings are consistent with reports from uraemic patients of reduced Na^+,K^+-ATPase activity in other tissues (Aparicio *et al.* 1991, Cole 1973, Edmondson *et al.* 1975, Vasarhelyi *et al.* 1996), abnormal extrarenal K^+ regulation (Huber *et al.* 1985, Sangkabutra *et al.* 2003)(Chapter 4), reduced muscle intracellular [K^+] (Bergström *et al.* 1983), muscle membrane depolarization (Cotton *et al.* 1979, Cunningham *et al.* 1971) and impaired muscle membrane excitability (Johansen *et al.* 2005).

Surprisingly, there was no difference in maximal in-vitro 3-O-MFPase activity between HD and RTx. It was hypothesised that Na^+,K^+-ATPase activity would be normal in RTx, based on previous studies showing normal or elevated Na^+,K^+-ATPase activity in erythrocytes after renal transplantation (Cole *et al.* 1975, Sigstrom 1981, Zannad *et al.* 1982). However, the present findings are consistent with others who found no change in erythrocyte Na^+,K^+-ATPase activity in HD patients following renal transplantation (Ferrer-Martinez *et al.* 1999). The Na^+,K^+-ATPase content of erythrocytes is very low (Wiley *et al.* 1977) and their role in extrarenal K^+ regulation is probably minor compared to that of skeletal muscle (Lindinger *et al.* 2003, Lindinger *et al.* 1999, Maassen *et al.* 1998, Sejersted *et al.* 2000). Thus, the depressed skeletal muscle Na^+,K^+-ATPase activity observed in HD and RTx in this study is likely of far greater significance to extrarenal K^+ regulation and muscle membrane excitability than any changes in erythrocytic Na^+,K^+-ATPase activity.

An important observation was that Na^+,K^+-ATPase content and isoform expression in skeletal muscle in HD and RTx did not differ from CON. Thus the depressed Na^+,K^+-ATPase activity in these patients does not appear to be due to reduced numbers of Na^+,K^+-ATPase, or markedly different isoform expression, but rather points to defective Na^+,K^+-ATPase. It should be noted that there was a ~2-fold difference between HD and CON in the relative abundance of the α_1 and α_3 isoforms, however this was not significant. Thus, it is possible that differences in isoform expression did exist but were not detectable due to the large variability of Western blotting. Whilst no previous studies have investigated Na^+,K^+-ATPase content in uraemic human skeletal muscle, Na^+,K^+-ATPase content was normal in uraemic rat skeletal muscle (Druml 1988). The positive association found between maximal in-vitro 3-O-MFPase activity and [^{3}H]ouabain binding site content supports previous

work (Fraser *et al.* 2002), and is not surprising as greater Na^+,K^+-ATPase content would increase maximal Na^+,K^+-ATPase activity.

This laboratory has recently shown that human skeletal muscle expresses the α_1, α_2, α_3, β_1, β_2, and β_3 Na^+,K^+-ATPase isoforms (Murphy *et al.* 2004). This study confirms this expression in skeletal muscle from HD and RTx, and demonstrates that their relative abundance did not differ from healthy control subjects. These results extend the findings of no difference in Na^+,K^+-ATPase isoform mRNA expression or protein abundance in skeletal muscle membrane fractions of nephrectomized and sham-operated rats (Greiber *et al.* 1994). Importantly, the present study utilised crude muscle homogenates, which include all intracellular and sarcolemmal membranes, whilst the previous study utilised membrane fractions (*ibid*), which may not be representative of the whole muscle (Hansen *et al.* 1988).

5.4.2 Possible mechanisms of impaired maximal in-vitro Na^+,K^+-ATPase activity

The normal muscle Na^+,K^+-ATPase content found in these patients suggests that their depressed maximal in-vitro Na^+,K^+-ATPase activity is due to reduced maximal turn-over rate per pump or grossly impaired activity in some pumps, rather than a lack of Na^+,K^+-ATPase units. This finding is consistent with a previous study in erythrocytes from HD patients that reported reduced ouabain-sensitive ^{86}Rb uptake but normal [^{3}H]ouabain binding site concentration (Fervenza *et al.* 1989). Given the numerous myopathies identified in HD and RTx, the cause of reduced Na^+,K^+-ATPase activity is likely to be multi-factorial, but might reflect altered lipid composition, presence of endogenous Na^+,K^+-ATPase inhibitors or uraemic toxins. Changes in the membrane lipid composition can alter Na^+,K^+-ATPase activity without altering Na^+,K^+-ATPase content (Wu *et al.* 2004) and abnormal membrane lipid composition has been found in erythrocytes from HD (Koorts *et al.* 2002, Vasarhelyi *et al.* 1996) and RTx (Phair *et*

al. 1989), and also in kidney, liver and testis microsomal membranes from rats following RTx (Lausada *et al.* 2005, Lausada *et al.* 2002). If membrane lipid composition is also abnormal in skeletal muscle from HD and RTx, this may explain the depressed maximal in-vitro Na^+,K^+-ATPase activity observed in this study, but this is yet to be determined. In uraemia, endogenous digitalis-like factors (Stokes *et al.* 1986, Vasarhelyi *et al.* 1996) and uraemic toxins (Kaji *et al.* 1998, Minkoff *et al.* 1972) in plasma have also been shown to cause depressed Na^+,K^+-ATPase activity, whilst in RTx recipients, the immunosuppressant drugs cyclosporine and tacrolimus inhibit Na^+,K^+-ATPase activity in heart (Mardini *et al.* 2001), erythrocytes (Huang *et al.* 1996) and kidney (Lea *et al.* 1994). Conversely, glucocorticoid based immunosuppressants, such as prednisolone, increase skeletal muscle Na^+,K^+-ATPase content (Ravn *et al.* 1997) and possibly also activity (McKenna *et al.* 2003a). Further research is required to determine the mechanisms inhibiting Na^+,K^+-ATPase activity in skeletal muscle from HD patients and RTx recipients.

5.4.3 Impaired exercise performance in HD and RTx with normal [Hb]

This is the first report directly demonstrating exacerbated muscle fatiguability during repeated dynamic contractions in HD or RTx with normal [Hb] compared to CON. Further, this study shows that fatiguability did not differ between HD and RTx. This is consistent with their decreased incremental exercise performance and 3-fold higher fatigue rate in HD compared to healthy controls during repeated incremental isometric contractions of the dorsiflexor muscles (Johansen *et al.* 2005). Muscle fatiguability was elevated in HD during repeated isometric hand-grip contractions compared to RTx (Moore *et al.* 1993a), which contrasts the results from this study. However, the HD had significantly lower Hct than RTx in the previous study (*ibid*), which may explain their higher fatiguability. The findings from this study also contrast those

from Chapter 4, in which fatiguability was not different between HD and CON. The lack of difference between HD and CON in Chapter 4 is probably due to a type II error as the sample size was small (n = 6). This is further supported by the finding that the magnitude of the difference between HD and CON was similar in this (1.7-fold) and the previous (1.8-fold) study.

This is the first study to compare $\dot{V}O_{2peak}$ in HD and RTx patients with similar [Hb], and shows that $\dot{V}O_{2peak}$ did not differ between these groups. In the present study, the $\dot{V}O_{2peak}$ of the HD and RTx groups was 74% and 76% of CON, respectively, which is within the range previously reported (Kettner Melsheimer *et al.* 1987, van den Ham *et al.* 2005) and confirms the poor exercise performance observed in HD and RTx patients, which persists despite normalisation of [Hb] (McMahon *et al.* 1999, van den Ham *et al.* 2000) and is consistent with their greater local muscle fatiguability. This study shows for the first time a triangular relationship between maximal exercise performance ($\dot{V}O_{2peak}$), muscle maximal in-vitro Na^+,K^+-ATPase activity (3-O-MFPase activity) and renal function (CrCl). This strongly suggests that impaired exercise performance in HD and RTx is related to their reduced muscle Na^+,K^+-ATPase activity, which is subsequent to their depressed kidney function. Endogenous digitalis-like substances within plasma inhibit Na^+,K^+-ATPase activity in uraemic patients (Stokes *et al.* 1986, Vasarhelyi *et al.* 1996) and their plasma concentration is inversely related to CrCl (Vasdev *et al.* 1987). Thus, those with the lowest CrCl are likely to have the highest concentration of circulating Na^+,K^+-ATPase inhibitors and therefore the lowest Na^+,K^+-ATPase activity. Since the Na^+,K^+-ATPase protects muscle membrane excitability and contractility, thus delaying fatigue during muscle contractions (Clausen 2003), depressed maximal in-vitro Na^+,K^+-ATPase

activity in HD and RTx may expedite the onset of fatigue and impair exercise performance and $\dot{V}O_{2peak}$.

In the present study, knee-extensor strength corrected for body mass was subnormal in HD and RTx but did not differ from CON when expressed relative to TMCSA. This suggests that skeletal muscle contractile function is normal in HD and RTx with normal [Hb] and that the deficit in strength relative to body mass may be due to reduced lean body mass in these patients (Heaf *et al.* 2004, Woodrow *et al.* 1996). This is consistent with this study's finding of reduced TMCSA relative to body mass in HD and RTx. We also report no difference in knee-extensor strength in RTx compared to HD, which supports previous studies (Kettner Melsheimer *et al.* 1987, Nyberg *et al.* 1995, van den Ham *et al.* 2005). Not surprisingly, $\dot{V}O_{2peak}$ was correlated with TMCSA and knee-extensor peak torque. Muscle CSA is a strong predictor of muscle strength, which has previously been shown to be related to $\dot{V}O_{2peak}$ in predialysis (Leikis *et al.* 2006) and HD (Diesel *et al.* 1990, Kettner Melsheimer *et al.* 1987). This is the first report of a relationship between muscle strength and $\dot{V}O_{2peak}$ in RTx, although $\dot{V}O_{2peak}$ has previously been correlated with TMCSA in these patients (Horber *et al.* 1987). This study also found an inverse relationship between $\dot{V}O_{2peak}$ and the fatigue index. These data, together with a lack of correlation between $\dot{V}O_{2peak}$ and [Hb], support the concept that skeletal muscle abnormalities play a vital role in limiting $\dot{V}O_{2peak}$ in HD and RTx.

Physical activity levels were not measured in this study, and these have been found to be reduced in HD compared to sedentary controls (***Johansen** et al.* 2000). Thus, the poor exercise performance in HD and RTx may be due to decreased physical activity levels in these patients compared to CON. Due to stringent patient screening criteria,

the HD and RTx recruited in this study were relatively healthy and probably considerably more active than the general renal patient population. It is therefore unlikely that the physical activity levels of the HD and RTx and sedentary controls in this study were markedly different. Nonetheless, differences cannot be ruled out.

5.4.4 Normal plasma K^+ regulation during exercise in HD and RTx

The higher plasma [K^+] at rest and persisting during submaximal exercise in HD is most likely due to their severely reduced renal K^+ excretion. Therefore the change in [K^+] ($\Delta[K^+]$) from rest during exercise was calculated. The present finding of normal $\Delta[K^+]$ during exercise and $\Delta[K^+].work^{-1}$ ratio at fatigue in HD and RTx contrasts with an earlier report from this laboratory of a two- and four-fold greater $\Delta[K^+]$ and $\Delta[K^+].work^{-1}$ ratio, respectively, in HD compared to CON (Sangkabutra *et al.* 2003) and also with the results of Chapter 4. As with these previous studies however, when all data were pooled a significant correlation was found between $\dot{V}O_{2peak}$ and $\Delta[K^+].work^{-1}$ ratio, suggesting a link between maximal exercise performance and K^+ regulation during exercise. The difference in $\dot{V}O_{2peak}$ between the patient and control groups in the previous studies was 37-44% (Sangkabutra *et al.* 2003)(Chapter 4), whereas in this study it was 24-26% and, as $\dot{V}O_{2peak}$ was related to the $\Delta[K^+].work^{-1}$ ratio in all studies, this may explain the contrasting K^+ regulation data. At fatigue, the plasma volume corrected $\Delta[K^+]$ was less in HD and RTx compared to CON and reflects their lower peak workrates and presumably lower muscle K^+ release during the incremental exercise test.

Changes in arterialised venous plasma [K^+] will not accurately reflect changes in net muscle K^+ efflux (Sejersted *et al.* 2000) as arterial and venous [K^+] may be influenced by K^+ uptake and dilution of blood from inactive tissues, and incomplete K^+ equilibration between the muscle cell, interstitium, and blood (Juel *et al.* 2000b). For

example, it has been shown that muscle interstitial [K^+] can exceed arterial or venous [K^+] by 2-5 mM during exercise (Green *et al.* 2000b, Juel *et al.* 2000b). This may then explain the apparently normal plasma K^+ regulation of HD and RTx during exercise despite their substantially reduced muscle maximal in-vitro Na^+,K^+-ATPase activity. Further studies investigating muscle K^+ release are required to determine whether extrarenal K^+ regulation is impaired during exercise in HD and RTx.

5.5 CONCLUSIONS

This study shows that maximal exercise performance remained significantly reduced in HD and RTx with normal [Hb], but was not different between these groups. This study also shows that plasma [K^+] regulation was not impaired despite depressed skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity in HD and RTx. The depressed activity is likely due to direct inhibition of Na^+,K^+-ATPase since skeletal muscle [^{3}H]ouabain binding site content and Na^+,K^+-ATPase isoform abundance were unchanged in HD and RTx. Finally, these results suggest a direct link between impaired maximal exercise performance, skeletal muscle maximal Na^+,K^+-ATPase activity, and renal function.

CHAPTER 6. DIGOXIN ADMINISTRATION, EXERCISE AND SKELETAL MUSCLE Na^+,K^+-ATPase CONTENT AND ACTIVITY IN HEALTHY HUMANS

6.1 INTRODUCTION

The skeletal muscle Na^+,K^+-ATPase protects muscle membrane excitability and is vital for skeletal muscle contractility (Nielsen *et al.* 2000). Therefore, any inhibition of Na^+,K^+-ATPase activity could potentially impair muscle contractility and exercise performance. Indeed, in isolated rat muscles, inhibition of the Na^+,K^+-ATPase by a high concentration of ouabain (10 μM) increased the rate of force decline by 3-fold (Nielsen *et al.* 1996). This laboratory has previously shown that skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity is depressed during fatiguing exercise (Aughey *et al.* 2006, Aughey *et al.* 2005, Fraser *et al.* 2002, Leppik *et al.* 2004, Murphy *et al.* 2006b, Petersen *et al.* 2005) and that maximal in-vitro Na^+,K^+-ATPase activity is related to $\dot{V}O_{2peak}$ (Fraser *et al.* 2002) and time to fatigue during submaximal cycling (Leppik *et al.* 2004). Furthermore, in Chapter 4 of this thesis, substantially depressed maximal in-vitro Na^+,K^+-ATPase activity in skeletal muscle of haemodialysis patients and renal transplant recipients was evident and was related to their impaired maximal incremental exercise performance. Thus, the exercise-induced depression in skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity may expedite the onset of fatigue. However, whether reduced skeletal muscle Na^+,K^+-ATPase activity actually impairs exercise performance in humans has not been thoroughly investigated.

Digoxin is a specific inhibitor of the Na^+,K^+-ATPase and is commonly used to treat patients with severe heart failure (Goldberg *et al.* 2007). The target organ of digoxin

is the left ventricular myocardium where it exerts a positive inotropic effect (Fozzard *et al.* 1985). However, only 3-4% of a given digoxin dose binds to the heart (Steiness 1978), with the largest proportion (~50%) binding to skeletal muscle, and the remainder distributed among the plasma and other tissues (Doherty *et al.* 1967, Steiness 1978). In congestive heart failure patients (CHF), a non-defined digoxin dose occupied 9% of skeletal muscle Na^+,K^+-ATPase, as measured by [^{3}H]ouabain binding following clearance of bound digoxin by digoxin antibody fragments (Schmidt *et al.* 1995). Furthermore, digoxin exacerbated K^+ loss from exercising muscles but time to fatigue was not altered (*ibid*). However, exercise performance is severely impaired in CHF patients due to numerous myopathies (Mettauer *et al.* 2006) that may have outweighed the effects of a small reduction in Na^+,K^+-ATPase content on muscle fatiguability. In healthy humans, two weeks of high dose digitalisation (0.50 $mg.d^{-1}$) had no effect on $\dot{V}O_{2peak}$, but Na^+,K^+-ATPase activity or content were not measured (Sundqvist *et al.* 1983). As two weeks is sufficient time for compensatory upregulation of Na^+,K^+-ATPase activity in guinea-pig myocardium (Bonn *et al.* 1978), Na^+,K^+-ATPase activity may not have been reduced at the time of testing. Conversely, several studies have found no evidence of compensatory upregulation of Na^+,K^+-ATPase content in cardiac or skeletal muscle from CHF patients (Schmidt *et al.* 1993a, Schmidt *et al.* 1991a, 1993b) but those studies were cross-sectional and lacked a pre-digitalis biopsy. Thus, it remains to be determined whether digoxin causes an inhibition of Na^+,K^+-ATPase activity and thus reduces maximal exercise performance in healthy humans.

This study therefore investigated the effects of 14 days of digoxin or placebo administration on skeletal muscle Na^+,K^+-ATPase content and maximal in-vitro activity and exercise performance in healthy humans. It was hypothesised that skeletal

muscle Na^+,K^+-ATPase content and maximal in-vitro activity, and time to fatigue during cycling exercise, would each be depressed following chronic digoxin administration.

6.2 METHODS

6.2.1 Subjects

Ten recreationally active subjects (one female, nine male; age, 26.1 ± 5.9 yr; height, 178.4 ± 9.1 cm; body mass 75.7 ± 11.3 kg, $\dot{V}O_{2peak}$, 3.67 ± 0.42 l.min^{-1}, mean ± SD) gave written informed consent and participated in the study. Sample size was calculated using Power and Precision software based on the expected Na^+, K^+-ATPase content means. Comparing by two-tailed t-test the expected muscle Na^+,K^+-ATPase content difference, of 300 pmol.g wet weight^{-1} for placebo, assuming a 10% lesser content with digoxin, SD within each group of 30 pmol.g wet weight^{-1}, α =0.05, a sample size of n = 10 per group yielded a power of 99%. All subjects underwent an initial medical examination to screen for abnormal plasma electrolyte concentrations, kidney function, rest and exercise ECG, and history of adverse cardiovascular events. All protocols and procedures were approved by the Victoria University Human Research Ethics Committee and Alfred Hospital Ethics Committee

6.2.2 Study design

Subjects were randomly allocated to either a digoxin (DIG) or placebo (CON) treatment group. Subjects were given either a typical clinical digoxin dose of 0.25 mg.d^{-1} or a placebo for 14 days. Trials were conducted in a crossover, double-blind, randomised, counterbalanced design. For ethical reasons, the attending medical practitioner was non-blinded. On the fourteenth day of the treatment period subjects performed an exercise test and had muscle biopsies taken. After a four week washout

period, the groups switched to the alternative treatment for a further two weeks and then repeated the exercise test and biopsies. Thus, for those subjects taking digoxin first, the effective digoxin clearance time was six weeks, i.e. the standard four week washout plus the two weeks of placebo treatment. Similarly, for the placebo group, the time between biopsies was six weeks.

6.2.3 Exercise test

Subjects cycled on an electrically braked cycle ergometer (Lode, Groningen, Holland) for 10 min at 33% $\dot{V}O_{2peak}$ followed by a two min pause, 10 min at 67% $\dot{V}O_{2peak}$, two min pause, and then continued until fatigue at 90% $\dot{V}O_{2peak}$. Subjects completed a total of four exercise tests. The first two were to determine the within-subject variability of the time to fatigue, and the final two trials were the digoxin or placebo experimental trials.

6.2.4 Muscle biopsies

After injection of a local anaesthetic into the skin and fascia (1% Xylocaine), a small incision was made and a muscle sample taken (~120mg) using a Stille biopsy needle. A muscle biopsy was taken from the middle third of the vastus lateralis at rest, immediately after the 67% $\dot{V}O_{2peak}$ exercise bout, at fatigue, and at three hours post-exercise (+3h). The sample was immediately frozen in liquid N_2 until later analysis of maximal in-vitro Na^+,K^+-ATPase activity (3-O-MFPase), and content ($[^3H]$ouabain binding site content).

6.2.5 Protein content

Skeletal muscle protein content was determined spectrophotometrically (Lowry *et al.* 1951). All Na^+,K^+-ATPase content and maximal in-vitro activity results were expressed relative to muscle protein content to correct for exercise-induced changes in muscle water content.

6.2.6 3-O-MFPase assay

Maximal in-vitro Na^+,K^+-ATPase activity was measured using the 3-O-MFPase assay (Fraser *et al.* 1998) as described in Chapter 3, except that the assay was performed on a different fluorometer (Photon Technology International, Birmingham, NJ). Excitation and emission wavelengths were 475 nm and 515 nm, respectively with 4 nm slit-widths. The intra-assay coefficient of variation (CV) for the 3-O-MFPase assay was 18%.

6.2.7 [^{3}H]ouabain binding site content

Skeletal muscle total Na^+,K^+-ATPase content was determined in quadruplicate by vanadate-facilitated [^{3}H]ouabain binding site content analysis (Nørgaard *et al.* 1984a, Nørgaard *et al.* 1983) as described in Chapter 3. The intra- and inter-assay CV for the [^{3}H]ouabain binding site assay were 9% and 14%, respectively.

As digoxin and ouabain bind competitively to the digitalis receptors on the Na^+,K^+-ATPase, the [^{3}H]ouabain binding assay will not detect any Na^+,K^+-pumps that are occupied by digoxin. Thus, the [^{3}H]ouabain binding assay was performed with and without prior incubation in digoxin antibody fragments (F_{ab}, Digibind®, GlaxoSmithKline, Melbourne, Australia), which has been shown to remove ~97% of bound digoxin from skeletal muscle (Schmidt *et al.* 1991b). Briefly, muscle samples were incubated for 16 h at 30ºC in tris-vanadate-sucrose (TVS) buffer containing 0.5 μM F_{ab}, after which the standard [^{3}H]ouabain binding site content assay was performed.

