Digoxin and Exercise Effects on Na^+,K^+-ATPase Isoform Gene and Protein Expression in Human Skeletal Muscle.

Submitted by

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DECLARATION

I, Xiaofei Gong, declare that the Master of Applied Science thesis entitled, *Digoxin and Exercise Effects on Na^+,K^+-ATPase Isoform Gene and Protein Expression in Human Skeletal Muscle* is no more than 60,000 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.

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List of Abbreviations

Na^+	sodium ion
K^+	potassium ion
ATP	adenosine triphosphate
[ ]	concentration of an ion
$[K^+]_i$	intracellular potassium concentration
$[K^+]_e$	extracellular potassium concentration
$[Na^+]_e$	extracellular sodium concentration
$[Na^+]_i$	intracellular sodium concentration
Ca^{2+}	calcium ion
Na^+,K^+-ATPase	sodium-potassium adenosine 5'triphosphatase
kDa	kilodaltons
[^{3}H]-ouabain binding	tritiated ouabain binding
DIG	digoxin
CON	control
cDNA	complementary deoxyribonucleic acid
T-tubules	tranverse tubules
CGRP	calcitonin gene-related peptide
3-*O*-MFP	3-*O*-methylfluoroscein phosphate
mRNA	messenger ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
ADP	Adenosine diphosphate
EDL	extensor digitorum longus
CHF	chronic heart failure

$\dot{V}O_{2peak}$	peak oxygen consumption
d	day
wks	weeks
wt	weight
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetraacetic acid
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid

ABSTRACT

This laboratory has shown that exercise in humans impairs skeletal muscle Na^+,K^+-ATPase maximal in vitro activity, whilst in isolated rat muscles, Na^+,K^+-ATPase inhibition with ouabain leads to early muscle fatigue. Hence, Na^+,K^+-ATPase function is likely to be important for skeletal muscle performance. Digoxin is a specific inhibitor of the Na^+,K^+-ATPase and is used to treat patients with severe heart failure. This thesis investigated whether in-vivo inhibition of Na^+,K^+-ATPase by digoxin adversely effected muscle performance and Na^+,K^+-ATPase isoform expression and protein abundance in skeletal muscle of healthy individuals.

Ten active, but not well-trained healthy volunteers (9 M, 1 F) gave written informed consent. Subjects performed incremental cycle ergometer exercise to measure $\dot{V}O_{2peak}$ and to determine 33, 67 and 90% $\dot{V}O_{2peak}$ workrates. Exercise tests were performed after taking digoxin (DIG, 0.25 $mg.d^{-1}$) or a placebo (CON) for 13 day (Cybex) or 14 day (cycling), in a randomised, counterbalanced, cross-over, double-blind design, with trials separated by at least 6 weeks. On day 13 subjects performed tests of quadriceps muscle strength and endurance of the dominant leg, on a Cybex isokinetic dynamometer (Cybex Norm 770, Henley Healthcare, USA). On day 14 subjects completed 10 min cycling at each of 33% and 67% $\dot{V}O_{2peak}$, then to fatigue at 90% $\dot{V}O_{2peak}$ on cycle ergometer (Lode Excalibur, Groningen, the Netherlands), with arterial blood sampling for plasma $[K^+]$ determinations. A muscle biopsy was taken at rest, after exercise at 67% and 90% $\dot{V}O_{2peak}$ and at 3 hr recovery. Muscle was analysed for Na^+, K^+-pump isoform (α_1-α_3, β_1-β_3) mRNA expression (real-time RT-PCR, GeneAmp 7500 Sequence Detection System) and whole homogenate protein abundance (immunoblotting, Kodak Digital Science Image Station 400_{CF}, Eastman Kodak Company, CT, USA).