To verify that use of F_{ab} would enable recovery of bound Na^+,K^+-ATPase and that the 16 h incubation would not affect [^{3}H]ouabain binding site content in healthy human skeletal muscle, and thus test the efficacy of the digoxin F_{ab} in this laboratory, two control experiments were conducted.

6.2.7.1 Recovery

Muscle samples from three healthy subjects were each divided into three groups, for incubation in digoxin, digoxin+F_{ab}, and control (n = 3 per group). The digoxin and digoxin+F_{ab} samples were incubated in 10 nM digoxin for 60 min at 37ºC. The digoxin+F_{ab} samples were subsequently incubated overnight in 0.5 μM digoxin F_{ab}, the control samples did not undergo any incubation. The digoxin, digoxin+F_{ab}, and control samples were then analysed for [^{3}H]ouabain binding site content. The clearance of bound digoxin was calculated from the [^{3}H]ouabain binding results of the digoxin, digoxin+F_{ab}, and control samples, using the formula: ((digoxin+F_{ab} – digoxin) ÷ (control – digoxin)) × 100

6.2.7.2 Incubation effects

To assess the effect of the 16 h overnight incubation, an additional three human skeletal muscle samples, from different subjects, were used. To compare the effect of incubation in F_{ab} versus incubation in standard TVS buffer, samples were incubated for 16 h at 30ºC in TVS buffer or buffer containing 0.5 μM F_{ab} (n = 3 per group). To determine if the 16 h incubation in F_{ab} or TVS buffer affected subsequent [^{3}H]ouabain binding, an additional 3 samples did not undergo any incubation (control). The TVS buffer, F_{ab}, and control samples were then analysed for [^{3}H]ouabain binding site content.

6.2.8 Skeletal muscle Na^+,K^+-ATPase activity-to-content ratio

To determine the exercise effects on Na^+,K^+-ATPase activity per pump, the ratio between 3-O-MFPase activity expressed per g protein and [^{3}H]ouabain binding site content expressed relative to muscle protein content without removal of bound digoxin (activity-to-content ratio) was calculated for combined CON and DIG data (n = 20).

6.2.9 Statistics

All data are presented as mean ± SD. Data were analysed using a repeated-measures two-way ANOVA (treatment, exercise), with two exceptions. For the digoxin F_{ab} verification experiments a one-way ANOVA was used, and for comparison of [^{3}H]ouabain binding site content performed with and without digoxin F_{ab}, a paired t-test was used. When significant treatment or exercise main effects were detected, pairwise comparisons were made using the Least Significant Difference (LSD) test. There were no significant treatment-by-exercise interactions. Statistical significance was accepted at $P < 0.05$.

6.3 RESULTS

6.3.1 Serum digoxin and exercise performance

Serum [digoxin] at rest on d 14 was 0.8 ± 0.2 nM in the digoxin trial and < 0.4 nM (detection limit) in the control trial.

The coefficient of variation for the time to fatigue during the two variability trials was low, being 5.9 ± 5.7%. Time to fatigue during exercise at 90% $\dot{V}O_{2peak}$ was not different between CON and DIG (254 ± 125 and 262 ± 156 s, respectively). There was also no difference in $\dot{V}O_2$ between DIG and CON during exercise (Table 6.1).

Table 6.1. Oxygen consumption ($L.min^{-1}$) during cycle exercise in digoxin (DIG) and control (CON) trials at 33%, 67% and 90% $\dot{V}O_{2peak}$.

Workrate	DIG	CON
33% $\dot{V}O_{2peak}$	1.21 ± 0.19	1.19 ± 0.22
67% $\dot{V}O_{2peak}$	2.63 ± 0.51	2.68 ± 0.54
90% $\dot{V}O_{2peak}$	3.61 ± 0.28	3.55 ± 0.44

Data expressed as mean ± SD, n = 10.

6.3.2 F_{ab} control experiments

Incubation of human skeletal muscle biopsy samples in 10 nM digoxin reduced [^{3}H]ouabain binding sites by 32% compared to control ($P < 0.05$, Figure 6.1). Following incubation in F_{ab}, the [^{3}H]ouabain binding site content was not different to control ($P = 0.92$). Thus the F_{ab} removed 96% of bound digoxin (i.e. 96% of the difference between control and digoxin) and enabled almost complete quantification of Na^+,K^+-ATPase (Figure 6.1). Overnight incubation in standard TVS buffer or in buffer containing F_{ab} had no effect on [^{3}H]ouabain binding site content, indicating that the overnight incubation did not result in any loss of Na^+,K^+-ATPase ($P = 0.93$, main effect, Figure 6.1).

6.3.3 Muscle protein content

6.3.3.1 Digoxin

The repeated-measures ANOVA revealed no effect of digoxin on skeletal muscle protein content.

6.3.3.2 Exercise

Skeletal muscle protein content was lower ($P < 0.01$ for both) at 67% (0.183 ± 0.009 g protein. g muscle wet weight^{-1}) and fatigue (0.182 ± 0.007 g protein. g muscle wet

weight^{-1}) compared to rest (0.197 ± 0.008 g protein. g muscle wet weight^{-1}) and +3 h (0.204 ± 0.007 g protein. g muscle wet weight^{-1}).

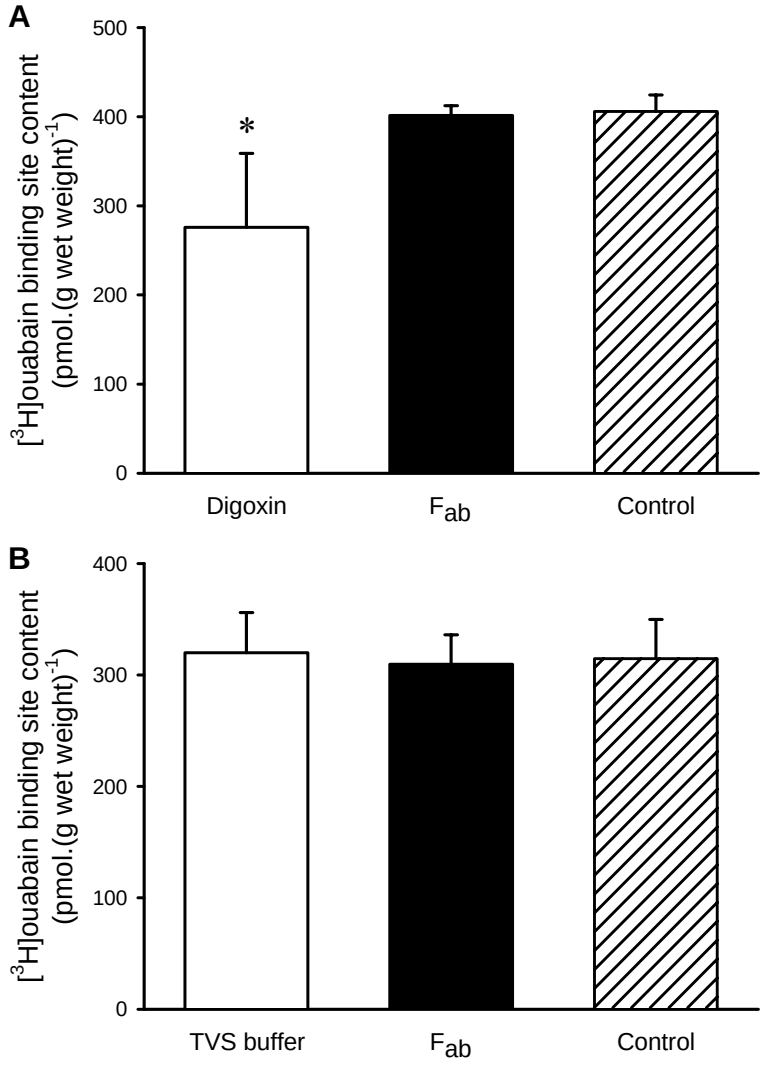

Figure 6.1. F_{ab} control experiments.

[^{3}H]ouabain binding site content, (A) in human skeletal muscle samples incubated in 10 nM digoxin for 60 min at 37ºC (Digoxin), followed by 16 h incubation in 0.5 µM digoxin F_{ab} (F_{ab}) or without digoxin incubation and F_{ab} wash (Control); (B) in human skeletal muscle samples (different from A) incubated for 16 h at 30ºC in tris-vanadate-sucrose (TVS) buffer (TVS buffer), or TVS buffer containing 0.5 µM F_{ab} (F_{ab}), or without any incubation (Control). Data are mean ± SD, n = 3. * less than F_{ab} and Control ($P < 0.05$).

6.3.4 Muscle [^{3}H]ouabain binding site content before clearance of bound digoxin by F_{ab}

6.3.4.1 Digoxin

[^{3}H]ouabain binding site content was not different between CON and DIG, whether expressed relative to muscle wet weight or per g protein.

6.3.4.2 Exercise

Expressed relative to muscle wet weight, [^{3}H]ouabain binding site content at 67% tended to be 10% higher than rest ($P < 0.06$) and was higher than +3h by 12% ($P < 0.01$**,** Figure 6.2). [^{3}H]ouabain binding at fatigue also tended to be higher than +3h ($P < 0.10$). Relative to muscle protein content, [^{3}H]ouabain binding site content showed a similar pattern to when expressed per gram wet weight (Figure 6.2). Compared to rest, [^{3}H]ouabain binding was increased by 21% at 67% ($P < 0.01$) and tended to be higher at fatigue ($P < 0.06$). [^{3}H]ouabain binding site at fatigue and 67% were also higher than +3h by 20% and 25%, respectively ($P < 0.01$).

6.3.5 [^{3}H]ouabain binding site content after clearance of bound digoxin by F_{ab}

6.3.5.1 Digoxin

[^{3}H]ouabain binding site content measured after clearance of bound digoxin by F_{ab} did not change as a result of chronic digoxin treatment when expressed relative to muscle wet weight or protein content (Figure 6.3).

6.3.5.2 Exercise

When expressed relative to muscle wet weight, [^{3}H]ouabain binding site content was not different between CON and DIG (Figure 6.3). When expressed per g protein, [^{3}H]ouabain binding site content at fatigue tended to be higher than rest ($P < 0.06$) and was higher than +3 h ($P < 0.05$, Figure 6.3).

6.3.6 Comparison of [^{3}H]ouabain binding site content with and without F_{ab}

Pooling of all [^{3}H]ouabain binding site measures performed without prior incubation in digoxin F_{ab} yielded a mean content of 364 ± 57 pmol.(g wet weight)$^{-1}$ (n = 80). This did not differ from pooled [^{3}H]ouabain binding site measures performed after incubation in digoxin F_{ab} 374 ± 35 pmol.(g wet weight)$^{-1}$ (n = 80, P < 0.60). Similarly, there was no difference in pooled [^{3}H]ouabain binding, without or with prior incubation in F_{ab}, when expressed relative to muscle protein content (1962 ± 417 and 1999 ± 300 pmol.g protein^{-1}, respectively, n = 80, P < 0.70).

6.3.7 Skeletal muscle maximal in-vitro 3-O-MFPase activity

6.3.7.1 Digoxin

Maximal in-vitro 3-O-MFPase activity was not different between CON and DIG when expressed relative to muscle wet weight, or protein content (Figure 6.4).

6.3.7.2 Exercise

Maximal in-vitro 3-O-MFPase activity, expressed relative to muscle wet weight, was depressed at fatigue by 15% compared to rest (P < 0.05) and had recovered at +3h (P < 0.01, Figure 6.4). When expressed relative to muscle protein content however, maximal in-vitro 3-O-MFPase activity did not change significantly as a result of exercise (Figure 6.4).

6.3.8 Skeletal muscle Na^+,K^+-ATPase activity-to-content ratio

The activity-to-content ratio was reduced at fatigue by 16% compared to rest (P < 0.05) and had recovered by +3 h (P < 0.05) (rest, 810 ± 220; 67%, 740 ± 160; fatigue, 680 ± 160; +3 h, 790 ± 160 min^{-1}).

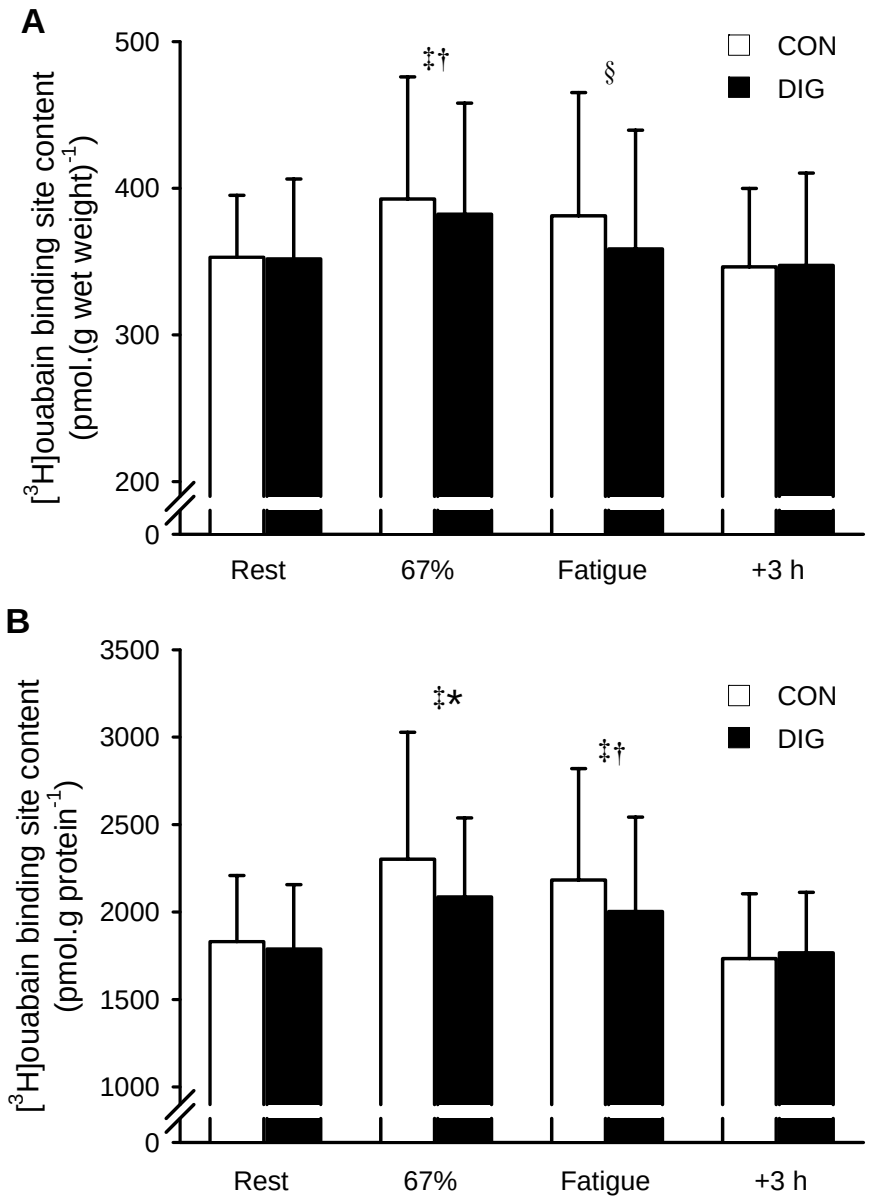

Figure 6.2. Effects of chronic digoxin treatment and acute exercise on Na^+,K^+-ATPase content in human skeletal muscle.

Skeletal muscle [^{3}H]ouabain binding site content expressed relative to (A) muscle wet weight and (B) muscle protein content, before, during and after cycling exercise in CON and DIG. Biopsies were taken at rest, after cycling at 67% $\dot{V}O_{2peak}$, immediately following cycling at 90% $\dot{V}O_{2peak}$ to fatigue, and at 3 h post-fatigue. Data are mean ± SD, n = 10. ‡ greater than +3 h ($P < 0.05$), * > rest ($P < 0.01$), † tended to be > rest ($P < 0.06$), § tended to be > +3h ($P < 0.1$).

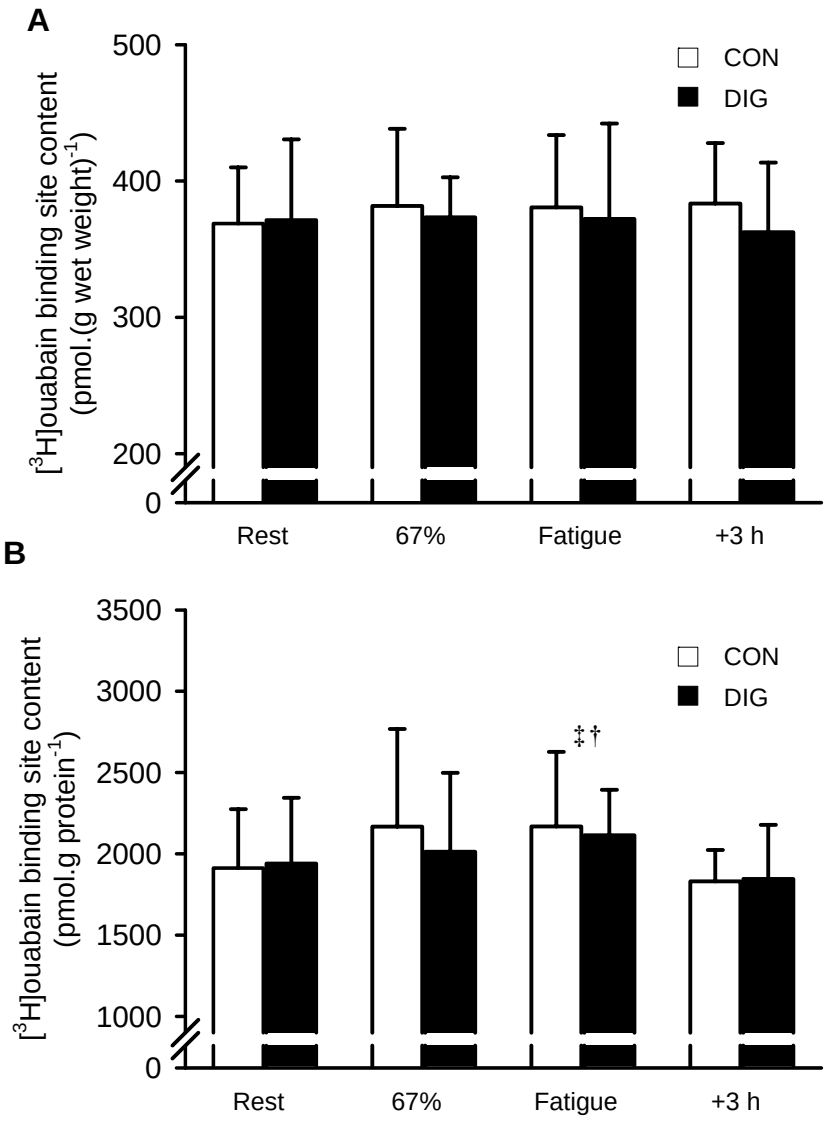

Figure 6.3. Effects of chronic digoxin treatment and acute exercise on Na^+,K^+-ATPase content in human skeletal muscle, measured after clearance of bound digoxin by F_{ab}.

Skeletal muscle [^{3}H]ouabain binding site content with prior incubation in digoxin Fab, expressed relative to (A) muscle wet weight and (B) muscle protein content, before, during and after cycling exercise in CON and DIG. Biopsies were taken at rest, after cycling at 67% $\dot{V}O_{2peak}$, immediately following cycling at 90% $\dot{V}O_{2peak}$ to fatigue, and at 3 h post-fatigue. Data are mean ± SD, n = 10. ‡ > +3h ($P < 0.05$), † tended to be > rest ($P < 0.06$).

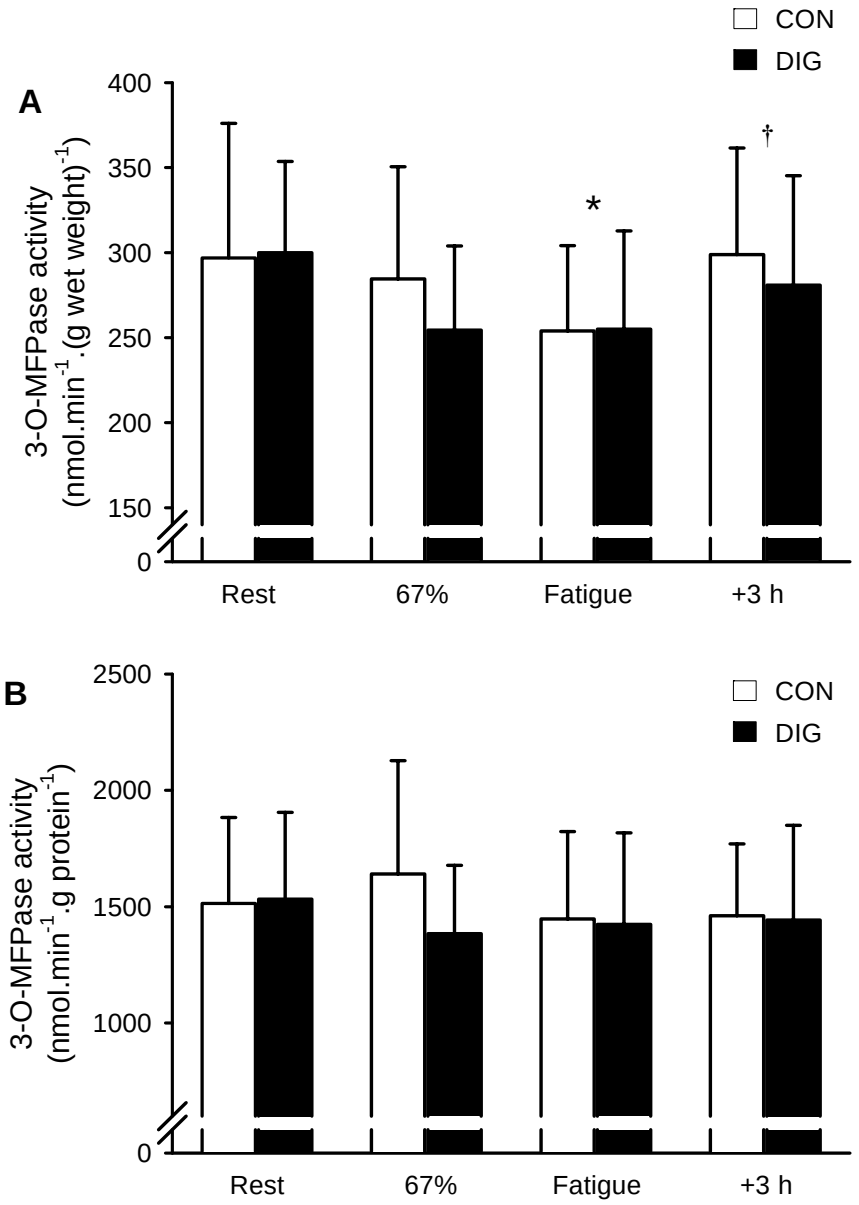

Figure 6.4. Effects of chronic digoxin treatment and acute exercise on maximal in-vitro Na^+,K^+-ATPase activity.

Skeletal muscle maximal in-vitro 3-O-MFPase activity before, during and after cycling exercise in CON and DIG, expressed relative to muscle wet weight (A) and muscle protein content (B). Biopsies were taken at rest, after cycling at 67% $\dot{V}O_{2peak}$, immediately following cycling at 90% $\dot{V}O_{2peak}$ to fatigue, and at 3 h post-fatigue. Data are mean ± SD, n = 10. * less than rest ($P < 0.05$), † greater than fatigue ($P < 0.01$).

6.4 DISCUSSION

This study investigated the effects of chronic digoxin administration on skeletal muscle Na^+,K^+-ATPase content and maximal in-vitro activity and exercise performance in healthy humans and reports two novel and surprising findings. Firstly, digoxin administration for 14 days did not affect skeletal muscle Na^+,K^+-ATPase content, maximal in-vitro activity, or exercise performance. Secondly, acute exercise of moderate duration caused a transient increase in skeletal muscle Na^+,K^+-ATPase content, as assessed by [^{3}H]ouabain binding, but did not depress maximal in-vitro activity.

6.4.1 Serum digoxin concentration

Recent findings indicate that a therapeutic serum [digoxin] between 0.65 – 1.15 nM is preferred for reducing mortality rates and hospitalisations and improving clinical symptoms in CHF patients (Ahmed *et al.* 2006, Wang *et al.* 2005). Thus, the serum [digoxin] of 0.8 ± 0.2 nmol.l^{-1} in this study was within the currently accepted clinical range. Furthermore, low serum [digoxin] was as effective as higher concentrations for the treatment of heart failure. The inotropic mechanism of action of digoxin on the heart is thought to be via inhibition of the myocardial Na^+,K^+-ATPase (Fozzard *et al.* 1985). Digoxin may also directly stimulate myocardial sarcoplasmic reticulum Ca^{2+} release (Nishio *et al.* 2004), however this has been disputed (Altamirano *et al.* 2006). This implies that low serum [digoxin] effectively inhibits myocardial Na^+,K^+-ATPase, however this has not been tested.

6.4.2 No effect of digoxin on Na^+,K^+-ATPase content or maximal in-vitro activity

6.4.2.1 Na^+,K^+-ATPase content

This is the first study to investigate the effects of digoxin administration on skeletal muscle Na^+,K^+-ATPase content in healthy humans. This study showed that

[^{3}H]ouabain binding site content, without prior incubation in digoxin F_{ab}, was not affected by digoxin. This surprising result suggests that compensatory upregulation of Na^+,K^+-ATPase content might have occurred following digoxin administration, as has previously been shown in erythrocytes in humans (Ford *et al.* 1979) and in pigs (Whittaker *et al.* 1983). However, if this had occurred, an increased [^{3}H]ouabain binding site content would be expected in the DIG samples measured after clearance of bound digoxin by incubation in F_{ab}. Yet, there was no difference between CON and DIG, suggesting that compensatory upregulation did not occur with digoxin. This laboratory has also separately investigated the effects of two weeks of chronic digoxin treatment on Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoform mRNA expression and protein abundance. In resting muscle, digoxin elevated total α mRNA expression (sum of α_1, α_2, and α_3) by 1.9-fold and total β mRNA expression (sum of β_1, β_2 and β_3) by 0.6-fold ($P < 0.05$ for both) (Gong *et al.* 2005). Furthermore, there was a tendency for digoxin to increase α_2 protein abundance by 44% ($P < 0.10$, *ibid*). These findings indicate that digoxin increased gene transcription or decreased gene degradation of the Na^+,K^+-ATPase α_1-α_3 and β_1-β_3 isoforms but, with the possible exception of the α_2 isoform, this did not translate into increased protein abundance. This corresponds with the apparent lack of effect of digoxin on the more functional measure of Na^+,K^+-ATPase content, but also suggests that digoxin did modulate Na^+,K^+-ATPase gene expression, lending support to the possibility of compensatory upregulation.

The lack of difference in [^{3}H]ouabain binding site content in the DIG samples before and after clearance of bound digoxin also suggests that there was no detectable occupancy of skeletal muscle Na^+,K^+-ATPase by digoxin. This was surprising, as the dose of digoxin given in this study (0.25 $mg.d^{-1}$, or ~3 $\mu g.kg^{-1}.d^{-1}$) was similar to doses used in other studies that have reported reduced [^{3}H]ouabain binding site

content following digoxin administration (Schmidt *et al.* 1995, Schmidt *et al.* 1991a, 1993b). However, those studies involved patients with heart failure, who probably had a lower muscle- to body-mass ratio than the young, recreationally active subjects used in this study (Strassburg *et al.* 2005). Therefore, in this study, the digoxin dose was likely dissipated over a larger muscle mass, thus minimising its effects. Unfortunately, skeletal muscle [digoxin] was not measured so this cannot be confirmed, however, in keeping with this, the serum [digoxin] in this study was lower than the serum [digoxin] (1.2 – 2.3 nM) in previous studies reporting digoxin occupancy of Na^+,K^+-ATPase (Schmidt *et al.* 1993a, Schmidt *et al.* 1995). Furthermore, healthy subjects taking a similar dose of digoxin (3.1 $\mu g.kg^{-1}.d^{-1}$) for 2-3 weeks had a serum [digoxin] of only 0.48 ± 0.16 nM and a skeletal muscle [digoxin] of 23.2 ± 11.8 nmol.kg dry $weight^{-1}$ (Jogestrand *et al.* 1981). Based on a muscle water content of 70 % w/w (Schmidt *et al.* 1993b) and a [^{3}H]ouabain binding site content of 364 pmol.g wet $weight^{-1}$, this corresponds to an occupancy of only ~2 % of total skeletal muscle Na^+,K^+-ATPase. Together, this suggests that a typical clinical dose of digoxin may not provide adequate digitalisation in healthy subjects and therefore may explain the lack of effect of digoxin in this study. In addition, it is possible that the inability to detect an effect of digoxin on Na^+,K^+-ATPase content may be due to the variability of the [^{3}H]ouabain binding site assay. Studies on heart failure patients have reported a skeletal muscle Na^+,K^+-ATPase occupancy by digoxin of 9, 13, and 35 % (Green *et al.* 2001, Schmidt *et al.* 1995, Schmidt *et al.* 1993b). As the variability of the [^{3}H]ouabain binding assay was 14 % in this study, it seems likely that the assay was not sufficiently sensitive to detect the small digoxin binding. It is also possible that with only 10 subjects the study lacked adequate power to detect a difference. However, the effect size of digoxin treatment was very small (η_p^2 = 0.031) and

therefore additional subjects would make little difference to the power or probability value.

6.4.2.2 Na^+,K^+-ATPase maximal in-vitro activity

This is the first study to investigate the effects of chronic digoxin administration on Na^+,K^+-ATPase activity in human skeletal muscle. This finding of no difference in skeletal muscle maximal in-vitro 3-O-MFPase activity between CON and DIG indicates that digoxin had no affect on Na^+,K^+-ATPase maximal activity and is in keeping with the lack of effect of digoxin on Na^+,K^+-ATPase content.

6.4.3 Lack of effect of digoxin on exercise performance

Neither time to fatigue nor $\dot{V}O_2$ during the incremental cycling test were affected by digitalisation. Additional experiments conducted on these subjects in this laboratory have also shown that digoxin had no effect on knee-extensor isokinetic peak torque (DIG 325 ± 67, CON 315 ± 70 Nm) or fatiguability (% decline in peak torque) during 50 maximal contractions (DIG 53 ± 9, CON 57 ± 10 %) (Gong *et al.* 2005). Muscle contractions increase muscle extracellular $[K^+]$ ($[K^+]_e$) (Sejersted *et al.* 2000), and high $[K^+]_e$ has been associated with depressed force development in isolated rat muscles (Clausen *et al.* 1993). Activation of the skeletal muscle Na^+,K^+-ATPase counters this muscle K^+ loss (Sejersted *et al.* 2000) and restores contractility in muscles paralysed by high $[K^+]_e$ (Clausen *et al.* 1993). This laboratory has previously shown that skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity is related to $\dot{V}O_{2peak}$ (Fraser *et al.* 2002) and time to fatigue during submaximal cycling (Leppik *et al.* 2004). In Chapter 3 of this thesis, skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity was depressed at fatigue and, in Chapter 4, substantially depressed maximal in-vitro Na^+,K^+-ATPase activity in skeletal muscle of haemodialysis patients and renal transplant recipients was evident and was related to

their impaired maximal incremental exercise performance. The lack of effect of digoxin on exercise performance is probably explained by the unchanged skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity in the present study. This is supported by data from a companion study performed in this laboratory, which also showed no effect of digoxin on peak arterial plasma [K^+] (DIG 5.40 ± 0.71, CON 5.26 ± 0.54 mM at fatigue) during the cycling exercise protocol reported here (Sostaric *et al.* 2005).

6.4.4 Effects of exercise on Na^+,K^+-ATPase content and maximal in-vitro activity

6.4.4.1 Na^+,K^+-ATPase content

A transient increase in [^{3}H]ouabain binding sites with exercise was detected when expressed per g protein or without removal of bound digoxin. This is the first time an increase in [^{3}H]ouabain binding has been reported following a single exercise bout of moderate duration. A 13 % increase in [^{3}H]ouabain binding has been found following a 100 km run lasting ~10 h, and was attributed to synthesis of new pumps (Overgaard *et al.* 2002). In the present study, there was a tendency for increased [^{3}H]ouabain binding site content after only 20 min of exercise, which is probably too rapid for Na^+,K^+-ATPase synthesis. An alternative explanation is that the increase was caused by translocation of Na^+,K^+-ATPase subunits to the sarcolemma from undefined intracellular stores, as previously reported following exercise in humans (Juel *et al.* 2000a) and rats (Juel *et al.* 2001, Tsakiridis *et al.* 1996). Translocation cannot be confirmed in this study however, as the [^{3}H]ouabain binding site analysis was performed on non-fractionated muscle biopsy samples, and thus does not allow differentiation between sarcolemmal, t-tubular and intracellular Na^+,K^+-ATPase. Another possibility is that the increased [^{3}H]ouabain binding site content was due to formation of functioning Na^+,K^+-ATPase from pre-existing individual

Na^+,K^+-ATPase subunits. Again, this possibility cannot be confirmed in the present study. This finding is at odds with Chapter 3 of this thesis and with several previous studies from this laboratory (Aughey *et al.* 2006, Aughey *et al.* 2005, Leppik *et al.* 2004, Murphy *et al.* 2006b), and elsewhere (Fowles *et al.* 2002b) that reported no change in [^{3}H]ouabain binding site content following exercise. It is possible that the exercise protocol employed in the present study may account for the disparity as this protocol has not previously been used. This seems unlikely however, given the diverse exercise protocols previously investigated, including dynamic (Chapter 3) and isometric (Fowles *et al.* 2002b) knee-extensions, and sprint (Aughey *et al.* 2006), incremental (Aughey *et al.* 2005), and prolonged cycling (Leppik *et al.* 2004, Murphy *et al.* 2006b). An alternative explanation, is that the previous studies have not corrected [^{3}H]ouabain binding results for likely fluid shifts during exercise (Chapter 3)(Fowles *et al.* 2002b, Leppik *et al.* 2004, Murphy *et al.* 2006b) or have not detected any fluid shifts, as determined by muscle protein content pre- and post-exercise (Aughey *et al.* 2006, Aughey *et al.* 2005). Again, this does not appear to be the cause of the discrepancy, as further analysis of the results from Chapter 3 show that [^{3}H]ouabain binding site content was still not different from rest at fatigue when expressed relative to muscle protein content (rest 1805 ± 504, fatigue 1893 ± 552 pmol.g^{-1} protein, $P < 0.80$). Thus, the reason for the different exercise effect on [^{3}H]ouabain binding between this and previous studies remains unclear, however it cannot be excluded that this result is an artefact caused by the variability of the assay.

6.4.4.2 Na^+,K^+-ATPase maximal in-vitro activity

Maximal in-vitro 3-O-MFPase activity expressed per g protein was not affected by exercise, suggesting that the depression in maximal in-vitro 3-O-MFPase activity per g wet weight was due to an exercise-induced increase in muscle water content.

Numerous studies have found reduced maximal in-vitro 3-O-MFPase activity following fatiguing exercise of varied intensity and duration which was still evident when results were expressed relative to muscle protein content (Aughey *et al.* 2006, Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Leppik *et al.* 2004, Murphy *et al.* 2006b, Sandiford *et al.* 2004). The reason for the lack of decrease in maximal in-vitro 3-O-MFPase activity in this study is unclear, but may be related to the different exercise protocol used in this study, which has not formerly been investigated. Again, this seems unlikely given the diverse exercise protocols previously employed that have caused depressed maximal in-vitro 3-O-MFPase activity, including one that used incremental cycling, albeit of a different duration (Aughey *et al.* 2005).

Given the increased Na^+,K^+-ATPase content during and immediately after exercise, maximal in-vitro Na^+,K^+-ATPase activity would be expected to follow a similar pattern, however maximal in-vitro Na^+,K^+-ATPase activity was unchanged. Thus, there was a reduction in the Na^+,K^+-ATPase activity-to-content ratio. Results from Chapter 3 and previous studies (Aughey *et al.* 2006, Aughey *et al.* 2005, Fowles *et al.* 2002b, Leppik *et al.* 2004, Murphy *et al.* 2006b), in which maximal in-vitro Na^+,K^+-ATPase activity was depressed despite unchanged Na^+,K^+-ATPase content, imply a similar occurrence. The cause of the reduced activity-to-content ratio has not yet been determined, but free radical damage may be involved, as this laboratory has shown that the exercise-induced depression in maximal in-vitro Na^+,K^+-ATPase activity was ameliorated by infusion of the anti-oxidant N-acetylcysteine (McKenna *et al.* 2006a). Previous studies have also speculated that increased intracellular Ca^{2+} concentrations may play a role (Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002).

6.5 CONCLUSIONS

Digoxin administration for 14 days elevated serum [digoxin] to therapeutic levels in healthy subjects. However, this did not alter skeletal muscle Na^+,K^+-ATPase content or maximal in-vitro activity or impair exercise performance. This might be due to inadequate digitalisation caused by their larger muscle mass compared to CHF patients. A surprising finding was that exercise caused a transient increase in Na^+,K^+-ATPase content, but no decline in in-vitro Na^+,K^+-ATPase maximal activity Exercise also depressed the Na^+,K^+-ATPase activity-to-content ratio. These results suggest that chronic administration of a typical digoxin dose does not affect skeletal muscle Na^+,K^+-ATPase or exercise performance in healthy humans.

CHAPTER 7. GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 GENERAL DISCUSSION

This thesis reports novel findings regarding the influence of acute exercise, renal disease, and digoxin on skeletal muscle Na^+,K^+-ATPase in humans. It also extends the current knowledge on the importance of skeletal muscle Na^+,K^+-ATPase for exercise performance in both health and disease. The findings of this thesis have been discussed in detail in their respective chapters. Thus, this chapter integrates where possible, and makes concluding comments regarding these findings. Additionally, this chapter identifies areas of importance for future research regarding skeletal muscle Na^+,K^+-ATPase that arise from this thesis.

7.1.1 Acute exercise effects on skeletal muscle Na^+,K^+-ATPase in-vitro maximal activity and content

The effects of acute exercise and recovery on the skeletal muscle Na^+,K^+-ATPase were examined during both brief intense exercise (Study 1, Chapter 3) and moderate duration, incremental exercise (Study 4, Chapter 6).

7.1.1.1 Acute exercise depresses muscle in-vitro Na^+,K^+-ATPase maximal activity

Conflicting results were found regarding the effect of acute exercise on muscle in-vitro Na^+,K^+-ATPase maximal activity. Whereas in-vitro Na^+,K^+-ATPase maximal activity was transiently depressed by 11% following ~6 min intense single-leg knee-extensions (Study 1), no change was found following ~24 min incremental cycling exercise (Study 4). This latter finding contrasts numerous previous studies, which reported depressed maximal in-vitro activity following acute exercise ((Aughey *et al.* 2006) and references therein). To determine whether this might reflect a type II error,

the data from Studies 1 and 4 were pooled. A paired t-test revealed a significant decrease ($P < 0.005$) in the pooled activity per g wet weight from rest (273 ± 64 nmol.min^{-1}.(g wet weight)$^{-1}$) to fatigue (237 ± 43 nmol.min^{-1}.(g wet weight)$^{-1}$). However, when expressed relative to muscle protein content no significant decrease was found (rest 1542 ± 347 nmol.min^{-1}.(g protein)$^{-1}$; fatigue 1443 ± 286 nmol.min^{-1}.(g protein)$^{-1}$, $P < 0.2$). However, the maximal in-vitro Na^+,K^+-ATPase activity analyses for Studies 1 and 4 were performed on different spectrofluorometers, therefore direct comparison of the results may not be valid. Thus, the maximal in-vitro Na^+,K^+-ATPase activity relative to muscle protein content at fatigue, was expressed as a fraction of the resting activity (i.e. fatigue/rest) for both Studies 1 and 4, and the results were pooled. A paired t-test revealed a significant 7.5 ± 13.3% decrease from rest to fatigue ($n = 22$, $P < 0.05$, Figure 7.1) in the muscle in-vitro Na^+,K^+-ATPase maximal activity ratio. Thus, the combined results from this thesis are consistent with previous studies, that acute exercise depresses in-vitro Na^+,K^+-ATPase maximal activity as measured by 3-O-MFPase activity (Aughey *et al.* 2006, Aughey *et al.* 2005, Fowles *et al.* 2002b, Fraser *et al.* 2002, Leppik *et al.* 2004, Murphy *et al.* 2006b). Therefore, the lack of change in maximal in-vitro Na^+,K^+-ATPase activity in Study 4 may reflect a type II error. Evidence from this laboratory suggests that reactive oxygen species may be involved in the exercise-induced depression in skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity (McKenna *et al.* 2006b). Elevated intracellular [Ca^{2+}] may also be speculated as a possible cause (Huang *et al.* 1982, Sulova *et al.* 1998).

The functional consequences of depressed muscle maximal in-vitro Na^+,K^+-ATPase activity with exercise have not yet been demonstrated. The potential consequences of depressed maximal Na^+,K^+-ATPase activity are shown in Figure 7.3. This thesis did

not determine whether reduced activity with exercise contributes to muscle fatigue, however some evidence is provided to suggest that depressed activity may be linked with muscle intracellular signalling pathways, and/or with exercise-induced muscle Na^+,K^+-ATPase upregulation.

7.1.1.2 Depressed muscle maximal in-vitro Na^+,K^+-ATPase activity and elevated gene expression with acute exercise

Study 1 suggests that acutely depressed maximal in-vitro Na^+,K^+-ATPase activity might be one factor underlying the exercise-induced upregulation of Na^+,K^+-ATPase content in skeletal muscle, which has been previously shown with very prolonged exercise (Overgaard *et al.* 2002) or exercise training (Green *et al.* 1993, McKenna *et al.* 1993). Study 1 reports a novel and interesting inverse correlation between the percentage change in 3-O-MFPase activity from rest to fatigue ($\%\Delta 3\text{-O-MFPase}_{rest\text{-}fatigue}$) and the percentage increase in mRNA expression at fatigue, for both the α_1 and α_2 Na^+,K^+-ATPase isoforms (Chapter 3). A potential mechanism is via the decrease in Na^+,K^+-ATPase activity causing direct protein-protein interactions of the Na^+,K^+-ATPase and it's neighbouring proteins, which triggers a signalling cascade, culminating in increased Na^+,K^+-ATPase gene transcription (Xie *et al.* 2002). Since the completion of Study 1 (Petersen *et al.* 2005), further evidence has emerged both in support of, and also in contrast to, this finding. A pool of non-pumping Na^+,K^+-ATPase was very recently identified in LLC-PK1 cells which was suggested to have additional roles, such as signal transduction (Liang *et al.* 2007). Conversely, a subsequent study from this laboratory found no correlation between the depression in Na^+,K^+-ATPase maximal activity and increased Na^+,K^+-ATPase isoform mRNA expression following prolonged exercise (Murphy *et al.* 2006b).