Serum digoxin was 0.7±0.1 nM at day 13 and 0.8±0.1 nM at day 14 (Mean±SEM) and was less than the lowest detection limit of 0.4 nM in control trials. There were no differences in $\dot{V}O_2$ or time to fatigue (DIG 262±156 vs CON 254 ±125 s) between DIG and CON during exercise. Arterial plasma [K^+] increased above rest at 67% $\dot{V}O_{2peak}$ and increased further at fatigue ($P < 0.05$). No significant differences were found in [K^+] between DIG and CON. Peak torque during dynamic isokinetic contractions was less at each increasing velocity ($P < 0.05$). No differences were found in muscle strength between DIG and CON. Similarly, there were no differences in the leg extensor fatigue index between trials (DIG 0.54±0.03 vs CON 0.57±0.03).

The mRNA expression of the α_1, α_2, α_3, β_1, β_1 or β_3 isoforms was not significantly changed by DIG. However, DIG increased the total α mRNA expression (sum of α_1, α_2, α_3) and also the total β mRNA expression (sum of β_1, β_2 and β_3) at rest by 1.9- and 0.6-fold, respectively ($P<0.05$). An exercise effect was observed for α_3 mRNA expression, which was 2.1-and 2.4-fold higher at 3 h post-exercise, than during exercise at 67% $\dot{V}O_{2peak}$ and fatigue, respectively ($P<0.05$). Similarly, β_3 mRNA expression was increased at 3 h post-exercise by 1.8-, 1.4- and 1.6-fold, compared to rest, 67% $\dot{V}O_{2peak}$ exercise and fatigue, respectively ($P<0.05$). No other significant changes with exercise or recovery were seen in mRNA expression.

In resting muscle, protein relative abundance was not significantly changed by digoxin for α_1 (1.14±0.19, $P=0.50$), α_3 (1.12±0.18, $P=0.52$), β_1 (1.19±0.18, $P=0.32$), β_2 (1.86±0.57, $P=0.17$), β_3 (0.85±0.17, $P=0.39$) compared to control (1.00) although a tendency was observed for an increase in α_2 with DIG (1.44±0.23, $P=0.096$). However, exercise affected both β_1 and β_3 isoform protein abundance. The β_1 protein abundance was increased at 3 h post-exercise by 2.2-and 1.5-fold compared to during exercise at 67% $\dot{V}O_{2peak}$ and fatigue,

respectively (P<0.05). Similarly, β_3 protein abundance was increased at 67% $\dot{V}O_{2peak}$ and 3 h post-exercise compared to rest, by 1.5-and 1.6-fold, respectively (P<0.05).

In summary, despite elevation of serum digoxin to therapeutic levels, quadriceps muscle strength, muscle fatiguability and arterial plasma [K^+] were each unchanged by DIG. Furthermore, digoxin treatment had only minimal effects on skeletal muscle Na^+,K^+-ATPase isoform mRNA expression and protein abundance in healthy individuals. Nonetheless Na^+,K^+-ATPase α subunit and β subunit total mRNA expression in resting muscle was increased with digoxin, suggesting an effect of digoxin on Na^+,K^+-ATPase gene expression. Whilst no significant change was detected in protein abundance of any isoform with digoxin in resting muscle, a tendency forwards an increase in α_2 protein abundance was observed (P=0.096). Together these suggest a possible compensatory upregulation with digoxin in muscle Na^+,K^+-ATPase in these healthy individuals.

Chapter 1. Introduction

The sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase) is ubiquitously expressed in mammalian cells. In skeletal muscle, Na^+,K^+-ATPase major regulatory functions include maintenance of trans-sarcolemmal [Na^+] and [K^+] gradients, membrane excitability and thus sustaining the capacity to undergo repeated contractions, i.e. exercise (Blanco and Mercer, 1998a, Clausen, 1986, Blanco and Mercer, 1998b). The Na^+,K^+-ATPase is made up of an α- and β-subunit, which form a functional αβ complex. The Na^+,K^+-ATPase exists as an αβ heterodimer and is under transcriptional and translational control. The four α (α_1, α_2, α_3 and α_4) and three β subunit isoforms (β_1, β_2 and β_3) of the Na^+,K^+-ATPase are expressed in a tissue- and developmental-specific manner (Blanco and Mercer, 1998b).