7.1.1.3 Increased Na^+,K^+-ATPase content with acute exercise

This thesis reports differing effects of acute exercise on muscle Na^+,K^+-ATPase content as assessed by [^{3}H]ouabain binding. In Study 1, the [^{3}H]ouabain binding site content did not change during, or up to 24 h after brief, intense exercise. Conversely, in Study 4, [^{3}H]ouabain binding increased immediately after moderate duration, incremental exercise to fatigue, returning to resting levels by 3 h post-exercise. It is unlikely that the difference between Studies 1 and 4 is due to the different exercise protocols (Chapter 6). To clarify whether this might reflect a type II error, the rest and fatigue data from studies 1 and 4 were pooled (as for section 7.1.1.1). The [^{3}H]ouabain binding site ratio was increased by 8.5 ± 18% at fatigue compared to rest (n = 24, $P < 0.05$, Figure 7.1). These combined results show for the first time that skeletal muscle Na^+,K^+-ATPase content was increased after acute exercise of only short to moderate duration in humans. Upregulation of [^{3}H]ouabain binding site content with acute exercise in humans had only previously been found after very prolonged (~10 h) exercise (Overgaard *et al.* 2002), whereas studies of shorter duration (~50 s - 75 min) found no change (Aughey *et al.* 2006, Aughey *et al.* 2005, Fowles *et al.* 2002b, Leppik *et al.* 2004, Murphy *et al.* 2006b).

In this thesis, the effect of exercise on Na^+,K^+-ATPase content appeared to be greater when the results were expressed relative to muscle protein content (effect size Study 1 = 0.30, Study 4 = 0.46) compared to muscle wet weight (effects size Study 1 = 0.10 , Study 4 = 0.30). This suggests that increased muscle water content during exercise may have diluted the increase in Na^+,K^+-ATPase content. However, some previous studies have still not detected increased Na^+,K^+-ATPase content when expressed relative to muscle protein content (Aughey *et al.* 2006, Aughey *et al.* 2005). Expressing results relative to muscle protein content may not be a reliable method of

correcting for muscle water shifts, as some studies have found unchanged muscle protein content with exercise (Aughey *et al.* 2006, Aughey *et al.* 2005), even though increased muscle water content would be likely (Sjøgaard 1986, Sjøgaard *et al.* 1985). However this may still be preferable to expressing results relative to muscle wet weight as muscle processing may result in water loss or gain. Therefore, an alternative method of correcting for muscle water shifts during exercise may be needed. Additionally, a meta-analysis of previous studies may be required to determine if the combined results from these studies indicate increased skeletal muscle Na^+,K^+-ATPase content with acute exercise.

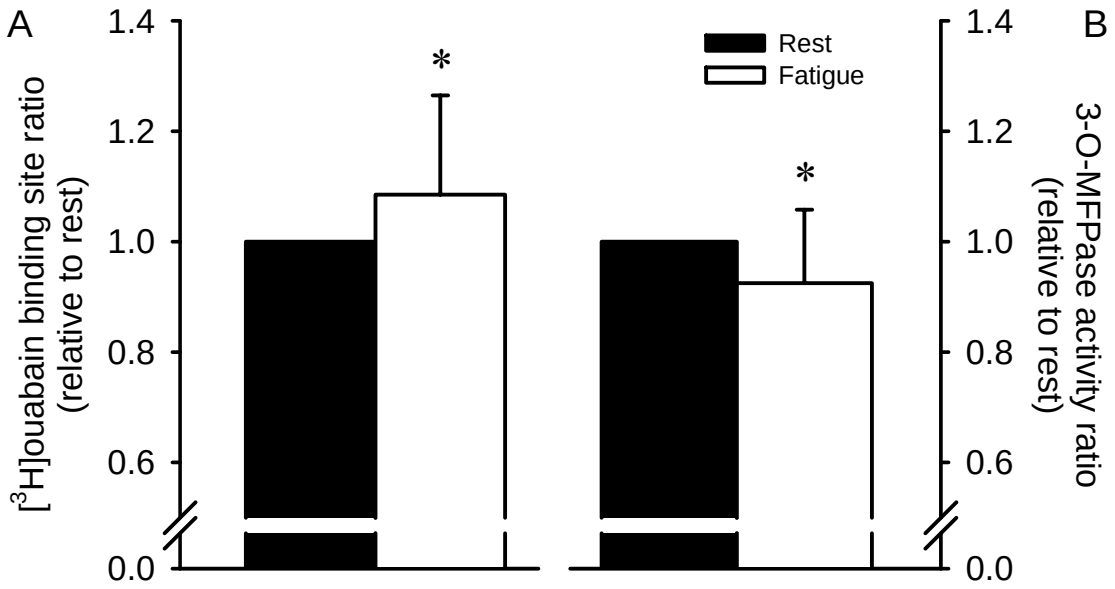

Figure 7.1. [^{3}H]ouabain binding sites and maximal 3-O-MFPase activity relative to rest, at rest and immediately after acute exercise.

Pooled [^{3}H]ouabain binding site content and 3-O-MFPase activity expressed relative to rest, before and immediately after fatiguing exercise induced by brief, intense single-leg knee-extensions (~ 6 min, Study 1), or moderate duration, incremental cycling exercise (~24 min, Study 4). Values are means ± SD, n = 24, * different from rest ($P < 0.05$).

7.1.2 Impaired skeletal muscle Na^+,K^+-ATPase, K^+ regulation, and exercise performance in renal disease

7.1.2.1 Depressed maximal in-vitro Na^+,K^+-ATPase activity

In the first investigation of Na^+,K^+-ATPase in skeletal muscle of humans with renal disease, Study 3 showed that skeletal muscle in-vitro Na^+,K^+-ATPase maximal activity was markedly depressed in HD and RTx compared to CON, despite unchanged content and isoform abundance. This is consistent with reports of depressed maximal in-vitro Na^+,K^+-ATPase activity in erythrocytes and leucocytes from uraemic patients (Aparicio *et al.* 1991, Edmondson *et al.* 1975) and skeletal muscle from uraemic rats (Bofill *et al.* 1994). The depressed muscle Na^+,K^+-ATPase maximal activity in HD and RTx might potentially contribute to their poor exercise performance (see Figure 7.3). This hypothesis is supported by the finding of moderate positive correlations between maximal activity and $\dot{V}O_{2peak}$, knee-extensor isometric PT, and time to fatigue for pooled HD, RTx and CON data. Acute exercise might further reduce maximal Na^+,K^+-ATPase activity in HD and RTx, thus potentially exacerbating the effects of their already depressed maximal activity on exercise performance. However this has yet to be determined.

7.1.2.2 Impaired K^+ regulation

Study 2 reported the novel finding that impaired plasma K^+ regulation during exercise was still evident even in EPO-treated HD. Conversely, plasma K^+ regulation was not impaired in EPO-treated HD or RTx in Study 3. This discrepancy might be explained by the lower $\dot{V}O_{2peak}$ of the HD in Study 2 relative to CON (~60% of CON), compared to the HD and RTx in Study 3 (~75% of CON). This is consistent with the even greater impairment in plasma K^+ regulation during exercise in anaemic HD,

whose $\dot{V}O_{2peak}$ was only ~44% of CON (Sangkabutra *et al.* 2003). Thus, a relationship appears to exist in HD between plasma K^+ regulation during exercise and $\dot{V}O_{2peak}$, which is supported by inverse correlations between $\Delta[K^+].work^{-1}$ ratio and $\dot{V}O_{2peak}$ in Studies 2 and 3.

The apparently normal plasma $[K^+]$ response during exercise in HD and RTx in Study 3 also contrasts with their depressed muscle Na^+,K^+-ATPase maximal activity. These contradictory results might be explained by the fact that arterial or arterialised venous plasma $[K^+]$ are not reliable indicators of muscle K^+ regulation (see Chapter 5).

7.1.2.3 Poor exercise performance in HD and RTx

This thesis supports findings from previous studies that incremental exercise performance is impaired in both HD and RTx (Kettner Melsheimer *et al.* 1987, van den Ham *et al.* 2005). Importantly, it also extends these findings by showing for the first time that each of $\dot{V}O_{2peak}$, knee-extensor peak torque and fatiguability were not different between HD and RTx with similar [Hb]. Furthermore, a novel finding in this thesis is that muscle fatiguability during repeated dynamic contractions is impaired in RTx. Conflicting evidence was obtained regarding muscle fatiguability in HD, with impaired fatiguability in Study 3, but not in Study 2. The lack of difference between HD and CON in Study 2 was probably due to a type II error as the sample size was small (n = 6). This is further supported by the finding that the magnitude of the difference between HD and CON was similar in Study 3 (1.7-fold) and Study 2 (1.8-fold). Finally, when the HD data from Studies 2 and 3 were pooled, the fatigue index was 1.7-fold higher than CON ($P < 0.001$). Muscle fatiguability during repeated dynamic contractions has not previously been investigated in HD, however these findings are consistent with both their (i) decreased incremental exercise performance and (ii) 3-fold higher rate of fatigue during repeated incremental isometric

contractions of the dorsiflexor muscles (Johansen *et al.* 2005) compared to healthy controls. Numerous factors are likely to contribute to the higher fatiguability in HD and RTx, including decreased muscle O_2 delivery (Sala *et al.* 2001), and muscle fibre abnormalities (Diesel *et al.* 1993).

7.1.3 No effect of digoxin on muscle Na^+,K^+-ATPase or exercise performance

Skeletal muscle Na^+,K^+-ATPase content and maximal in-vitro activity were not significantly depressed following administration of a typical clinical digoxin dose for 14 days in healthy humans. This result is surprising as serum [digoxin] reached low therapeutic levels, and a similar dose was previously reported to occupy 13% of muscle Na^+,K^+-ATPase in patients with chronic heart failure (Schmidt *et al.* 1993b). The healthy, recreationally active subjects used in Study 4 probably had a larger muscle mass than the heart failure patients used previously (*ibid*), and therefore the digoxin dose may have been inadequate to occupy a significant number of pumps. This is also supported by the lower serum [digoxin] in these healthy subjects compared to the heart failure patients (1.2 – 2.3 nM) (*ibid*). This cannot be confirmed however, as skeletal muscle [digoxin] was not measured in Study 4. Additionally, the apparent lack of effect of digoxin on Na^+,K^+-ATPase content and activity may have been due to the high variability of the [^{3}H]ouabain binding (CV = 14%) and 3-O-MFPase (CV = 18%) assays.

The unchanged Na^+,K^+-ATPase content and activity following digoxin administration could also have been caused by compensatory upregulation of Na^+,K^+-ATPase, as previously reported in human erythrocytes (Cumberbatch *et al.* 1981) and rat hind-limb muscle (Wai Ching Li *et al.* 1993). There is still no evidence for this in human skeletal muscle however, and the results from Study 4 cannot confirm this. However, upregulation is suggested by a companion study from this laboratory, which reported

increased Na^+,K^+-ATPase isoform mRNA expression after chronic digoxin administration (Gong *et al.* 2005).

Chronic digoxin administration had no effect on time to fatigue or $\dot{V}O_2$ during an incremental cycling test, and this probably reflects the lack of effect of digoxin on muscle Na^+,K^+-ATPase content and activity. This contrasts a report of worsened repeat running performance following digoxin (Bruce *et al.* 1968). However, the digoxin dose was 6-fold higher in the previous study and muscle Na^+,K^+-ATPase content or activity, whilst not measured (*ibid*), would be expected to be markedly effected. Thus, whether direct skeletal muscle Na^+,K^+-ATPase inhibition impairs exercise performance in healthy humans was not resolved in this study and remains to be determined.

7.1.4 Relationship of skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity with exercise performance

A relationship between skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity and exercise performance was identified in Studies 3 and 4. Maximal in-vitro Na^+,K^+-ATPase activity was significantly correlated with $\dot{V}O_{2peak}$ when all data were pooled in Study 3 and in Study 4, but not in Study 1. When data from all subjects in Studies 1, 3, and 4 were pooled, significant correlations were found between $\dot{V}O_{2peak}$ and each of Na^+,K^+-ATPase in-vitro maximal activity ($r = 0.49$, $P < 0.001$, $n = 50$, Figure 7.2) and content ($r = 0.32$, $P < 0.05$, $n = 52$). However, no such correlations were found for pooled data from renal disease patients or healthy subjects, individually. Thus, the combined results of this thesis suggest a link between maximal incremental exercise performance and skeletal muscle Na^+,K^+-ATPase activity. A relationship between Na^+,K^+-ATPase in-vitro maximal activity and $\dot{V}O_{2peak}$ has also been reported in a previous study (Fraser *et al.* 2002). It should be noted that maximal

Na^+,K^+-ATPase activity explained only 24% ($r^2 = 0.24$) of the variance in $\dot{V}O_{2peak}$. Thus, numerous other factors must also be involved. As $\dot{V}O_{2peak}$ is determined by peak cardiac output and arterio-venous O_2 difference, any factors which influence these variables will contribute to the variance in $\dot{V}O_{2peak}$.

A diagram of the proposed link between skeletal muscle Na^+,K^+-ATPase activity and exercise performance is shown in Figure 7.3. Depressed Na^+,K^+-ATPase activity may exacerbate muscle K^+ efflux during muscle contraction (Hallén *et al.* 1994), resulting in elevated $[K^+]_I$, reduced $[K^+]_i$, and decreased trans-membranous $[K^+]$ gradient (Sejersted *et al.* 2000). This reduces membrane potential (Sjøgaard *et al.* 1985), impairs muscle contractility, and increases muscle fatiguability (Clausen *et al.* 1991).

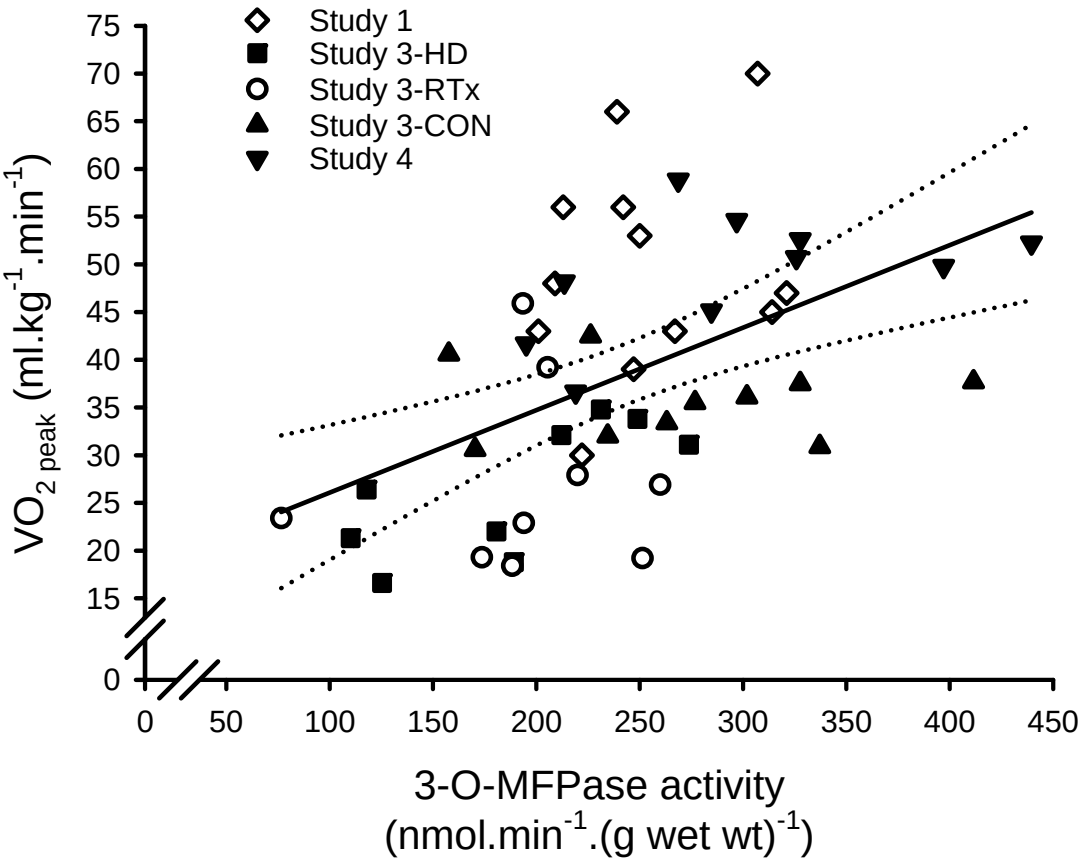

Figure 7.2. Relationship between skeletal muscle maximal 3-O-MFPase activity and $\dot{V}O_{2peak}$ for combined data from Studies 1, 3, and 4.

Study 1 (▼), *n* = 12; Study 3-HD (■), haemodialysis, *n* = 9; Study 3-RTx (○), renal transplant, *n* = 9; Study 3-CON (▲), healthy controls, *n* = 10; Study 4 (◇), *n* = 10. Regression line is for pooled data (solid line, *n* =50, *r* = 0.49, *P* < 0.001, y = 0.9x + 17). Dotted curves indicate 95% confidence intervals.

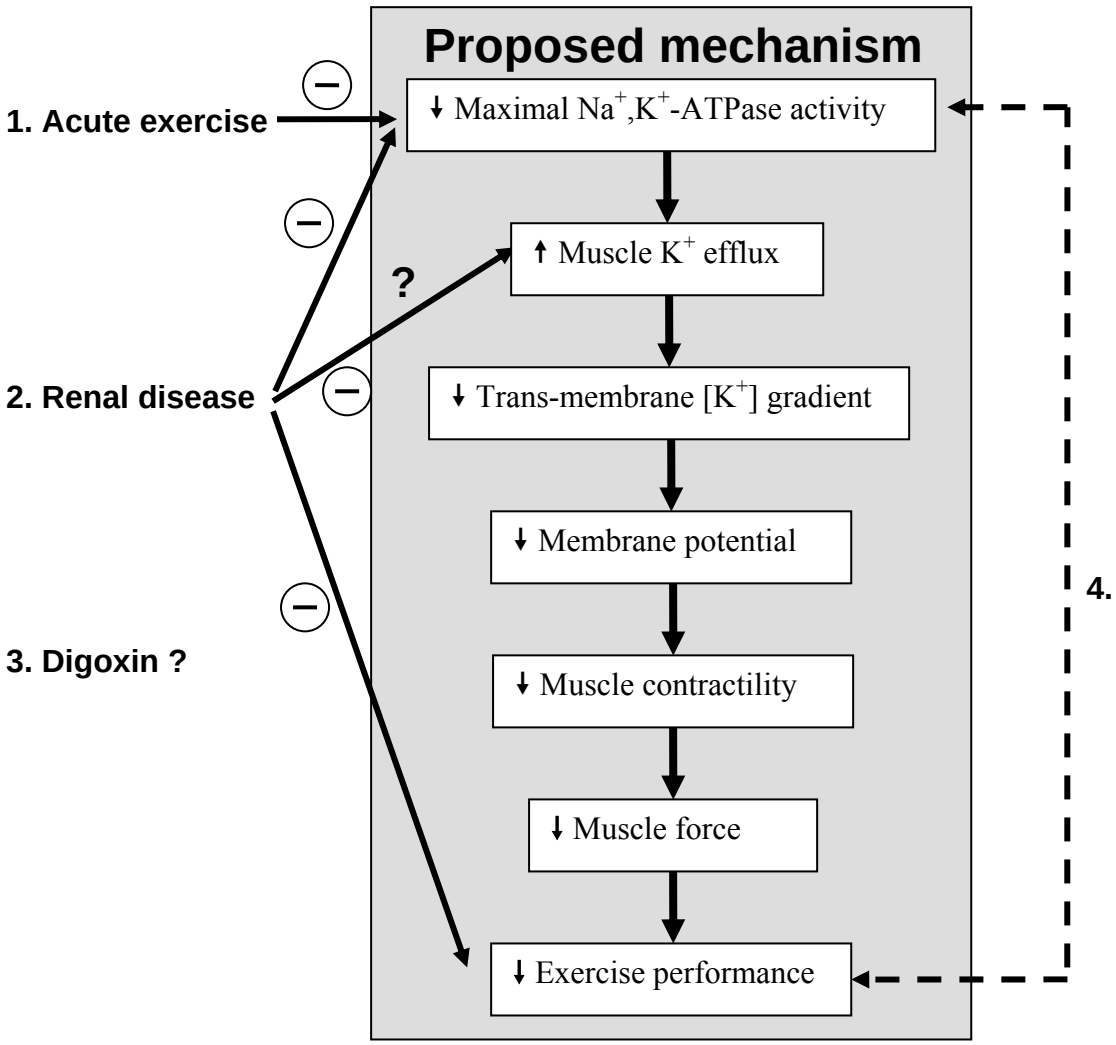

Figure 7.3. Effects of acute exercise, renal disease, and digoxin on skeletal muscle Na^+,K^+-ATPase and related effects on plasma K^+ and muscle performance.

For details of proposed mechanism see text. Solid arrows indicate a direct effect. Dashed arrow indicates a relationship between two variables.1, acute exercise depressed muscle maximal Na^+,K^+-ATPase activity. 2, renal disease decreased maximal Na^+,K^+-ATPase activity, and impaired maximal incremental exercise performance, but the effects on muscle K^+ efflux (reflected by elevated plasma $[K^+]$) were equivocal. 3, the effects of digoxin on muscle Na^+,K^+-ATPase and subsequent effects on exercise performance remain to be determined. 4, maximal Na^+,K^+-ATPase activity was related to maximal incremental exercise performance for pooled data from Studies, 1, 3, and 4.

7.2 CONCLUSIONS

The major conclusions from this thesis are:

Study 1, Chapter 3

1. Acute brief intense exercise transiently depressed skeletal muscle Na^+,K^+-ATPase maximal in-vitro activity at fatigue, which had recovered by 3 hours post-exercise.
2. Skeletal muscle Na^+,K^+-ATPase content was unchanged immediately, and up to 24 h after, acute brief intense exercise.
3. The reduction in Na^+,K^+-ATPase maximal in-vitro activity immediately following an acute bout of brief intense exercise was correlated with the increases in the mRNA expression of the Na^+,K^+-ATPase α_1 and α_2, but not α_3 isoforms.