Na^+,K^+-ATPase is intimately involved in muscle excitability and force development (Clausen, 2003). Several studies have shown a decreased maximal rate of Na^+,K^+-ATPase enzyme activity with fatigue induced by intense exercise (Aughey *et al.*, 2005, Fraser *et al.*, 2002) and isometric contractions (Fowles *et al.*, 2002b). These studies implicate depressed Na^+,K^+-ATPase activity as an important site for fatigue during exercise.

An additional functional consequence of depressed Na^+,K^+-ATPase activity is increased gene expression. Non-toxic concentrations of ouabain, that cause partial inhibition of Na^+,K^+-ATPase and an increase in cardiac contractility, also stimulated myocyte growth and protein synthesis (Xie and Askari, 2002). Ouabain induced a number of early response proto-oncogenes, activated transcription factors activator protein 1 (AP-1) and NF-B, and induced or repressed the transcription of several late-response cardiac marker genes (Xie and Askari, 2002). Thus inhibition of Na^+,K^+-

ATPase activity may also be involved in gene expression at least in myocardial tissue. Recently studies from this laboratory investigated the Na^+,K^+-ATPase isoforms expressed in human skeletal muscle with acute and chronic exercise (Murphy *et al.*, 2004, Murphy *et al.*, 2006b) and found a relationship between depressed Na^+,K^+-ATPase activity and increased Na^+,K^+-ATPase mRNA after intense exercise (Petersen *et al.*, 2005).

A common drug used for patients with chronic heart failure is digoxin, which inhibits Na^+,K^+-ATPase, and consequently increases the force of contraction of the heart (Lichtstein, 1995, Rose and Valdes, 1994). Digoxin inhibits Na^+,K^+-ATPase in the sarcolemma of all tissues, with about 50% of the total amount of digoxin in the body bound to skeletal muscle and only 3-4% to the heart muscle (Steiness, 1978). Thus digoxin is also expected to have marked effects on skeletal muscle. In heart failure patients, digoxin blocked ~13% of Na^+,K^+-ATPase in skeletal muscle and exacerbated muscle K^+ loss during cycling exercise (Schmidt *et al.*, 1993a). Furthermore there was no compensatory upregulation of Na^+,K^+-ATPase with chronic digitalisation in myocardium (Schmidt *et al.*, 1993b).

There have been no previous investigations into the effects of digoxin on Na^+,K^+-ATPase in skeletal muscle in healthy individuals. Furthermore, no studies have investigated digoxin effects on muscle fatigue in humans. Finally, the limited work to date examining the effects of digoxin on muscle strength is ambiguous. Hence little is known about the effects of digoxin on Na^+,K^+-ATPase regulation during exercise in human muscle.

This study provides new knowledge regarding the effects of digoxin and exercise on Na^+,K^+-ATPase in human skeletal muscle, within applications for understanding muscle fatigue and treatment in older patients with chronic heart failure.

This thesis was to investigated the effects of digoxin and exercise on Na^+,K^+-ATPase isoform protein and gene expression in skeletal muscle in healthy volunteers. Further, the thesis explored whether digoxin reduced muscle strength and increased fatiguability during exercise.

Chapter 2. Review of Literature

2.1 Na^+,K^+-ATPase in skeletal muscle

2.1.1 Na^+,K^+-ATPase structure and isoform function

The Na^+,K^+-ATPase was first identified by Skou (Skou, 1957), who was awarded the Nobel Prize in chemistry for this discovery in 1997. He found that the Na^+,K^+-ATPase regulated active transport of Na^+ and K^+ across cell membranes. In skeletal muscle, the Na^+,K^+-ATPase major regulatory function is maintenance of intracellular to extracellular [Na^+] and [K^+] gradients, which are vital for membrane excitability and contractility (Blanco and Mercer, 1998b).