Study 2, Chapter 4

4. Haemodialysis patients (HD) treated with erythropoietin (EPO) had reduced $\dot{V}O_{2peak}$ and muscle strength and exhibited a tendency for higher fatiguability compared to healthy controls (CON).
5. Plasma $[K^+]$, $\Delta[K^+]$, the rate of rise in plasma $[K^+]$ and the $\Delta[K^+].work^{-1}$ ratio during incremental exercise were each higher in EPO-treated HD than CON.
6. Stationary cycle training for six weeks in HD increased time to fatigue and work done, but not $\dot{V}O_{2peak}$, muscle strength and fatiguability, or quality of life. Plasma $[K^+]$ during incremental exercise was not decreased after training, however the $\Delta[K^+].work^{-1}$ ratio was reduced.

Study 3, Chapter 5

7. $\dot{V}O_{2peak}$, muscular strength, and fatiguability during dynamic contractions were reduced in both HD and renal transplantation recipients (RTx) compared to CON. However, each of these did not differ between RTx and EPO-treated HD with similar [Hb].
8. The $\Delta[K^+]$, the rate of rise in $[K^+]$, and the $\Delta[K^+].work^{-1}$ ratio during incremental exercise did not differ between EPO-treated HD, RTx, and CON.
9. Skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity was depressed in both HD and RTx, but did not differ between them, whilst muscle Na^+,K^+-ATPase content and isoform abundance were unchanged in HD and RTx, compared to CON.
10. Impaired skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity in HD, and in pooled data for HD, RTx, and CON, was related to $\dot{V}O_{2peak}$ and time to fatigue during incremental exercise, and knee-extensor isometric PT.

Study 4, Chapter 6

11. Administration of a typical clinical digoxin dose for two weeks elevated serum [digoxin] in healthy humans but did not depress skeletal muscle Na^+,K^+-ATPase content or maximal in-vitro activity.
12. Acute incremental, moderate duration exercise increased Na^+,K^+-ATPase content but did not depress maximal in-vitro activity. However, combined data from Studies 1 and 4 indicates that acute exercise of short to moderate duration increased Na^+,K^+-ATPase content and depressed maximal in-vitro activity.
13. Time to fatigue during cycling exercise, was not depressed following chronic digoxin administration.

7.3 RECOMMENDATIONS FOR FUTURE RESEARCH

7.3.1 Acute exercise effects on skeletal muscle Na^+,K^+-ATPase

This thesis found that acute exercise transiently depressed skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity, however the consequences of this have not yet been elucidated and require further investigation. Studies should investigate relationships between depressed maximal activity with exercise and muscle excitability (M-wave amplitude and area) and muscle performance.

Reactive oxygen species may be involved in the exercise-induced depression in skeletal muscle maximal in-vitro Na^+,K^+-ATPase activity (McKenna *et al.* 2006b). Elevated intracellular $[Ca^{2+}]$ also inhibits Na^+,K^+-ATPase activity (Huang *et al.* 1982, Sulova *et al.* 1998). Investigation of both Na^+,K^+-ATPase activity and intracellular $[Ca^{2+}]$ after exercise or stimulation in intact animal muscles will allow identification of a potential relationship. Other possible mechanisms such as elevated muscle temperature could also be investigated in exercising humans.

Study 1 suggested a link between depressed Na^+,K^+-ATPase activity with brief intense exercise and increased Na^+,K^+-ATPase isoform mRNA expression. However no such relationship was found with prolonged exercise (Murphy *et al.* 2006b), possibly due to the smaller sample size (n = 11) and variability of the mRNA measures. Thus, further studies, employing large sample sizes and a range of exercise protocols, are needed to verify if such a link exists. Furthermore, mechanistic studies are needed to determine the potential signalling pathways involved, such as those previously identified in other tissues (Xie *et al.* 2002).

7.3.2 Impaired skeletal muscle Na^+,K^+-ATPase, K^+ regulation and exercise performance in renal disease

The results of this thesis do not allow determination of the mechanisms contributing to reduced skeletal muscle Na^+,K^+-ATPase activity in renal disease. This thesis speculated that altered muscle membrane lipid composition, presence of endogenous Na^+,K^+-ATPase inhibitors or uraemic toxins, or immunosuppressive steroids might be involved. In-vitro studies will allow investigation of the acute effects of these factors on maximal Na^+,K^+-ATPase activity. Interventions, such as modifying the dose or type of prescribed immunosuppressants, should reveal any chronic effects.

Arterial or arterialised venous plasma $[K^+]$ was used as a gross indicator of extrarenal K^+ regulation, thus also reflecting muscle K^+ regulation, in renal disease in this thesis. However plasma $[K^+]$ is not a reliable indicator of muscle K^+ fluxes during and after exercise. Furthermore, plasma $[K^+]$ during exercise in HD was impaired in Study 2, but not in Study 3. Therefore, more detailed measures of K^+ dynamics in renal disease are required. Measurement of arteriovenous $[K^+]$ differences and muscle blood flow (Sostaric *et al.* 2005) are required to determine if muscle K^+ fluxes during and after exercise are impaired in renal disease. Microdialysis techniques could also reveal potential elevations in muscle $[K^+]_I$ (Juel *et al.* 2000b).

Na^+,K^+-ATPase content and K^+ regulation during exercise are improved after training in healthy subjects (Green *et al.* 1993, McKenna *et al.* 1993). K^+ regulation during exercise was also improved with training in HD in Study 2. However, whether this was due to increased muscle Na^+,K^+-ATPase content was not investigated. This is potentially important, as this thesis indicated that impaired skeletal muscle Na^+,K^+-ATPase activity was linked with the poor exercise performance observed in renal disease. Therefore, future research should determine if exercise training

increases skeletal muscle Na^+,K^+-ATPase content and activity in renal disease and whether this contributes to their improved exercise performance. Combined resistance and endurance training causes substantial increases in $\dot{V}O_{2peak}$ in HD (Kouidi *et al.* 2004), and each of these also increase Na^+,K^+-ATPase content in healthy humans (Green *et al.* 1999a). Therefore, the effects of combined resistance and endurance training on muscle K^+ regulation and Na^+,K^+-ATPase in renal disease warrant investigation.

7.3.3 Digoxin effects on skeletal muscle Na^+,K^+-ATPase and exercise performance

The reason for the lack of effect of chronic digoxin administration on muscle Na^+,K^+-ATPase activity and content in Study 4 is unclear. This thesis speculated that the digoxin dose may have been inadequate for significant inhibition of muscle Na^+,K^+-ATPase or compensatory upregulation of skeletal muscle Na^+,K^+-ATPase may have occurred. Whether digoxin causes compensatory upregulation in skeletal muscle is equivocal and remains to be determined in future studies. That aside, the effects of skeletal muscle Na^+,K^+-ATPase inhibition on exercise performance in healthy humans are also yet to be determined. Future studies should investigate the effects of acute Na^+,K^+-ATPase inhibition by oral digoxin administration, with repeated measures at 2, 12 and 24 h to avoid possible compensatory upregulation. Radioimmunoassay of skeletal muscle [digoxin] should also be performed to ensure adequate digitalisation.

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APPENDIX 1 SUBJECT INFORMATION SHEETS, CONSENT FORMS, AND RISK FACTOR QUESTIONNAIRES

APPENDIX 1.1 SUBJECT INFORMATION SHEETS

Appendix 1.1.1 Subject information sheet for study one.

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Footscray Park Campus
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SUBJECT INFORMATION SHEET

"Effects of acute exercise on skeletal muscle Na^+,K^+-pumps in humans: content, activity and gene expression"

Investigators:
Associate Professor Michael McKenna, Ms Kate Murphy and Aaron Petersen,
School of Human Movement, Recreation and Performance, Victoria University of Technology, Footscray, Victoria and Dr Rodney Snow, David Cameron-Smith, School of Health Sciences, Deakin University, Burwood, Victoria.

Aim of the study:
This study will investigate the effects of exercise on a key enzyme located in your muscle cells, known as the sodium, potassium pump. This enzyme helps to prevent your muscles from becoming fatigued, but we know little about its role in exercise. The first aim of this study is to find out whether exercise causes an increased or decreased availability of these pumps in your muscle. The second aim is to see whether a single bout of exercise is sufficient to cause the genes in your muscle cells to start producing new sodium, potassium pumps, which might then allow improvement in subsequent exercise sessions. Results from this project will yield considerable social benefits by enhancing our understanding of the acute responses of muscle to exercise, factors influencing muscle fatigue, and the initial steps underlying normal training responses. Possible future applications may include the use of even single exercise bouts to enhance muscle function in patients before and after surgery.

Subject participation:
Should you choose to participate, as a subject you will be free to withdraw from the study at any time, without any adverse effects, reactions or discrimination. Your total time involvement would be from 3-4 weeks.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be asked to undertake each of the three tests described below. You will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Campus of Victoria University of Technology on five separate occasions for an exercise test. Whilst each test is tiring, you will recovery from this very quickly. You will be asked to perform three different types of exercise tests:

(i) maximal aerobic fitness (VO_2 peak): this test involves continuous exercise on a cycle ergometer with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. The test is completed when you

become too tired to continue (wish to stop), or unless we stop the test due to you having an abnormal response to exercise, such as unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath. The most common event associated with maximal exercise tests is fainting. This will be prevented using our standard laboratory procedures. In the unlikely event of emergency situations, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications and the Western Hospital is minutes away by ambulance.

(ii) maximal leg muscle strength: this test involves contractions of your thigh muscles on a Cybex dynamometer, with your leg kicking speed controlled at a moderate speed.

(iii) leg muscle fatigue: this test involves repeated kicking contractions at 45% of your maximal leg strength, performed every 2 seconds, until your thigh muscles fatigue.

Order of Exercise Tests:

Should you choose to participate, the tests will be conducted in the following order. On the first visit to the laboratory you will be required to perform the VO_2 peak test. After a 20 minute rest you will then be asked to do a practise trial of the leg strength and the leg fatigue tests, to familiarise you with these tests. You will then repeat the leg strength and the leg fatigue test on three more visits to the laboratory, so that we can measure how much your leg strength and fatigue varies. On your fifth visit, we will ask you to repeat the leg fatigue test again. During this test, muscles biopsy samples will be taken from your thigh muscle, and blood samples will be taken from a hand vein, at rest, and during exercise, as described below.

Blood Samples:

Should you choose to participate, blood samples will be taken as described. At specific intervals throughout the exercise test a small blood sample (each 5 ml) will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the teflon tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed into the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein). A total of approximately fifty (50) ml of blood will be taken during the test. (Normally 400 ml is taken when you donate at the Blood Bank). Each time a blood sample is taken, a small volume of sterile heparinised saline (1-2 ml) will be injected to clear the catheter. Catheterisation of subjects can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. Blood samples will be analysed for metabolites, electrolytes and pH.

Muscle Biopsies and Muscle Fatigue Testing:

Should you choose to participate, muscle biopsy samples will be taken as described. On your fifth visit to the Exercise Physiology Laboratory, a muscle biopsy will be taken from your thigh muscle, at rest, immediately after you stop the fatigue test, and then 3 hours later. You will be asked to return to the laboratory for the final time (sixth visit) the day after the test, to have the last muscle biopsy taken at 24 hours after the end of the test. Thus a total of four biopsies will be taken over a 24 hour period. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of enzymes and energy sources. An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable, but will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. Many people experience pain during this part of the procedure but this lasts only for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a grain of rice. This poses no long term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months.

Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. You should not exercise for 24 hours after biopsies and you should avoid heavy knocks. It is common for subjects to experience some mild soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement.

The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. The whole procedure will be performed under sterile conditions by a qualified medical practitioner. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection (for example, puss, tenderness and/or redness), significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

Contact Numbers:

Aaron Petersen	**W: (03) 9688-4066**	**H: (03) 9388-1165**
Kate Murphy	**Mobile: 0411-763-614**	**H: (03) 9438-3030**
Assoc.Prof. Michael McKenna	**W: (03) 9688-4499**	**H (03) 5422-6089**
Dr Rod Snow	**W: (03) 9251-7315**	
Dr David Cameron-Smith	**W: (03) 9244-6502**	

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. M. McKenna Ph. (03) 54226089). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 1.1.2 Subject information sheet for study two

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PARTICIPANT INFORMATION

The effects of endurance training on K^+ regulation, exercise performance, and quality of life in patients on haemodialysis.

Investigators:

Associate Professor Michael McKenna and Mr Aaron Petersen, School of Human Movement, Recreation and Performance, Victoria University;
Dr Lawrence McMahon, Dr Murray Leikis and Ms Annette Kent, Department of Nephrology, Royal Melbourne Hospital

Aims of the study:

This study aims to determine whether potassium regulation is improved after exercise training in patients on haemodialysis. Impaired potassium regulation has been found to occur in patients on haemodialysis and may be partially responsible for their poor exercise capacity. This study also aims to determine if exercise capacity and quality of life will be improved and whether these improvements are associated with enhanced potassium regulation. It is important that we gain a greater understanding of the benefits and effects of exercise training on patients with kidney failure. This may then enable us to design more effective rehabilitation programmes to improve the general health and quality of life of kidney failure patients.

Study Overview

Should you choose to participate in this study you will be asked to perform a number of exercise tests and 6 weeks of stationary cycle training (details of the exercise tests and training programme are provided below). Initially you will be asked to perform a maximal aerobic fitness test, a leg muscle strength test, a leg muscle fatigue test, and complete a quality of life questionnaire. There will then be a 6-week period during which you will just perform your normal daily activities. You will then be asked to perform all the tests again so we can see if any change occurred during the previous six weeks. Following the second series of tests you will be asked to begin a 6-week stationary cycle training programme (see below for details). Finally, after the 6-week training period, you will be asked to perform the tests one last time. In total you will be required to visit the lab on six separate occasions as well as performing 18 half-hour cycle training sessions.

Volunteer participation:

As a volunteer, you are free to withdraw from the study at any time, without any adverse effects, reactions or discrimination.

Cycle Training

The stationary cycle training programme will last for six weeks and will involve three sessions per week (18 sessions in total) of approximately 30 minutes duration each. The cycling will be performed on a cycle ergometer, which is designed to be positioned in front of your normal dialysis chair. This allows you to cycle while sitting in the dialysis chair and undergoing your normal dialysis therapy. The training programme will be designed especially for you depending on your initial level of fitness. The first training sessions will be quite easy but will gradually be made more difficult as your body adapts to the training. Initially you may only be able to cycle for a few minutes at a time before becoming tired. This is quite normal, however, during the following six weeks, we expect that your fitness will improve until you are capable of cycling continuously for 30 minutes. You will be free to stop cycling at any time during a training session either to rest briefly or if you feel you can't continue with the

session. Should you experience any chest pain, light-headedness, severe shortness of breath, or any other unusual discomfort related to or caused by the exercise then you should stop cycling and inform the supervising personnel. An exercise physiologist will be in attendance at all of your training sessions while a physician will attend the first two sessions and then be on call for the remainder of the sessions.

Exercise Testing Procedures:

Should you decide to participate in this study, you will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Park Campus of Victoria University on 6 separate occasions for an exercise test. Whilst each test is tiring, you will recover from this very quickly. You will be asked to perform three different types of exercise tests and complete a quality of life questionnaire.

(i) maximal aerobic fitness (VO_2 peak): this test involves continuous exercise on a stationary cycle with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. A mouthpiece and mask will be worn during the test so we can measure your exhaled breath. The test is completed when you become too tired to continue (wish to stop), or we stop the test due to you having an unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath. The most common event associated with maximal exercise tests is fainting. The chance of this occurring will be minimised using our standard laboratory procedures. In the unlikely event of an emergency situation, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications, a medical practitioner will be in attendance and the Western Hospital is minutes away by ambulance.

(ii) maximal leg muscle strength: this test involves contractions of your thigh muscles on a muscle strength testing machine, with your leg kicking speed controlled at a moderate speed. After a warm up you will perform 3 kicking motions at various speeds. These tasks are easily performed and pose no unusual risk.

(iii) leg muscle fatigue: this test involves 30 repeated kicking contractions at your maximal leg strength, performed every 2 seconds, which will cause your thigh muscles to tire.

(iv) quality of life questionnaire: this questionnaire contains 36 questions about factors that may influence your quality of life. The questionnaire takes approximately 5 – 10 minutes to complete.

Order of Exercise Tests:

On the first visit to the laboratory you will be required to complete the quality of life questionnaire and perform the VO_2 peak test. After a 30-minute rest you will then be asked to practise the leg strength and leg fatigue tests, to familiarise you with these tests. You will be asked to practise the leg strength and the leg fatigue test on three more visits to the laboratory, so that we can measure how much your leg strength and fatigue varies. Six weeks later you will then be asked to return to the laboratory to repeat the quality of life questionnaire, VO_2 peak test, and leg strength and leg fatigue tests. You will then begin six weeks of stationary cycle training, at the end of which you will be asked to repeat the quality of life questionnaire, VO_2 peak test, and leg strength and leg fatigue tests one last time. Therefore, should you decide to participate, your total time contribution would be approximately five hours of laboratory tests plus nine hours of cycle training.

Blood Samples:

At specific intervals throughout the cycle exercise test a small blood sample (each 5 ml) will be taken via a catheter placed into the arterio-venous fistula in your arm. The catheter consists of a needle and plastic tubing. The tubing is fed over the top of the needle (which has punctured the fistula) and into the fistula. The needle is then withdrawn, leaving only the plastic tubing in your fistula for the remainder of the experiment. A tap (stopcock) is placed at the end of the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple punctures of the fistula. A total of approximately seventy-five (75) ml of blood will be taken during the test, this is less than one-fifth of the amount taken when donating blood. Each time a blood sample is taken, a small volume of sterile heparinised saline (1-2 ml) will be injected to clear the catheter and keep it patent. In total, not more than 80 units of heparin will be administered. This is a very small dose. Catheterisation can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. Blood samples will be analysed for metabolites, electrolytes and pH.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your cooperation.

Contact Numbers:

Assoc. Prof. Michael McKenna W: 9688-4499 H: 5422-6089
Dr Lawrence McMahon W: 9342-8353
Aaron Petersen W: 9688-4207

Any queries about your participation in this project may be directed to the researcher (Name: Assoc.Prof. Michael McKenna ph. (03) 9688-4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 1.1.3 Subject information sheet for healthy controls in study three.

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VICTORIA UNIVERSITY OF TECHNOLOGY
Participant Information for healthy control participants

Muscle electrolytes, metabolism and exercise performance in patients with kidney failure and kidney transplant.

Investigators:
Associate Professor Michael McKenna and Mr Aaron Petersen, School of Human Movement, Recreation and Performance, Victoria University;
Dr Lawrence McMahon, Dr Murray Leikis and Ms Annette Kent, Department of Nephrology, Royal Melbourne Hospital

Aims of the study:
This study aims to determine some factors that may cause reduced exercise performance in people with kidney failure or those who have had a kidney transplant. We investigate whether the control of muscle potassium, sodium and calcium levels, as well as energy metabolism, are impaired in patients with kidney failure or kidney transplant by comparing these factors to those of healthy control participants. Through this study we hope to improve understanding of the causes of early fatigue in haemodialysis and kidney transplant patients during exercise. This fatigue occurs in all people including healthy people and those with kidney failure. This may then lead to future investigations to improve their exercise capacity and quality of daily living.

Volunteer participation:
As a volunteer, you are free to withdraw from the study at any time, without any adverse effects, reactions or discrimination. Your total time involvement will be from 3-4 weeks.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Park Campus of Victoria University on five separate occasions for an exercise test. Whilst each test is tiring, you will recover from this very quickly. You will be asked to perform three different types of exercise tests:
(i) maximal aerobic fitness (VO_2 peak): this test involves continuous exercise on a stationary cycle with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. A mouthpiece and mask will be worn during the test so we can measure your exhaled breath. The test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath. The most common event associated with maximal exercise tests is fainting. The chance of this occurring will be minimised using our standard laboratory procedures. In the unlikely event of an emergency situation, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications, a medical practitioner will be in attendance and the Western Hospital is minutes away by ambulance.

(ii) maximal leg muscle strength: this test involves contractions of your thigh muscles on a muscle strength testing machine, with your leg kicking speed controlled at a moderate speed. After a warm up you will perform 3 kicking motions at various speeds. These tasks are easily performed and pose no unusual risk.
(iii) leg muscle fatigue: this test involves 30 repeated kicking contractions at your maximal leg strength, performed every 2 seconds, which will cause your thigh muscles to tire.
Order of Exercise Tests:
On the first visit to the laboratory you will be required to perform the VO_2 peak test. After a 30 minute rest you will then be asked to practise the leg strength and leg fatigue tests, to familiarise you with these tests. You will be asked to practise the leg strength and the leg fatigue test on three more visits to the laboratory, so that we can measure how much your leg strength and fatigue varies. Immediately prior to your final leg strength and fatigue test a muscle biopsy will be taken from your non-exercising leg. The first visit to the laboratory will last approximately one hour, all subsequent visits will last about 30 minutes. Therefore, should you decide to participate, your total time contribution would be approximately three hours.

CT scan
To help us better assess your muscle size, participants will be asked to undergo a single CT scan of the leg prior to the study. The CT scanner allows us to look at any part of your body in cross section (like looking at each sliced loaf of bread in turn). Using the CT scanner we will determine the amount (area) of muscle in your leg. CT scanning is similar to having a normal x-ray, except that the equipment is larger and more complicated. You will be asked to lie on a table, which is moved into the centre of a machine that looks like a square doughnut. The CT scanner makes a slight buzzing sound as it operates. The CT scan will take about 5 minutes. It will be performed at the Western Hospital, at either the Footscray or Sunshine campus, depending on your preference.
During the research protocol you will be exposed to a tiny amount of radiation (<0.1 mSv). This amount of radiation is about the same as two weeks of natural background radiation. All people on earth are exposed to background radiation. This background radiation comes from the sun, the earth, the air and all around us
The ill effects at very high doses of radiation have been well documented, for example increased life threatening cancer rates and sometimes death has been reported in populations exposed to nuclear explosions or in patients undergoing radiotherapy treatment. However, at tiny or trivial doses of radiation, similar to those being received from being a participant in this research, the risk of a life threatening cancer is about 1 in 250,000. This model is based on a conservative approach and the actual risk may be a lot smaller. Compared to other risks in everyday life this risk is considered negligible. For example: this theoretical risk is approximately the same as smoking 8 cigarettes, travelling 400 km by car, or travelling 4,000 km by commercial aircraft.

Blood Samples:
At specific intervals throughout the cycle exercise test a small blood sample (each 5 ml) will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and plastic tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the plastic tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed at the end of the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple punctures of the vein. A total of approximately seventy-five (75) ml of blood will be taken during the test. Each time a blood sample is taken, a small volume of sterile heparinised saline (1-2 ml) will be injected to clear the catheter and keep it patent. In total, not more than 30 units of heparin will be administered. This is a very small dose Catheterisation can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. Blood samples will be analysed for metabolites, electrolytes and pH.

Muscle Biopsies:
The muscle biopsy will be taken in the Exercise Physiology Laboratory at Victoria University of Technology, by a doctor from the Nephrology Department, Royal Melbourne Hospital. A biopsy will

be taken from your thigh muscle whilst you are awake, with a local anaesthetic in your thigh. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of enzymes and energy sources. An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm (1/4 inch) long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable, but will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. Many people experience pain during this part of the procedure but this lasts only for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a grain of rice. This poses no long term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. You should not exercise for 24 hours after biopsies and you should avoid heavy knocks to the area. It is common for participants to experience some mild soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these disappear within a week. The whole procedure will be performed under sterile conditions by a qualified medical practitioner. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy and assist in obtaining any necessary treatment.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your cooperation.

Contact Numbers:

Assoc. Prof. Michael McKenna W: 9688-4499 H: 5422-6089
Dr Lawrence McMahon W: 9342-8353
Aaron Petersen W: 9688-4066

Any queries about your participation in this project may be directed to the researcher (Name: Assoc.Prof. Michael McKenna ph. (03) 9688-4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 1.1.4 Subject information sheet for haemodialysis patients and renal transplant recipients in study three

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VICTORIA UNIVERSITY OF TECHNOLOGY
Participant Information for patients with kidney failure or kidney transplant

Muscle electrolytes, metabolism and exercise performance in patients with kidney failure and kidney transplant.

Investigators:
Associate Professor Michael McKenna and Mr Aaron Petersen, School of Human Movement, Recreation and Performance, Victoria University;
Dr Lawrence McMahon, Dr Murray Leikis and Ms Annette Kent, Department of Nephrology, Royal Melbourne Hospital

Aims of the study:
This study aims to determine some factors that may cause reduced exercise performance in people with kidney failure or those who have had a kidney transplant. We investigate whether the control of muscle potassium, sodium and calcium levels, as well as energy metabolism, are impaired. Through this study we hope to improve understanding of the causes of early fatigue in haemodialysis and kidney transplant patients during exercise. This fatigue occurs in all people including healthy people and those with kidney failure. This may then lead to future investigations to improve their exercise capacity and quality of daily living.

Volunteer participation:
As a volunteer, you are free to withdraw from the study at any time, without any adverse effects, reactions or discrimination. Your total time commitment will involve five sessions over a 3-4 week period.

Exercise Testing Procedures:
Should you decide to participate in this study, you will be required to attend the Exercise Physiology Laboratory (Room L305, Building L), at the Footscray Park Campus of Victoria University on five separate occasions for an exercise test. Whilst each test is tiring, you will recover from this very quickly. You will be asked to perform three different types of exercise tests:
(i) maximal aerobic fitness (VO_2 peak): this test involves continuous exercise on a stationary cycle with the exercise intensity (effort) being progressively increased. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. A mouthpiece and mask will be worn during the test so we can measure your exhaled breath. The test is completed when you become too tired to continue (wish to stop), or unless we stop the test due to you having an unusual heart rhythm, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath. The most common event associated with maximal exercise tests is fainting. The chance of this occurring will be minimised using our standard laboratory procedures. In the unlikely event of an emergency situation, two members of the research team have current CPR (cardio pulmonary

resuscitation) qualifications, a medical practitioner will be in attendance and the Western Hospital is minutes away by ambulance.

(ii) maximal leg muscle strength: this test involves contractions of your thigh muscles on a muscle strength testing machine, with your leg kicking speed controlled at a moderate speed. After a warm up you will perform 3 kicking motions at various speeds. These tasks are easily performed and pose no unusual risk.

(iii) leg muscle fatigue: this test involves 30 repeated kicking contractions at your maximal leg strength, performed every 2 seconds, which will cause your thigh muscles to tire.

Order of Exercise Tests:

On the first visit to the laboratory you will be required to perform the VO_2 peak test. After a 30 minute rest you will then be asked to practise the leg strength and leg fatigue tests, to familiarise you with these tests. You will be asked to practise the leg strength and the leg fatigue test on three more visits to the laboratory, so that we can measure how much your leg strength and fatigue varies. Immediately prior to your final leg strength and fatigue test a muscle biopsy will be taken from your non-exercising leg. The first visit to the laboratory will last approximately one hour, all subsequent visits will last about 30 minutes. Therefore, should you decide to participate, your total time contribution would be approximately three hours.

CT scan

To help us better assess your muscle size, participants will be asked to undergo a single CT scan of the leg prior to the study. The CT scanner allows us to look at any part of your body in cross section (like looking at each sliced loaf of bread in turn). Using the CT scanner we will determine the amount (area) of muscle in your leg. CT scanning is similar to having a normal x-ray, except that the equipment is larger and more complicated. You will be asked to lie on a table which is moved into the centre of a machine that looks like a square doughnut. The CT scanner makes a slight buzzing sound as it operates. The CT scan will take about 5 minutes. It will be performed at the Western Hospital, at either the Footscray or Sunshine campus, depending on your preference.

During the research protocol you will be exposed to a tiny amount of radiation (<0.1 mSv). This amount of radiation is about the same as two weeks of natural background radiation. All people on earth are exposed to background radiation. This background radiation comes from the sun, the earth, the air and all around us.

The ill effects at very high doses of radiation have been well documented, for example increased life threatening cancer rates and sometimes death has been reported in populations exposed to nuclear explosions or in patients undergoing radiotherapy treatment. However, at tiny or trivial doses of radiation, similar to those being received from being a participant in this research, the risk of a life threatening cancer is about 1 in 250,000. This model is based on a conservative approach and the actual risk may be a lot smaller. Compared to other risks in everyday life this risk is considered negligible. For example: this theoretical risk is approximately the same as smoking 8 cigarettes, travelling 400 km by car, or travelling 4,000 km by commercial aircraft.

These risks can be compared with the chance anybody has of dying from cancer, which is about 1 in four.

Blood Samples:

At specific intervals throughout the cycle exercise test a small blood sample (each 5 ml) will be taken via a catheter placed into a vein in the back of your hand. The catheter consists of a needle and plastic tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the plastic tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed at the end of the tubing so that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiplepunctures of the vein. A total of approximately seventy-five (75) ml of blood will be taken during the test. Each time a blood sample is taken, a small volume of sterile heparinised saline (1-2 ml) will be injected to clear the catheter and keep it patent. In total, not more than 30 units of heparin will be administered. This is a very small dose Catheterisation can cause slight discomfort and can lead to bruising and infection. However, the use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor. Blood samples will be analysed for metabolites, electrolytes and pH.

Muscle Biopsies:

The muscle biopsy will be taken in the Exercise Physiology Laboratory at Victoria University of Technology, by a doctor from the Nephrology Department, Royal Melbourne Hospital. A biopsy will be taken from your thigh muscle whilst you are awake, with a local anaesthetic in your thigh. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of enzymes and energy sources. An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm (1/4 inch) long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable, but will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. Many people experience pain during this part of the procedure but this lasts only for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a grain of rice. This poses no long term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. You should not exercise for 24 hours after biopsies and you should avoid heavy knocks to the area. It is common for participants to experience some mild soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these disappear within a week. The whole procedure will be performed under sterile conditions by a qualified medical practitioner. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy and assist in obtaining any necessary treatment.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your cooperation.

Contact Numbers:

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Any queries about your participation in this project may be directed to the researcher (Name: Assoc.Prof. Michael McKenna ph. (03) 9688-4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 1.1.5 Example of standard consent form

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CONSENT FORM FOR SUBJECTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study which will investigate the effects of exercise on a key enzyme located in your muscle cells, known as the sodium, potassium pump.

CERTIFICATION BY SUBJECT
I, ...
of .....

certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the experiment entitled: "Effects of acute exercise on skeletal muscle Na^+,K^+-pumps in humans: content, activity and gene expression" being conducted at Victoria University of Technology by:
Associate Professor Michael McKenna, Dr Rodney Snow, Dr David Cameron-Smith, Ms Kate Murphy and Mr Aaron Petersen.
I certify that the objectives of the experiment, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by:
Associate Professor Michael McKenna.

I have been informed of the risks involved and am freely participating in this study.
Procedures:
1. Pre-experiment subject screening
2. Maximal incremental exercise test on a cycle ergometer
3. Thigh muscle strength testing on a Cybex isokinetic dynamometer
4. Venous catheterisation, arterialisation and blood sampling in the muscle fatigue test
5. Muscle biopsies at rest and following the muscle fatigue test.

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: **.....** }

Witness other than the experimenter: } **Date: .....**

..... }

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. M. McKenna Ph. (03) 54226089). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MC, Melbourne, 8001 (telephone no: 03-9688 4710).

Appendix 1.1.6 Example of standard cardiovascular risk factor questionnaire

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CARDIOVASCULAR RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:
"Effects of uraemia on skeletal muscle ion regulation, metabolism, and exercise performance."

you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: _____ Date: _____

Age: _____ years Weight: _____ kg Height: _____ cms

Give a brief description of your average activity pattern in the past 2 months:
_____
_____
_____

Circle the appropriate response to the following questions.

1.	Are you overweight?	Yes	No	Don't know
2.	Do you smoke?	Yes	No	Social
3.	Does your family have a history of premature cardiovascular problems (eg. heart attack, stroke)?	Yes	No	Don't Know
4.	Are you an asthmatic	Yes	No	Don't Know
5.	Are you a diabetic?	Yes	No	Don't Know
6.	Do you have a high blood cholesterol level?	Yes	No	Don't Know
7.	Do you have high blood pressure?	Yes	No	Don't Know
8.	Are you on any medication?	Yes	No	
	If so, what is the medication? _____			
9.	Do you think you have any medical complaint or any other reason which you know of which you think may prevent you from participating in strenuous exercise?	Yes		No

If Yes, please elaborate _____

I, _____, believe that the answers to these questions are true and correct.

Appendix 1.1.7 Example of standard muscle biopsy questionnaire

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MUSCLE BIOPSY QUESTIONNAIRE

NAME: _____

ADDRESS: _____
DATE: _____ **AGE:** _____ **years**

1. Have you or your family suffered from any tendency to bleed excessively ? (eg. haemophilia) or bruise very easily ?

   Yes No Don't Know

   If yes, please elaborate..._____
   _____

2. Are you allergic to local anaesthetic?

   Yes No Don't Know

   If yes, please elaborate..._____
   _____

3. Do you have any skin allergies?

   Yes No Don't Know

   If yes, please elaborate..._____
   _____

4. Have you any other allergies?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

5. Are you currently on any medication?

   Yes No Don't Know

   If yes, what is the medication? _____
   _____

6. Do you have any other medical problem?

   Yes No

   If yes, please elaborate... _____
   _____

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: _____ Date: _____

Appendix 1.1.8 Example of subject revocation from research form

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REVOCATION OF CONSENT FORM FOR SUBJECTS INVOLVED IN RESEARCH
Used for participants who wish to withdraw from the project

I, ...

of (address), ...

.....

hereby wish to WITHDRAW my consent to participate in the research proposal described in the Plain Language Statement for the study called:
The effects of endurance training on K^+ regulation, exercise performance, and quality of life in patients on haemodialysis.
and understand that such withdrawal WILL NOT jeopardise any treatment or my relationship with Victoria University.

Any data already collected may/may not be included in the research project.

Signature: ..... Date: .....

APPENDIX 2 INDIVIDUAL DATA

APPENDIX 2.1 INDIVIDUAL DATA FOR STUDY ONE

Appendix 2.1.1 Subject physical characteristics

Subject	Age (years)	Height (cm)	Bodymass (kg)	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)
1	28	180	82	66
2	21	173	90	47
3	20	179	61	53
4	40	179	76	70
5	18	171	72	39
6	20	180	78	62
7	26	179	86	45
8	26	173	76	30
9	27	179	86	45
10	29	181	81	56
11	21	168	58	48
12	19	177	61	56
13	21	180	76	59
14	37	164	59	43
15	18	159	56	43
n	**15**	**15**	**15**	**15**
mean	**25**	**175**	**73**	**51**
SD	**7**	**7**	**11**	**11**

Appendix 2.1.2 Exercise data from brief intense exercise test

Subject	Time to fatigue (s)	Total work (J)	Target work/ rep. (J)	Actual work/ rep. (J)	No. of repetitions
1	192	12687	99	101	125
2	189	8802	59	59	148
3	159	5450	53	55	99
4	891	25552	41	42	603
5	788	44721	82	89	504
6	596	27850	72	72	385
7	420	22298	78	89	250
8	312	12245	63	66	186
9	119	4221	46	56	76
10	181	5042	39	39	128
11	615	18630	41	42	442
12	83	3040	61	52	59
13	98	6371	92	95	67
14	514	25142	68	74	342
15	118	5321	60	61	87
n	**15**	**15**	**15**	**15**	**15**
mean	**352**	**15158**	**64**	**66**	**233**
SD	**268**	**11946**	**18**	**20**	**178**

Appendix 2.1.3 [^{3}H]ouabain binding to skeletal muscle before and after brief intense exercise (pmol.(g wet weight)$^{-1}$)

Subject	Rest	Fatigue	+3 h	+24 h
1	164	239	212	207
2	229	256	205	260
3	356	445	405	377
4	354	361	328	343
5	316	240	224	278
6	341	359	347	329
7	297	291	288	263
8			263	182
9	239	239	376	280
10	327	328	317	366
11	285	304	275	232
12	346	342	415	332
13	281	322	285	278
14	251	195	266	232
15	283	302	319	276
n	**14**	**14**	**15**	**15**
mean	**291**	**302**	**302**	**282**
SD	**56**	**65**	**65**	**57**

Appendix 2.1.4 Skeletal muscle maximal 3-O-MFPase activity before and after brief intense exercise (pmol.min^{-1}.(g wet weight)$^{-1}$)

Subject	Rest	Fatigue	+3 h	+24 h
1	239	206	244	251
2	321	277	272	223
3	250	217	235	251
4	307	301	331	286
5	247	256	245	231
6		208		251
7			210	188
8	222	220	210	227
9	314	249	240	258
10	213	186	219	208
11	209	201	211	216
12	242	207	243	233
13		221		215
14	267	212	240	252
15	201	180	193	201
n	**12**	**14**	**13**	**15**
mean	**253**	**224**	**238**	**233**
SD	**42**	**34**	**35**	**26**

Appendix 2.1.5 Brief intense exercise effects on Na^+,K^+-ATPase α_1, α_2 and α_3 mRNA expression in human skeletal muscle

	α_1				α_2				α_3			
Subject	**Rest**	**Fat**	**+3 h**	**+24 h**	**Rest**	**Fat**	**+3 h**	**+24 h**	**Rest**	**Fat**	**+3 h**	**+24 h**
1	1.00	5.62	9.75	3.24	1.00	3.48	0.53	0.38	1.00	3.35	0.10	1.59
2	1.00	0.53	1.25	4.93	1.00	0.20	0.78	1.76	1.00	3.35	5.46	1.20
3	1.00	0.09	0.16	0.37	1.00	0.06	0.08	1.04	1.00	3.35	1.51	0.26
4	1.00	4.39	1.28	6.96	1.00	10.56	1.47	0.71	1.00	0.88	1.08	0.09
5	1.00	3.24	1.63	7.39	1.00	5.24	6.02	7.76	1.00	1.47	0.46	1.38
6	1.00	0.15	0.12	0.04	1.00	0.04	0.02	0.15	1.00	0.23	0.11	0.59
7	1.00	2.27	1.07	0.65	1.00	4.48	8.94	4.48	1.00	10.27	2.29	1.46
8	1.00	2.86	0.56	1.38	1.00	0.39	0.93	0.20	1.00	1.05	4.79	2.33
9	1.00	2.46	1.13	3.56	1.00	3.48	0.80	2.27	1.00	3.35	1.11	2.57
10	1.00	4.75	1.04	5.69	1.00	6.16	13.41	2.07	1.00	2.01	0.49	0.75
11	1.00	0.47	0.42	1.15	1.00	1.85	8.78	0.73	1.00	7.39	6.19	0.93
12	1.00	4.72	2.27	6.75	1.00	9.02	7.04	0.74	1.00	6.87	8.94	1.67
13	1.00	0.66	1.62	0.19	1.00	1.22	4.48	0.19	1.00	0.01	2.86	0.37
14	1.00	2.24	0.37	3.11	1.00	2.57	9.42	2.19	1.00	3.35	2.86	1.62
n	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**
mean	**1.00**	**2.46**	**1.62**	**3.24**	**1.00**	**3.48**	**4.48**	**1.76**	**1.00**	**3.35**	**2.73**	**1.20**
SD	**0.00**	**1.90**	**2.42**	**2.70**	**0.00**	**3.33**	**4.43**	**2.10**	**0.00**	**2.96**	**2.68**	**0.75**

Appendix 2.1.6 Brief intense exercise effects on Na^+,K^+-ATPase β_1, β_2 and β_3 mRNA expression in human skeletal muscle

	β_1				β_2				β_3			
Subject	**Rest**	**Fat**	**+3 h**	**+24 h**	**Rest**	**Fat**	**+3 h**	**+24 h**	**Rest**	**Fat**	**+3 h**	**+24 h**
1	1.00	2.28	0.97	1.10	1.00	0.88	0.54	1.05	1.00	3.51	1.54	1.24
2	1.00	0.83	0.30	1.23	1.00	0.98	0.19	1.33	1.00	2.58	1.18	1.04
3	1.00	0.61	0.17	1.92	1.00	0.40	0.74	0.58	1.00	1.01	1.04	0.23
4	1.00	12.69	1.38	0.57	1.00	1.84	4.64	0.23	1.00	2.55	0.42	0.06
5	1.00	3.45	1.96	0.73	1.00	1.37	0.30	0.93	1.00	0.95	3.01	0.82
6	1.00	0.37	0.26	0.07	1.00	0.25	0.20	0.86	1.00	0.94	0.40	1.13
7	1.00	1.85	2.84	1.00	1.00	4.35	3.66	0.98	1.00	5.98	6.70	6.63
8	1.00	0.04	0.65	0.13	1.00	0.18	1.94	0.85	1.00	0.15	10.41	0.36
9	1.00	2.28	2.21	4.31	1.00	2.72	0.88	2.69	1.00	2.04	0.60	0.39
10	1.00	0.60	2.68	3.21	1.00	3.81	1.96	3.09	1.00	0.89	0.43	0.44
11	1.00	1.24	11.16	0.69	1.00	3.71	3.46	1.33	1.00	1.42	7.84	0.53
12	1.00	1.71	10.41	0.39	1.00	5.17	4.71	0.48	1.00	4.99	2.37	0.91
13	1.00	2.36	2.82	0.52	1.00	8.98	1.94	2.06	1.00	0.95	2.85	0.19
14	1.00	1.58	1.68	1.33	1.00	3.43	2.02	2.10	1.00	0.59	1.05	0.60
n	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**	**14**
mean	**1.00**	**2.28**	**2.82**	**1.23**	**1.00**	**2.72**	**1.94**	**1.33**	**1.00**	**2.04**	**2.85**	**1.04**
SD	**0.00**	**3.14**	**3.50**	**1.20**	**0.00**	**2.44**	**1.61**	**0.85**	**0.00**	**1.73**	**3.17**	**1.65**

Appendix 2.1.7 Plasma [K^+] (mmol.l^{-1}) before, during and after brief intense exercise

Subject	Rest	1 min	Fatigue	+1 min	+2 min	+5 min	+10 min
1	3.9	4.0	4.4	3.7	3.3	3.3	3.5
2	4.1	4.5	4.7	4.0	3.6	3.7	3.9
3	3.6	3.8	4.0	3.6	3.5	3.4	3.4
4	4.1	3.9	4.3	3.2	3.0	2.9	3.3
5	3.8	3.7	4.0	3.9	3.4	3.2	3.6
6	3.9	4.3	4.4	3.6	3.4	3.5	3.7
7	4.4	4.6	4.3	3.7	3.9	3.9	4.0
8	3.7	4.0	4.2	3.7	3.7	3.5	3.7
9	3.8	3.8	4.2	3.7	3.7	3.5	4.1
10	4.1	4.6	4.8	4.2	3.5	3.8	
11	3.9	4.0	3.8	3.6	3.8	3.8	3.8
12	3.7	3.9	4.6	3.6	3.4	3.2	3.7
13	4.2	4.4	4.6	3.7	3.7	3.6	3.9
14	3.9	4.4			3.8	3.9	
15	3.8	4.0	4.5	3.6	3.6	3.6	3.9
n	**15**	**15**	**14**	**14**	**15**	**15**	**13**
mean	**3.9**	**4.1**	**4.3**	**3.7**	**3.5**	**3.5**	**3.7**
SD	**0.2**	**0.3**	**0.3**	**0.2**	**0.2**	**0.3**	**0.2**

APPENDIX 2.2 INDIVIDUAL DATA FOR STUDY TWO

Appendix 2.2.1 Subject physical characteristics

		Subject	Age (years)	Body mass (kg)	Height (cm)	BMI	[Hb] ($g.dl^{-1}$)	Hct (%)
CON	Baseline	1	51	81.0	1.79	25.3	14.4	41.9
		2	36	81.9	1.86	23.7	14.8	42.0
		3	50	85.8	1.77	27.5	15.6	43.9
		4	44	88.0	1.73	29.4	15.1	41.8
		5	46	59.9	1.68	21.2	12.6	36.1
		6	29	56.0	1.75	18.3	16.5	45.6
n			**6**	**6**	**6**	**6**	**6**	**6**
mean			**43**	**75.4**	**1.76**	**24.2**	**14.8**	**41.9**
SD			**9**	**13.8**	**0.06**	**4.1**	**1.3**	**3.2**
HD	Baseline	1	37	64.3	1.67	23.1	11.8	33.8
		2	34	69.4	1.58	27.8	12.8	36.7
		3	26	70.0	1.78	22.1	13.8	41.9
		4	52	59.1	1.74	19.5	13.9	40.6
		5	59	80.5	1.80	24.8	12.9	39.1
		6	44	70.2	1.71	24.0	12.3	35.6
n			**6**	**6**	**6**	**6**	**6**	**6**
mean			**42**	**68.9**	**1.71**	**23.6**	**12.9**	**37.9**
SD			**12**	**7.1**	**0.08**	**2.8**	**0.8**	**3.1**
	Pre-train	1	37	64.1	1.67	23.0	11.45	32.1
		2	34	71.6	1.58	28.7	12.45	36.8
		3	26	68.3	1.78	21.6	13.8	41.9
		4	52	60.3	1.74	19.9	14.3	42.1
		5	59	80.8	1.80	24.9	12.65	38.9
		6	44	70.5	1.71	21.8	12.3	35.6
n			**6**	**6**	**6**	**6**	**6**	**6**
mean			**42**	**69.3**	**1.71**	**23.3**	**12.8**	**37.9**
SD			**12**	**7.1**	**0.08**	**3.1**	**1.0**	**3.9**
	Post-train	1	37	63.1	1.67	22.6	12.9	36.5
		2	34	72.1	1.58	28.9	13.1	37.6
		3	26	68.7	1.78	21.7	13.8	41.9
		4	52	60.7	1.74	20.0	13.95	39.8
		5	59	81.1	1.80	25.0	12.25	37.5
		6	44	71.2	1.71	22.0	12.3	35.6
n			**6**	**6**	**6**	**6**	**6**	**6**
mean			**42**	**69.5**	**1.71**	**23.4**	**13.1**	**38.1**
SD			**12**	**7.3**	**0.08**	**3.1**	**0.7**	**2.3**

Appendix 2.2.2 $\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$) in CON and before and after training in HD

	CON	**HD**		
Subject	Baseline	Baseline	Pre-train	Post-train
1	41.0	29.8	26.7	31.8
2	44.7	17.8	20.9	21.7
3	35.6	35.4	33.5	31.2
4	37.2	23.9	27.8	29.5
5	32.1	17.8	20.0	17.9
6	33.7	16.2	19.3	19.5
n	**6**	**6**	**6**	**6**
mean	**37.4**	**23.5**	**24.7**	**25.3**
SD	**4.7**	**7.7**	**5.6**	**6.3**

Appendix 2.2.3 Time to fatigue (s) during an incremental cycle test in CON and before and after training in HD.