The Na^+,K^+-ATPase is a transmembranous protein, composed of two subunits, a catalytic α subunit involved in the splitting of ATP, with a molecular mass of approximately 100-112 kDa and a regulatory β subunit, of approximately 40-60 kDa. A further regulatory γ subunit is found in some tissues, approximately 6.5 kDa (Mercer *et al.*, 1993). The α and β subunits form a functional $\alpha\beta$ complex, which is the minimal functional Na^+,K^+-ATPase unit (Lingrel, 1992, Levenson, 1994). The α subunit, which pumps Na^+ and K^+, contains binding sites for Na^+, K^+, ATP, and the cardiac glycosides digoxin and ouabain, as well as being the catalytic subunit (Lingrel *et al.*, 1994, Lingrel, 1992, Jorgensen, 1982). The β subunit is necessary for the structural and functional maturation of the α subunit and the enzyme activity, as well as its transport to the plasma membrane (Geering, 1991, Noguchi *et al.*, 1990).

The Na^+,K^+-ATPase is a multi-gene regulated enzyme. The Na^+,K^+-ATPase subunits are expressed in various isoforms, which can be detected using specific antibodies (Murphy *et al.*, 2004). Four isoforms of the Na, K^+-ATPase α, and three isoforms of the β subunits have been identified, which are the α_1, α_2, α_3, α_4, β_1, β_2 and β_3 (Blanco and Mercer, 1998b). All seven isoforms are expressed in mammalian cells (Hundal *et*

al., 1994, Blanco and Mercer, 1998b). Each of α_1, α_2, α_3, β_1, β_2 and β_3 isoforms have recently been identified in human skeletal muscle (Murphy *et al.*, 2006b, Murphy *et al.*, 2004). The four α and three β of the Na, K^+-ATPase are expressed in a tissue-, developmental-specific manner, which indicates a variety of isoform-specific functions (Orlowski and Lingrel, 1988, Blanco and Mercer, 1998b).

2.1.2 Localization and quantification of Na^+,K^+-ATPase

The most commonly used method to quantify of Na^+,K^+-ATPase, [^{3}H]-ouabain binding, is based on the cardiac glycoside ouabain binding to the α-subunit of the enzyme, with a one-for-one molecular stoichiometry (Hansen, 1984). Using [^{3}H]-ouabain binding analysis, human vastus lateralis muscle Na^+,K^+-ATPase content mainly ranges from 260 to 340 pmol.(g wet wt)$^{-1}$ (Nielsen and Clausen, 2000, Clausen, 2003).

The major part of the enzyme activity was associated with the sarcolemma. Measurement of [^{3}H]-ouabain binding showed that in sarcolemma, the density of Na^+,K^+-ATPase is about 3,350 molecules/μm^2 in the soleus of 4-wk-old rats (Clausen and Hansen, 1974) and 2,500 molecules/μm^2 in frog sartorius (Venosa and Horowicz, 1981) and 1,000-1,800 molecules/μm^2 in muscles from 8-wk-old pigs (Harrison *et al.*, 1994). Early measures found that there were some Na^+,K^+-ATPase activity and specific ouabain binding sites also in the transverse tubules, but at a considerably lower density (Lau *et al.*, 1977, Lau *et al.*, 1979, Narahara *et al.*, 1979, Seiler and Fleischer, 1982). Direct evidence that T-tubular membranes bind ^{3}H-ouabain and contain Na^+,K^+-ATPase was shown in membrane isolated from rabbit sacrospinalis skeletal muscle and identified by their morphological association with the terminal cisternae (Caswell *et al.*, 1976, Lau *et al.*, 1979). In intact frog sartorius muscle, measurements of the content of ^{3}H-ouabain binding sites in T-tubules showed the value at 215 pmol mg protein^{-1} (Jaimovich *et al.*, 1986), whilst in rabbit skeletal

muscle, values were 169 pmol mg protein^{-1} (Jaimovich *et al.*, 1986). Na^+,K^+-ATPase are clearly therefore located both in the sarcolemma and t-tubules.

In cell cultures prepared from chick embryo leg muscles, the Na^+,K^+-ATPase was also found in an intracellular pool, that corresponded to around 60% of the total amount of the enzyme present in the cells, by using immunolabelling techniques (Fambrough *et al.*, 1987, Wolitzky and Fambrough, 1986). However, no one has yet identified the precise localization of this intracellular pool and the magnitude appears to be surprisingly large.