	CON	**HD**		
Subject		Baseline	Pre-train	Post-train
1	1165	626	593	676
2	1100	378	432	442
3	1074	729	726	740
4	1027	487	553	600
5	670	458	460	509
6	664	390	431	452
n	**6**	**6**	**6**	**6**
mean	**950**	**511**	**533**	**570**
SD	**224**	**139**	**116**	**123**

Appendix 2.2.4 Peak workrate (W) during an incremental cycle test in CON and before and after training in HD.

	CON	**HD**		
Subject		Baseline	Pre-train	Post-train
1	1165	626	593	676
2	1100	378	432	442
3	1074	729	726	740
4	1027	487	553	600
5	670	458	460	509
6	664	390	431	452
n	**6**	**6**	**6**	**6**
mean	**950**	**511**	**533**	**570**
SD	**224**	**139**	**116**	**123**

Appendix 2.2.5 Total work done (kJ) during an incremental cycle test in CON and before and after training in HD.

	CON	**HD**		
Subject	Baseline	Baseline	Pre-train	Post-train
1	179	165	150	180
2	160	105	120	120
3	152	195	195	195
4	140	135	150	165
5	612	120	120	135
6	601	105	120	120
n	**6**	**6**	**6**	**6**
mean	**125**	**39**	**41**	**46**
SD	**52**	**20**	**17**	**19**

Appendix 2.2.6 Knee-extensor fatigue index (%) in CON and before and after training in HD.

	CON	**HD**		
Subject	Baseline	Baseline	Pre-train	Post-train
1	8	29	30	30
2	17	25	25	34
3	11	6	18	9
4	14	24	22	25
5	14	26	22	11
6		29	31	33
n	**5**	**6**	**6**	**6**
mean	**13**	**23**	**25**	**24**
SD	**4**	**9**	**5**	**11**

Appendix 2.2.7 Knee-extensor isokinetic peak torque (Nm) in CON and before and after training in HD.

		0º.s^{-1}	**60º.s^{-1}**	**120º.s^{-1}**	**180º.s^{-1}**	**240º.s^{-1}**	**300º.s^{-1}**	**360º.s^{-1}**
CON	Baseline	215	185	178	157	134	114	104
		287	259	208	178	151	130	113
		256	222	186	160	133	115	106
		238	212	179	148	130	113	100
		170	127	87	70	59	51	44
		167	175	134	109	96	74	79
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**222**	**197**	**162**	**137**	**117**	**99**	**91**
SD		**48**	**45**	**44**	**40**	**34**	**30**	**26**
HD	Baseline	168	125	112	95	84	72	62
		106	106	83	74	62	55	52
		248	181	136	107	94	76	71
		155	111	81	60	46	40	37
		130	109	94	78	61	49	45
		171	116	82	80	69	59	50
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**163**	**125**	**98**	**82**	**69**	**59**	**53**
SD		**48**	**28**	**22**	**17**	**17**	**14**	**12**
	Pre-train	165	142	118	97	102	85	65
		98	92	84	69	57	47	45
		214	140	120	98	80	58	66
		155	101	79	62	47	37	36
		140	125	95	74	67	58	52
		148	128	99	88	60	58	47
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**153**	**121**	**99**	**81**	**69**	**57**	**52**
SD		**38**	**21**	**17**	**15**	**20**	**16**	**12**
	Post-train	157	125	109	104	89	81	78
		100	100	86	76	64	55	54
		218	186	152	130	102	88	74
		155	100	82	64	51	41	36
		162	147	128	100	81	63	46
		163	117	100	91	75	57	50
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**159**	**129**	**110**	**94**	**77**	**64**	**56**
SD		**37**	**33**	**27**	**23**	**18**	**17**	**16**

Appendix 2.2.8 Knee-extensor isokinetic peak torque relative to body mass ($Nm.kg^{-1}$) in CON and before and after training in HD.

		0°.s^{-1}	**60°.s^{-1}**	**120°.s^{-1}**	**180°.s^{-1}**	**240°.s^{-1}**	**300°.s^{-1}**	**360°.s^{-1}**
CON	Baseline	2.65	2.28	2.20	1.94	1.65	1.41	1.28
		3.50	3.16	2.54	2.17	1.84	1.59	1.38
		2.98	2.59	2.17	1.86	1.55	1.34	1.24
		2.70	2.41	2.03	1.68	1.47	1.28	1.13
		3.49	2.61	1.79	1.44	1.21	1.05	0.90
		2.98	3.13	2.39	1.95	1.71	1.32	1.41
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**3.05**	**2.70**	**2.19**	**1.84**	**1.57**	**1.33**	**1.22**
SD		**0.37**	**0.37**	**0.27**	**0.25**	**0.22**	**0.18**	**0.19**
HD	Baseline	2.60	1.94	1.74	1.47	1.31	1.12	0.96
		1.53	1.53	1.20	1.07	0.89	0.79	0.75
		3.54	2.59	1.94	1.53	1.34	1.09	1.01
		2.61	1.88	1.37	1.01	0.78	0.68	0.62
		1.61	1.35	1.16	0.96	0.76	0.61	0.55
		2.44	1.65	1.17	1.14	0.98	0.84	0.71
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**2.39**	**1.82**	**1.43**	**1.20**	**1.01**	**0.85**	**0.77**
SD		**0.75**	**0.43**	**0.33**	**0.24**	**0.26**	**0.21**	**0.19**
	Pre-train	2.57	2.22	1.84	1.51	1.59	1.33	1.01
		1.37	1.28	1.17	0.96	0.80	0.66	0.63
		3.13	2.05	1.76	1.43	1.17	0.85	0.97
		2.57	1.68	1.31	1.03	0.78	0.61	0.60
		1.73	1.54	1.18	0.91	0.83	0.71	0.64
		2.10	1.82	1.40	1.25	0.85	0.82	0.67
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**2.25**	**1.76**	**1.44**	**1.18**	**1.00**	**0.83**	**0.75**
SD		**0.64**	**0.34**	**0.29**	**0.25**	**0.32**	**0.26**	**0.19**
	Post-train	2.49	1.98	1.73	1.65	1.41	1.28	1.24
		1.39	1.39	1.19	1.05	0.89	0.76	0.75
		3.17	2.71	2.21	1.89	1.48	1.28	1.08
		2.55	1.65	1.35	1.05	0.84	0.68	0.59
		2.00	1.81	1.58	1.23	1.00	0.78	0.57
		2.29	1.64	1.40	1.28	1.05	0.80	0.70
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**2.31**	**1.86**	**1.58**	**1.36**	**1.11**	**0.93**	**0.82**
SD		**0.60**	**0.46**	**0.36**	**0.34**	**0.27**	**0.28**	**0.27**

Appendix 2.2.9 Plasma [K^+] (mmol.l^{-1}) before, during and after an incremental cycle test, in CON and before and after training in HD.

		Rest	1	2	3	4	5	6	Fat	F+1	F+2	F+5	F+10
CON	Baseline	3.8	4.0	4.2					6.5	5.3	4.7	3.5	3.6
		3.9	4.0	4.0		4.0		4.1	6.2	5.2	4.4		
		4.2	4.5	4.5	4.6	4.6	4.7	4.8	6.3	6.1	5.5	4.4	4.5
		4.1	4.5	4.6	4.9			4.7	6.9	6.1	5.7	4.4	4.3
		3.9	4.2	4.2	4.3	4.5	4.6	4.7	6.5	5.1	4.8	4.1	4.0
		3.8	3.8	3.8	3.8	3.8	3.9	4.0	5.0				
n		**6**	**6**	**6**	**4**	**4**	**3**	**5**	**6**	**5**	**5**	**4**	**4**
mean		**4.0**	**4.1**	**4.2**	**4.4**	**4.2**	**4.4**	**4.5**	**6.2**	**5.6**	**5.0**	**4.1**	**4.1**
SD		**0.2**	**0.3**	**0.3**	**0.5**	**0.4**	**0.4**	**0.4**	**0.7**	**0.5**	**0.5**	**0.4**	**0.4**
HD	Baseline	4.6	4.9	4.8	4.9	5.1	5.3	5.2	5.9	4.7	4.3	4.4	4.9
		5.2	5.7	5.8	5.9	5.9	6.1	6.4	6.1	5.2	4.7	4.8	5.4
		4.7	5.1	5.1	5.1	5.2	5.3	5.4	6.5	5.4	4.9	4.7	4.8
		4.7	5.1	5.2	5.3	5.6	5.6	5.8	6.6	5.4	4.6	4.4	4.9
		5.3	5.6	5.8	5.7	6.0	6.0	6.4	6.6	5.6	5.2	4.9	5.4
		3.8	4.1	4.2	4.4	4.6	4.8	5.0	4.8	4.2	4.2	4.1	4.0
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**4.7**	**5.1**	**5.2**	**5.2**	**5.4**	**5.5**	**5.7**	**6.1**	**5.1**	**4.7**	**4.5**	**4.9**
SD		**0.5**	**0.6**	**0.6**	**0.5**	**0.5**	**0.5**	**0.6**	**0.7**	**0.5**	**0.4**	**0.3**	**0.5**
	Pre-train	4.4	4.8	4.8	4.7	4.8	4.8	5.0	5.4	4.0	3.7	3.9	4.5
		5.1	5.3	5.4	5.5	5.8	5.7	6.0	6.0	4.8	4.5	4.4	4.8
		4.6	4.9	5.0	5.0	5.0	5.1	5.2	6.5	5.1	4.6	4.5	4.7
		4.9	4.9	4.9	4.9	5.0	5.3	5.6	6.2	5.2	4.5	4.3	4.8
		5.4	5.9	5.8	5.8	6.0	5.9	6.1	6.5	5.6	5.0	4.6	5.4
		3.7	4.1	4.2	4.3	4.5	4.7	4.9	5.2	4.5	4.1	3.9	3.9
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**4.7**	**5.0**	**5.0**	**5.0**	**5.2**	**5.3**	**5.5**	**6.0**	**4.9**	**4.4**	**4.3**	**4.7**
SD		**0.6**	**0.6**	**0.5**	**0.6**	**0.6**	**0.5**	**0.5**	**0.5**	**0.6**	**0.4**	**0.3**	**0.5**
	Post-train	4.9	5.1	5.0	5.1	5.1	5.0	5.1	5.6	4.4	4.0	4.1	4.5
		5.3	5.6	5.8	5.7	5.9	5.9	6.1	5.7	4.9	4.6	4.7	5.6
		4.6	4.8	4.9	4.9	4.9	5.1	5.2	6.0	4.9	4.7	4.6	4.8
		3.5	3.8	3.7	3.9	4.0	4.1	4.3	5.7	4.5	3.7	3.2	3.4
		4.9	5.4	5.3	5.3	5.5	5.6	5.6	6.0	5.2	4.7	4.3	4.9
		3.9	4.2	4.3	4.4	4.5	4.8	5.0	5.2	4.3	4.0	4.0	4.1
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
mean		**4.5**	**4.8**	**4.8**	**4.9**	**5.0**	**5.1**	**5.2**	**5.7**	**4.7**	**4.3**	**4.1**	**4.5**
SD		**0.7**	**0.7**	**0.7**	**0.7**	**0.7**	**0.6**	**0.6**	**0.3**	**0.3**	**0.4**	**0.6**	**0.7**

Appendix 2.2.10 $\Delta[K^+]$.work^{-1} ratio (nmol.l^{-1}. J^{-1}) during an incremental cycle test in CON and before and after training in HD.

	CON	**HD**		
Subject	Baseline	Baseline	Pre-train	Post-train
1	10	23	21	12
2	6	44	34	14
3	7	26	27	19
4	12	56	30	44
5	25	45	37	31
6	11	46	54	45
n	**6**	**6**	**6**	**6**
mean	**12**	**40**	**34**	**27**
SD	**7**	**13**	**11**	**15**

APPENDIX 2.3 INDIVIDUAL DATA FROM STUDY THREE

Appendix 2.3.1 Subject physical characteristics

Subject	Age (years)	Bodymass (kg)	Height (cm)	BMI ($kg.m^{-2}$)	[Hb] ($g.l^{-1}$)	Hct (%)	Creatinine clearance ($ml.min^{-1}$)
CON							
1	47	87.6	187	25	14.1	41.6	111.4
2	51	81.0	179	25	14.4	41.9	84.0
3	36	81.9	186	24	14.8	42.0	131.4
4	50	85.8	177	28	15.6	43.9	105.9
5	35	82.4	182	25	15.9	42.9	133.5
6	44	88.0	173	29	15.1	41.8	115.9
7	32	48.7	158	20	13.3	37.7	92.0
8	46	59.9	168	21	12.6	36.1	84.4
9	29	56.0	175	18	16.5	45.6	130.1
10	28	52.3	166	19	12.7	36.6	96.0
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**39.8**	**72.4**	**175**	**23.4**	**14.5**	**41.0**	**108.5**
SD	**8.8**	**16.0**	**9**	**3.7**	**1.3**	**3.2**	**19.2**
HD							
1	45	107.2	180	33	11.7	33.5	17.2
2	46	69.8	176	23	14.5		7.9
3	33	67.0	158	27	14.0	40.9	8.2
4	30	65.3	173	22	11.9	32.7	7.9
5	45	91.3	184	27	11.6	32.9	11.5
6	41	68.6	154	29	14.1	42.7	7.8
7	52	102.5	180	32	14.4	40.9	10.2
8	37	60.8	174	20	15.7	47.6	5.9
9	40	74.2	186	21	12.3	35.5	13.5
10	23	61.7	183	18	13.0	38.4	8.6
n	**10**	**10**	**10**	**10**	**10**	**9**	**10**
mean	**39.2**	**76.8**	**175**	**25.2**	**13.3**	**38.3**	**9.9**
SD	**8.6**	**17.1**	**11**	**5.0**	**1.4**	**5.12**	**3.4**
RTx							
1	35	70.7	183	21	12.5	37.5	57.3
2	49	99.0	175	32	14.7	40.7	85.6
3	25	63.4	153	27	12.3	35.7	85.0
4	31	66.1	161	26	14.1	39.6	98.8
5	39	90.5	185	26	13.1	38.4	66.4
6	55	93.6	184	28	13.2	39.6	61.4
7	54	63.5	159	25	12.9	35.6	63.7
8	48	54.2	157	22	13.6	38.1	47.6
9	36	79.8	178	25	14.6	43.4	113.8
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**
mean	**41.3**	**75.6**	**171**	**25.8**	**13.4**	**38.7**	**75.5**
SD	**10.6**	**15.7**	**13**	**3.3**	**0.9**	**2.5**	**21.6**

Appendix 2.3.2 Skeletal muscle [^{3}H]ouabain binding site content (pmol.(g wet weight)$^{-1}$)

Subject	CON	HD	RTx
1	283	317	293
2	389	311	263
3	266	171	198
4	310	369	301
5	290		208
6	267	182	316
7	345	241	334
8	232	378	294
9	264	340	271
10	189	252	
n	**10**	**9**	**9**
mean	**284**	**285**	**275**
SD	**56**	**77**	**46**

Appendix 2.3.3 Skeletal muscle maximal 3-O-MFPase activity (nmol.min^{-1}.(g wet weight)$^{-1}$)

Subject	CON	HD	RTx
1	328	189	260
2	158	212	174
3	226	110	77
4	277	231	194
5	412		205
6	302	125	188
7	337	181	251
8	170	249	194
9	263	274	220
10	235	118	
n	**10**	**9**	**9**
mean	**271**	**188**	**196**
SD	**78**	**60**	**53**

Appendix 2.3.4 Skeletal muscle Na^+,K^+-ATPase isoform relative protein abundance (arbitrary units)

	α_1	α_2	α_3	β_1	β_2	β_3
CON						
1	2.0	0.9	6.9	3.3	0.9	1.1
2	7.3	1.0	10.4	1.9	2.7	0.5
3	2.7	1.4	8.6	3.5	1.7	0.8
4	3.4	0.7	4.3	1.5	1.7	1.1
5	2.9	2.2	2.7	1.3	3.6	0.5
6	0.3	2.1	1.1	1.3	1.1	0.5
7	0.7	2.2	2.4	3.9	1.3	0.7
8	1.0	1.0	1.0	1.0	1.0	1.0
9	0.2	1.4	7.6	2.1	0.6	0.4
10	0.5	0.8	4.9	1.4	2.3	1.2
n	**10**	**10**	**10**	**10**	**10**	**10**
mean	**2.1**	**1.4**	**4.8**	**2.0**	**1.8**	**0.7**
SD	**2.3**	**0.6**	**3.4**	**1.0**	**0.9**	**0.3**
HD						
1	0.7	1.5	2.0	1.9	1.0	1.0
2	0.2	1.7	2.0	1.1	2.1	1.2
3	0.7	0.3	1.4	0.9	1.0	0.1
4	3.7	2.4	5.1	1.3	2.1	0.2
5						
6	1.4	3.1	5.5	1.8	1.1	0.4
7	1.3	1.9	4.0	1.4	1.2	1.3
8	0.5	1.9	3.0	3.0	1.0	0.6
9	2.4	2.0	2.6	1.3	1.8	1.3
10	1.0	0.6	1.3	1.7	0.8	0.3
n	**9**	**9**	**9**	**9**	**9**	**9**
mean	**1.3**	**1.7**	**3.0**	**1.6**	**1.4**	**0.7**
SD	**1.1**	**0.9**	**1.5**	**0.6**	**0.5**	**0.5**
RTx						
1	1.7	1.5	3.9	3.9	0.4	0.4
2	3.1	0.3	5.3	1.9	0.5	0.7
3	7.7	1.4	4.1	4.3	1.1	0.4
4	3.2	1.8	2.5	1.7	1.1	1.0
5	0.9	0.4	4.1	1.7	1.3	0.3
6	0.8	1.0	3.0	5.6	1.3	0.1
7	2.0	2.9	1.7	1.0	1.4	2.7
8	0.9	0.9	2.7	0.8	2.7	0.4
9	0.2	0.9	1.8	1.6	4.2	1.1
n	**9**	**9**	**9**	**9**	**9**	**9**
mean	**2.3**	**1.2**	**3.2**	**2.5**	**1.5**	**0.8**
SD	**2.3**	**0.8**	**1.2**	**1.7**	**1.2**	**0.8**

Appendix 2.3.5 Incremental exercise test data

	$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	Peak power (Watts)	Time to fatigue (s)
CON			
1	37.5	300	1147
2	40.6	300	1165
3	42.5	285	1100
4	35.5	270	1074
5	37.7	255	975
6	36.1	270	1027
7	30.9	135	495
8	30.6	180	670
9	33.4	180	664
10	32.0	180	681
n	**10**	**10**	**10**
mean	**35.7**	**236**	**900**
SD	**4**	**60**	**246**
HD			
1	18.8	165	630
2	32.1	180	722
3	21.3	120	435
4	34.8	180	711
5	26.9	195	780
6	16.6	90	315
7	22.0	180	720
8	33.8	165	620
9	31.1	195	794
10	26.4	150	554
n	**10**	**10**	**10**
mean	**26.4**	**162**	**628**
SD	**7**	**34**	**155**
RTx			
1	26.9	165	618
2	19.3	165	610
3	23.4	135	499
4	45.9	255	1020
5	39.2	240	950
6	18.4	150	560
7	19.2	105	369
8	22.9	120	430
9	27.9	165	660
n	**9**	**9**	**9**
mean	**27.0**	**167**	**635**
SD	**10**	**51**	**220**

Appendix 2.3.6 Thigh muscle cross-sectional area (cm^2)

Subject	CON	HD	RTx
1	139.4	121.1	104.3
2	146.0	114.5	146.8
3	134.9	99.1	106.6
4	169.8	118.7	142.1
5	151.4		135.2
6	159.3	92.3	127.6
7	85.1	152.1	91.4
8	106.2	109.2	88.3
9	119.5	125.0	118.5
10	99.1	86.9	
n	**10**	**9**	**9**
mean	**131.1**	**113.2**	**117.9**
SD	**27.7**	**19.6**	**21.5**

Appendix 2.3.7 Knee-extensor fatigue index (%)

Subject	CON	HD	RTx
1	12	25	22
2	8	23	19
3	17	27	17
4	11	31	12
5	25	27	9
6	14	24	31
7	13	25	31
8	14	29	42
9		25	31
10	16	15	
n	**9**	**10**	**9**
mean	**15**	**25**	**24**
SD	**5**	**4**	**11**

Appendix 2.3.8 Knee-extensor peak isokinetic torque data relative to bodymass ($Nm.kg^{-1}$)

	0°.s^{-1}	**60°.s^{-1}**	**120°.s^{-1}**	**180°.s^{-1}**	**240°.s^{-1}**	**300°.s^{-1}**	**360°.s^{-1}**
CON							
1	3.13	2.55	2.12	1.76	1.50	1.28	1.13
2	2.65	2.28	2.20	1.94	1.65	1.41	1.28
3	3.50	3.16	2.54	2.17	1.84	1.59	1.38
4	2.98	2.59	2.17	1.86	1.55	1.34	1.24
5	3.78	3.18	2.56	2.28	1.77	1.56	1.34
6	2.70	2.41	2.03	1.68	1.47	1.28	1.13
7	2.07	1.93	1.17	1.33	1.13	0.99	0.82
8	2.84	2.12	1.45	1.17	0.98	0.85	0.73
9	2.98	3.13	2.39	1.95	1.71	1.32	1.41
10	3.27	2.45	1.89	1.49	1.15	0.98	0.84
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**2.99**	**2.58**	**2.05**	**1.76**	**1.48**	**1.26**	**1.13**
SD	**0.48**	**0.44**	**0.45**	**0.35**	**0.30**	**0.25**	**0.25**
HD							
1	1.86	1.05	0.88	0.80	0.65	0.54	0.52
2	2.80	1.81	1.40	1.27	1.10	0.97	0.87
3	1.54	1.36	1.27	1.07	0.97	0.85	0.73
4	3.15	2.95	2.32	1.88	1.63	1.41	1.20
5	1.98	1.56	1.25	1.05	0.73	0.51	0.49
6	1.12	1.03	0.87	0.69	0.56	0.44	0.42
7	1.62	1.63	1.42	1.27	1.05	0.86	0.75
8	3.63	2.71	2.29	1.71	1.43	1.27	1.09
9	3.06	2.76	2.65	2.22	1.74	1.68	1.24
10	2.85	2.42	2.04	1.75	1.40	1.19	1.04
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**2.36**	**1.93**	**1.64**	**1.37**	**1.13**	**0.97**	**0.83**
SD	**0.84**	**0.73**	**0.64**	**0.50**	**0.41**	**0.41**	**0.30**
RTx							
1	2.40	1.87	1.49	1.20	1.00	0.79	0.69
2	2.00	1.32	1.13	0.96	0.78	0.62	0.46
3	2.16	2.22	1.78	1.42	1.14	1.06	0.95
4	3.27	2.56	2.16	1.85	1.55	1.24	1.07
5	2.33	1.73	1.45	1.13	1.08	0.94	0.85
6	2.37	1.98	1.58	1.28	1.08	0.91	0.79
7	1.86	1.60	1.22	0.94	0.81	0.72	0.61
8	2.23	1.98	1.67	1.45	1.25	1.10	0.97
9	2.34	2.04	1.67	1.35	1.17	0.99	0.82
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**
mean	**2.33**	**1.92**	**1.57**	**1.29**	**1.10**	**0.93**	**0.80**
SD	**0.40**	**0.36**	**0.31**	**0.28**	**0.23**	**0.20**	**0.19**

Appendix 2.3.9 Knee-extensor isokinetic peak torque relative to thigh muscle cross-sectional area ($Nm.cm^{-2}$)

	0°.s^{-1}	**60°.s^{-1}**	**120°.s^{-1}**	**180°.s^{-1}**	**240°.s^{-1}**	**300°.s^{-1}**	**360°.s^{-1}**
CON							
1	1.97	1.60	1.33	1.10	0.94	0.80	0.71
2	1.47	1.27	1.22	1.08	0.92	0.78	0.71
3	2.13	1.92	1.54	1.32	1.12	0.96	0.84
4	1.51	1.31	1.10	0.94	0.78	0.68	0.62
5	2.06	1.73	1.39	1.24	0.96	0.85	0.73
6	1.49	1.33	1.12	0.93	0.81	0.71	0.62
7	1.19	1.10	0.67	0.76	0.65	0.56	0.47
8	1.60	1.20	0.82	0.66	0.56	0.48	0.41
9	1.40	1.46	1.12	0.91	0.80	0.62	0.66
10	1.73	1.29	1.00	0.79	0.61	0.51	0.44
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**1.65**	**1.42**	**1.13**	**0.97**	**0.81**	**0.70**	**0.62**
SD	**0.31**	**0.26**	**0.26**	**0.21**	**0.18**	**0.16**	**0.14**
HD							
1	1.65	0.93	0.78	0.71	0.57	0.48	0.46
2	1.71	1.10	0.86	0.77	0.67	0.59	0.53
3	1.04	0.92	0.86	0.73	0.66	0.58	0.49
4	1.73	1.62	1.28	1.04	0.90	0.77	0.66
5							
6	0.83	0.76	0.65	0.51	0.42	0.33	0.31
7	1.09	1.10	0.96	0.85	0.71	0.58	0.51
8	2.02	1.51	1.27	0.95	0.80	0.70	0.60
9	1.82	1.64	1.58	1.32	1.03	1.00	0.74
10	2.02	1.72	1.45	1.24	0.99	0.85	0.74
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**
mean	**1.55**	**1.26**	**1.07**	**0.90**	**0.75**	**0.65**	**0.56**
SD	**0.44**	**0.37**	**0.33**	**0.26**	**0.20**	**0.20**	**0.14**
RTx							
1	1.62	1.27	1.01	0.81	0.68	0.54	0.47
2	1.35	0.89	0.76	0.65	0.53	0.42	0.31
3	1.28	1.32	1.06	0.84	0.67	0.63	0.56
4	1.52	1.19	1.01	0.86	0.72	0.58	0.50
5	1.56	1.16	0.97	0.75	0.72	0.63	0.57
6	1.74	1.45	1.16	0.94	0.80	0.67	0.58
7	1.29	1.11	0.85	0.66	0.56	0.50	0.43
8	1.37	1.22	1.02	0.89	0.76	0.67	0.59
9	1.57	1.37	1.13	0.91	0.78	0.67	0.55
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**
mean	**1.48**	**1.22**	**1.00**	**0.81**	**0.69**	**0.59**	**0.51**
SD	**0.16**	**0.16**	**0.12**	**0.11**	**0.09**	**0.09**	**0.09**

Appendix 2.3.10 Plasma [K^+] (mmol.l^{-1}) before, during and after incremental exercise test

	Rest	**1**	**2**	**3**	**4**	**5**	**Fat**	**F+1**	**F+2**	**F+5**	**F+10**
CON											
1	4.11	4.39	4.30	4.38	4.42	4.46	5.80	5.22	4.50	3.86	4.06
2	3.79	3.88	4.08				5.50	4.51			
3	3.90	3.78	3.88		3.75		4.90	4.24	3.60		
4	4.22	4.29	4.36	4.41	4.46	4.35	5.22	5.07	4.67	3.86	4.03
5	3.72	4.01	3.89	3.99	4.09	4.14	5.13	4.52	4.08	3.85	3.90
6	4.07	4.29	4.30	4.54			5.72	5.00	4.67	3.89	3.94
7	4.06	4.18	4.41	4.53	4.57	5.13	5.33	4.23	3.65	3.59	3.78
8	3.94	4.05	4.09	4.14	4.37	4.28	5.48	4.45	4.16	3.65	3.68
9	3.84	3.94	3.78	3.77	3.87	3.85	4.52				
10	3.94	4.05	4.15	4.28	4.28	4.42	5.69	5.11	4.19	3.62	3.78
n	**10**	**10**	**10**	**8**	**8**	**7**	**10**	**9**	**8**	**7**	**7**
mean	**3.96**	**4.09**	**4.12**	**4.25**	**4.23**	**4.38**	**5.33**	**4.70**	**4.19**	**3.76**	**3.88**
SD	**0.2**	**0.2**	**0.2**	**0.3**	**0.3**	**0.4**	**0.4**	**0.4**	**0.4**	**0.1**	**0.1**
HD											
1	3.77	4.05	3.87	4.10	4.12	4.29	4.87	3.81	3.52	3.44	3.74
2	5.07	5.32	5.43	5.51	5.60	5.65					5.24
3	5.35	5.41	5.55	5.65	5.75	5.92	6.07	5.17	4.93	4.87	5.12
4	4.43	4.46	4.43	4.68	4.72	4.74	5.54	4.04	3.53	3.34	3.59
5	4.86	5.08	4.99	5.08	5.06	5.09	5.61		4.24		5.04
6											
7	7.32	7.43	7.30	7.21	7.22	7.20	7.17	6.20	6.11	5.98	6.91
8	5.94	6.13	6.21	6.22	6.36	6.28	6.87	5.64	5.11	5.27	5.70
9	4.67	4.95	4.87	5.03	5.16	5.13	5.84	4.87	4.49	4.45	4.84
10	4.93	4.90	4.85	5.11	5.15	5.22	5.64	4.86	4.36	4.30	4.96
n	**9**	**9**	**9**	**9**	**9**	**9**	**8**	**7**	**8**	**7**	**9**
mean	**5.15**	**5.30**	**5.28**	**5.40**	**5.46**	**5.50**	**5.95**	**4.94**	**4.53**	**4.52**	**5.01**
SD	**1.0**	**1.0**	**1.0**	**0.9**	**0.9**	**0.9**	**0.7**	**0.8**	**0.9**	**1.0**	**1.0**
RTx											
1	4.52	4.85	4.85	4.89	4.97	4.91	5.30	4.30	4.14	4.63	4.95
2	4.05	4.18	4.29	4.36	4.31	4.32	4.42	4.41	4.04	3.78	4.04
3	4.61	4.45	4.37	4.63	4.77	4.85	5.16	4.06	3.71	3.84	4.26
4	3.81	3.96	3.84	3.85	3.84	3.97	5.24	4.27	3.81		3.89
5	4.56	4.77	4.74	4.67	4.67	4.47	5.78	4.52	3.88	3.92	4.36
6	4.05	4.29	4.18	4.28	4.27	4.37	5.16	4.40	3.83	3.51	3.84
7	4.33	4.60	4.56	4.76	5.03	5.16	5.37	4.49	4.03	3.99	4.34
8	4.02	4.12	4.12	4.18	4.33	4.59	5.07	4.32	3.84	3.53	3.88
9	4.01	3.99	4.07	4.14	4.17	4.20	4.65	4.47	4.13	4.15	4.66
n	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**9**	**8**	**9**
mean	**4.21**	**4.36**	**4.33**	**4.42**	**4.48**	**4.54**	**5.13**	**4.36**	**3.94**	**3.92**	**4.25**
SD	**0.3**	**0.3**	**0.3**	**0.3**	**0.4**	**0.4**	**0.4**	**0.1**	**0.2**	**0.4**	**0.4**

APPENDIX 2.4 INDIVIDUAL DATA FROM STUDY FOUR

Appendix 2.4.1 Subject physical characteristics

Subject	Age (years)	Body mass (kg)	Height (cm)	$\dot{V}O_{2peak}$
1	28	67.6	175	54.6
2	21	73.1	181	45.1
3	20	75.8	179	36.6
4	21	64.6	174	48.2
5	40	103.2	189	58.8
6	30	80.6	180	52.6
7	23	66.8	173	52.2
8	24	82.7	188	49.8
9	26	74.6	159	41.7
10	27	68.5	188	50.7
n	**10**	**10**	**10**	**10.0**
mean	**26**	**75.8**	**178**	**45.5**
SD	**6**	**11.3**	**9**	**13.3**

Appendix 2.4.2 Serum digoxin concentration at day 14 in digoxin (DIG) and control (CON) trials ($nmol.l^{-1}$).

Subject	CON	DIG
1	0.7	<0.4
2		<0.4
3	1.1	
4	0.8	<0.4
5	0.5	<0.4
6	0.9	<0.4
7	0.8	<0.4
8	0.6	<0.2*
9	0.7	<0.2*
10	0.8	<0.2*
n	**9**	
mean	**0.8**	
SD	**0.2**	

* in later trials the detection limit was revised from <0.4nM to <0.2 nM.

Appendix 2.4.3 Time to fatigue during cycling test in digoxin (DIG) and control (CON) trials (s).

Subject	CON	DIG
1	609	438
2	182	262
3	290	179
4	223	210
5	246	272
6	121	112
7	108	78
8	194	343
9	197	199
10	451	445
n	**10**	**10**
mean	**262**	**254**
SD	**156**	**125**

Appendix 2.4.4 Oxygen consumption during cycling exercise at 33, 67, and 90% $\dot{V}O_{2peak}$ in digoxin (DIG) and control (CON) trials ($l.min^{-1}$).

	CON			DIG		
Subject	**33%**	**67%**	**90%**	**33%**	**67%**	**90%**
1	1.00	2.12	3.52	1.00	2.26	3.58
2	1.13	2.67	3.41	1.22	2.71	3.83
3	1.11	2.54	3.64	1.11	2.68	3.47
4	1.04	2.38	3.22	0.98	2.33	3.01
5	1.26	2.82	3.91	1.34	2.87	4.11
6	1.30	3.50	3.95	1.47	3.64	3.99
7	1.54	3.10		1.27	3.06	
8	1.53	3.01	3.64	1.50	3.06	4.22
9	1.14	1.77		0.87	1.67	2.05
10	1.04	2.35	3.19	1.14	2.49	3.65
n	**10**	**10**	**8**	**10**	**10**	**9**
mean	**1.21**	**2.63**	**3.61**	**1.19**	**2.68**	**3.55**
SD	**0.20**	**0.51**	**0.26**	**0.21**	**0.54**	**0.42**

Appendix 2.4.5 Digoxin and exercise effects on skeletal muscle protein content (mg protein. mg muscle wet weight^{-1}).

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	0.23	0.20	0.17	0.21	0.20	0.20	0.18	0.15
2	0.17	0.14	0.15		0.17	0.15	0.16	0.22
3	0.15	0.14	0.15	0.18	0.15	0.14	0.18	0.17
4	0.18	0.15	0.17	0.18	0.19	0.16	0.17	0.21
5	0.20	0.20	0.19	0.22	0.23	0.22	0.19	0.20
6	0.18	0.16	0.16	0.19	0.19	0.19	0.17	0.19
7	0.22		0.18	0.20	0.21		0.17	0.19
8	0.19	0.18	0.18	0.21	0.20	0.19	0.18	0.19
9	0.20	0.22	0.22	0.26	0.22	0.21	0.23	0.24
10	0.24	0.22	0.22	0.21	0.23	0.22	0.21	0.24
n	**10**	**9**	**10**	**9**	**10**	**9**	**10**	**10**
mean	**0.20**	**0.18**	**0.18**	**0.21**	**0.20**	**0.19**	**0.18**	**0.20**
SD	**0.03**	**0.03**	**0.02**	**0.03**	**0.03**	**0.03**	**0.02**	**0.03**

Appendix 2.4.6 Digoxin and exercise effects on skeletal muscle [^{3}H]ouabain binding site content before clearance of bound digoxin by F_{ab} (pmol.(g wet weight^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	394	419	426	322	301	343	361	312
2	363	404	396	346	424	431	362	365
3	377	350	326	311	285	291	269	281
4	347	497	412	384	348	394	392	421
5	321	471	403	346	409	503	408	411
6	429	497	548	456	429	452	481	390
7	368	393	419	356	373	382	400	365
8	312	259	285	341	291	328	293	290
9	285	280	250	245	329	264	206	238
10	333	357	347	356	330	435	414	401
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**353**	**393**	**381**	**346**	**352**	**382**	**359**	**347**
SD	**42**	**83**	**84**	**54**	**54**	**76**	**81**	**63**

Appendix 2.4.7 Digoxin and exercise effects on skeletal muscle [^{3}H]ouabain binding site content before clearance of bound digoxin by F_{ab} (pmol.(g protein^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	1724	2121	2482	1563	1473	1715	2054	2107
2	2133	2988	2628	1734	2566	2873	2326	1681
3	2445	2426	2147	1744	1864	2079	1467	1640
4	1896	3358	2420	2107	1859	2463	2360	2047
5	1645	2299	2086	1734	1769	2295	2202	2010
6	2383	3203	3432	2370	2208	2379	2801	2084
7	1676	2302	2275	1807	1772	2086	2287	1935
8	1641	1415	1618	1623	1469	1728	1638	1513
9	1394	1269	1156	927	1473	1265	903	995
10	1373	1643	1591	1731	1441	1974	1991	1661
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**1831**	**2302**	**2184**	**1734**	**1789**	**2086**	**2003**	**1767**
SD	**378**	**725**	**637**	**371**	**367**	**453**	**540**	**346**

Appendix 2.4.8 Digoxin and exercise effects on skeletal muscle [^{3}H]ouabain binding site content after clearance of bound digoxin by F_{ab} (pmol.(g wet weight^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	350	290	363	348	368	281	321	322
2	377	460	438	373	435	438	393	413
3	329	335	272	368	390	370	401	387
4	345	359	448	379	333	323	401	408
5	411	470	418	373	452	485	452	421
6	448	412	422	400	427	343	354	337
7	326	371	423	379	324	372	322	390
8	382	337	351	330	295	271	388	260
9	395	362	345	435	422	439	441	328
10	325	318	337	348	361	400	362	359
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**369**	**371**	**382**	**373**	**381**	**372**	**384**	**363**
SD	**41**	**59**	**57**	**29**	**53**	**70**	**44**	**51**

Appendix 2.4.9 Digoxin and exercise effects on skeletal muscle [^{3}H]ouabain binding site content after clearance of bound digoxin by F_{ab} (pmol.(g protein^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	1532	1468	2115	1689	1801	1405	1827	2175
2	2215	3402	2907	1831	2633	2920	2525	1902
3	2134	2322	1791	2063	2551	2643	2186	2259
4	1885	2426	2632	2079	1779	2019	2415	1984
5	2107	2294	2164	1831	1955	2213	2439	2059
6	2489	2656	2643	2079	2197	1805	2061	1801
7	1485	2168	2297	1924	1539	1778	1841	2067
8	2009	1841	1992	1570	1489	1428	2169	1356
9	1932	1640	1596	1645	1890	2104	1932	1371
10	1340	1463	1545	1692	1577	1816	1741	1487
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**1913**	**2168**	**2168**	**1840**	**1941**	**2013**	**2114**	**1846**
SD	**362**	**600**	**459**	**191**	**403**	**485**	**280**	**332**

Appendix 2.4.10 Digoxin and exercise effects on skeletal muscle maximal 3-O-MFPase activity (nmol.min^{-1}.(g wet weight^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	297	295	268	245	294	246	259	224
2	285	244	243	299	293	310	272	260
3	219	294	245	259	336	200	279	306
4	214	197	181	285	204	202	211	217
5	269	322	284	321	291	353	313	374
6	328	433	351	371	354	280	336	376
7	440	285	310	351	400	255	312	346
8	397	208	229	332	293	212	194	256
9	195	276	214	359	274	225	212	227
10	326	292	216	166	263	262	163	223
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**297**	**285**	**254**	**299**	**300**	**254**	**255**	**281**
SD	**79**	**66**	**50**	**63**	**54**	**49**	**58**	**64**

Appendix 2.4.11 Digoxin and exercise effects on skeletal muscle maximal 3-O-MFPase activity (pmol. min^{-1}.(g protein^{-1}))

	CON				DIG			
Subject	**Rest**	**67%**	**Fatigue**	**+3 h**	**Rest**	**67%**	**Fatigue**	**+3 h**
1	1299	1492	1560	1189	1437	1228	1472	1514
2	1673	1806	1613	1461	1776	2069	1750	1197
3	1421	2038	1611	1450	2198	1429	1519	1788
4	1167	1332	1064	1563	1088	1264	1271	1053
5	1377	1571	1469	1493	1257	1610	1688	1830
6	1820	2794	2196	1930	1823	1476	1957	2008
7	2002	1640	1682	1782	1899	1384	1783	1835
8	2088	1136	1298	1581	1477	1118	1084	1333
9	954	1249	991	1358	1227	1079	929	948
10	1343	1345	992	809	1148	1187	784	924
n	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
mean	**1514**	**1640**	**1448**	**1461**	**1533**	**1384**	**1424**	**1443**
SD	**369**	**487**	**375**	**309**	**372**	**293**	**394**	**406**