2.1.3 General mechanisms for acute and chronic regulation of the Na^+,K^+-ATPase

In skeletal muscle, the Na^+,K^+-ATPase is under acute and chronic regulation by several factors, such as muscle contraction, numerous hormones and chronic exercise training.

2.1.3.1 Muscle contraction and excitation

In resting muscle, the Na^+,K^+-ATPase activity is at only a low level, but this increases rapidly with muscle contraction (Everts and Clausen, 1994). In rat soleus muscle, excitation leads to a rapid and pronounced increase in Na^+,K^+-ATPase activity by up to 15-fold (Everts and Clausen, 1994). The Na^+,K^+-ATPase may increase as much as 22-fold after muscle contractions lasting only 10 seconds (Nielsen and Clausen, 2000, Everts and Clausen, 1994).

Muscle contraction leads to an efflux of K^+ and an influx of Na^+ into the muscle. Therefore, contractile activity induces a net K^+ loss and Na^+ gain in human muscle (Sejersted and Sjøgaard, 2000). Several studies demonstrated that the Na^+,K^+-ATPase in working muscle cannot keep the pace with the Na^+ and K^+ leaks by the evidence of increases in both muscle intracellular sodium concentration ($[Na^+]_i$) and extracellular potassium concentration ($[K^+]_e$) (Nielsen *et al.*, 2004a, Nielsen and Clausen, 1997, Clausen and Everts, 1989). During exhaustive muscle contractions, muscle $[Na^+]_i$ and

$[K^+]_e$ may double, thereby decreasing the membrane potential and reducing muscle membrane excitability (Balog and Fitts, 1996, Sejersted and Sjøgaard, 2000, Vøllestad *et al.*, 1994). In rat soleus muscle, excitation by electrical stimulations at 120 HZ has been found to produce up to an 18-22-fold increase in the rate of net Na^+ extrusion via Na^+,K^+-ATPase (McKenna *et al.*, 2003, Nielsen and Clausen, 1997, Everts and Clausen, 1994). The activation of the Na^+,K^+-ATPase is also demonstrated by the rapid decline in plasma $[K^+]$ or muscle interstitial $[K^+]$ after intense exercise in human muscle (Sejersted and Sjøgaard, 2000, Juel *et al.*, 2000c, Green *et al.*, 2000). Increasing $[K^+]_e$ from 4 mM to 20 or 50 mM was also shown to increase the activation of the Na^+,K^+-ATPase by 16% and 28%, respectively (Everts and Clausen, 1994).

2.1.3.2 Hormonal Na^+,K^+-ATPase activation

Several hormones are involved in both acute and chronic Na^+,K^+-ATPase regulation, such as insulin, calcitonin gene-related peptide (CGRP), thyroid hormones (Andersen *et al.*, 1993, Clausen, 2003).

2.1.3.2.1 Insulin

Insulin stimulates the Na^+,K^+-ATPase. In isolated rat soleus muscle, insulin stimulated a 45% increase in Na^+ efflux and a 25% increase in K^+ uptake (Clausen and Flatman, 1987). In rat diaphragm muscle, insulin was also found to decrease $[Na^+]_i$ (Creese, 1968). Another study has also reported that insulin increased ouabain-sensitive K^+ uptake and the rate of [^{3}H]-ouabain binding by 74% (Weil *et al.*, 1991). This effect was blocked by ouabain suggesting insulin induced Na^+,K^+-ATPase activation. In frog skeletal muscle, insulin induced acute Na^+,K^+-ATPase activation by increasing the rate of [^{3}H]-ouabain binding (Omatsu-Kanbe and Kitasato, 1990). In human muscle, insulin stimulated K^+ net uptake from the plasma (Zierler and Rabinowitz, 1964, Ferrannini *et al.*, 1988).

2.1.3.2.2 calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) acutely augments the contractile response of skeletal muscle to both direct and indirect stimulation (Fleming *et al.*, 1993). In a rat phrenic nerve-hemidiaphragm preparation, CGRP increased the twitch contraction during intense stimulation (Uchida *et al.*, 1990). In the isolated rat soleus muscle, stimulation at intensities 50-100 times the threshold increased significantly the amount of CGRP release from the muscle (Sakaguchi *et al.*, 1991). Releasing of CGRP induce the increase in activation of the Na^+,K^+-ATPase and may contribute to the excitation-induced force recovery in stimulated rat muscle (Nielsen *et al.*, 1998). This study also found that decreasing CGRP content by capsaicin pre-treatment or prior denervation prevented both the excitation-induced force recovery and the drop in intracellular Na^+ (Nielsen *et al.*, 1998).

2.1.3.2.3 Thyroid hormones

Thyroid hormones (T_3) is considered as an endocrine factor which effects regulation of Na^+,K^+-ATPase activity (Lei *et al.*, 2004, Izmail-Beigi and Edelman, 1970). In rats, the differences in transmembrane Na^+ and K^+ concentration were increased in both liver and diaphragm by the administration of triiodothyronine, indicating that thyroid hormone activates Na^+ extrusion and K^+ accumulation, either by increasing the local concentration of ATP or by direct stimulation of the Na^+,K^+-ATPase (Izmail-Beigi and Edelman, 1970). Several studies confirmed the finding that T_3 treatment increases Na^+,K^+-ATPase activity in either thyroidectomized or euthyroid rats (Ismail-Beigi and Edelman, 1971, Asano *et al.*, 1996). The Na^+,K^+-ATPase content, measured by the [^{3}H]-ouabain binding site, in extensor digitorum longus, diaphragm, gastrocnemius and soleus muscles from hyperthyroid rats were respectively 2.6, 3.5, 5.1 and 9.8 times higher than those hypothyroid rats, which also indicates that the

effect of thyroid hormones is more pronounced on slow-twitch than on fast-twitch fibres (Kjeldsen *et al.*, 1986).

2.1.4 Acute exercise effect on Na^+,K^+-ATPase activity

Several studies have investigated the effects of acute exercise on Na^+,K^+-ATPase activity in muscles, measured by maximal 3-*O*-MFPase activity. In rat soleus, EDL, white vastus lateralis and red vastus lateralis muscles, there was a 12% depression in maximal 3-*O*-MFPase activity after 2 h of submaximal running followed by an additional 45 min of low-intensity running (Fowles *et al.*, 2002a).

In human muscles, the maximal *in-vitro* Na^+,K^+-ATPase activity, as measured by 3-*O*-MFPase activity, decreased 17% following 50 maximal knee extension contractions (Fraser *et al.*, 2002). During prolonged exercise, Na^+,K^+-ATPase activity was reported to be 21% depressed after 30 min of cycling (Sandiford *et al.*, 2004). In another submaximal cycling exercise study, muscle 3-*O*-MFPase activity was decreased by 11% after 45 min cycling and by 13% at fatigue which occurred after ~72 min (Leppik *et al.*, 2004). In trained subjects, the muscle maximal in vitro 3-*O*-MFPase activity declined by 12% and 13% immediately following incremental exercise (Aughey *et al.*, 2005). In another study, prolonged submaximal at 75% $\dot{V}O_{2peak}$ to fatigue, decreased maximal 3-*O*-MFPase activity by 18.9% (Murphy *et al.*, 2006b). Repeated isokinetic single-leg knee extensions decreased 3-*O*-MFPase activity by 10.7% at fatigue (Petersen *et al.*, 2005). Thus exercise depresses maximal in-vitro Na^+,K^+-ATPase activity.

2.2 Expression and Location of Na^+, K^+-ATPase Isoforms in Mammalian Skeletal Muscle.

2.2.1 Na^+, K^+-ATPase isoform gene and protein expression

2.2.1.1 Isoform gene transcripts

In rats, skeletal muscle has been reported to express α_1, α_2, α_3, β_1 and β_2 gene transcripts (Hundal *et al.*, 1993, Orlowski and Lingrel, 1988, Thompson *et al.*, 2001), but the α_4 and β_3 gene transcripts have not been probed. The Na^+, K^+-ATPase gene transcripts vary in different tissues. In brain, the α_3 mRNA was found to be the predominant α isoform transcript, whilst α_1 mRNA was the major transcript expressed in heart and kidney. In contrast to these other tissues, skeletal muscle expressed predominantly α_2 mRNA (Orlowski and Lingrel, 1988).

Recently, several studies have reported the Na^+, K^+-ATPase gene transcripts expressed in human skeletal muscle, with detection of the α_1, α_2 and β_1 gene transcripts (Nordsborg *et al.*, 2003a), the α_4 gene transcript (Keryanov and Gardner, 2002), and the β_3 gene transcript (Malik *et al.*, 1998). Our research group has recently investigated Na^+, K^+-ATPase gene transcripts in human muscle, measuring mRNA by real-time RT-PCR, detecting each of the α_1, α_2, α_3, β_1, β_2 and β_3 gene transcripts (Murphy *et al.*, 2004, Murphy *et al.*, 2006b). Another study also reported detection of the α_4 gene transcript in human skeletal muscle, but at unreliable low level (Nordsborg *et al.*, 2005).

2.2.1.2 Isoform protein abundance

The α_2 isoform is the dominant α-subunit (~75-80%) in skeletal muscle (Lingrel, 1992, Sweadner, 1989). In rats, skeletal muscle expresses each of the α_1, α_2, β_1, β_2 and β_3 proteins in a fibre-type dependent manner (Hundal *et al.*, 1993). The protein expression of the α_3 isoform could not be detected by immunoblotting in sarcolemmal

or intracellular membrane fractions, in rat hindlimb, soleus, EDL, white gastrocnemius and diaphragm muscle (Hundal *et al.*, 1994, Thompson and McDonough, 1996), since the protein expression of the α_3 was too low to be detected. The α_1, α_2 and β_1, but not β_2 isoform proteins were reported in healthy human muscle, whilst α_3 and β_3 were not probed (Juel *et al.*, 2000a). In humans, amputated limb muscle expressed α_3 but did not express the β_2 isoform (Hundal *et al.*, 1994). Our research group has more recently investigated Na^+,K^+-ATPase protein abundance in human muscle, measuring by western blotting, detecting each of α_1, α_2, α_3, β_1, β_2, and β_3 proteins (Murphy *et al.*, 2004). Neither absolute the relative amounts of these proteins cannot be discerned by this technique.

2.2.2 Isoform specific localisation in muscle

From membrane fractionation analyses, Na^+,K^+-ATPase α_1 was mainly located at the plasma membrane, in both red and white muscle; whilst the α_2 isoform was principally located in the surface membrane, it was also present in T-tubules and at other intracellular sites (Clausen, 2003, Hundal *et al.*, 1992, Hundal *et al.*, 1993, Hundal *et al.*, 1994). In human soleus muscle, the α_1 isoform protein abundance in an intracellular membrane fraction accounted for only 4% of that in the plasma fraction (Hundal *et al.*, 1994). The α_3 subunit was detected in the plasma membranes but the lack of good antibodies to this isoform at that time prevented an immunocytochemical analysis of its localization (Hundal *et al.*, 1994). In rat mixed hindlimb muscle, 80% of the β_1 isoforms were located in the intracellular fraction, while 20% were located in the sarcolemmal fraction (Hundal *et al.*, 1992). In human muscle, the β_1 isoform was mainly located with the sarcolemmal fraction, but with about 33% present in the intracellular fraction (Hundal *et al.*, 1994, Juel *et al.*, 2000a). The β_2 isoform was found to be predominantly located in the intracellular fraction in rat muscle (Hundal

et al., 1992). However, the cellular localisation of the β_2 and β_3 isoforms is unknown in human muscle.

2.2.3 Difficulties in measuring isoform expression

Recently more studies investigated the Na^+,K^+-ATPase mRNA expression and protein abundance (Murphy *et al.*, 2006b, Thompson and McDonough, 1996, Sun *et al.*, 1999, Tsakiridis *et al.*, 1996, Murphy *et al.*, 2004). However, there are difficulties in interpreting findings in those studies that utilised membrane fractionation, due to poor membrane recovery and lack of quantitation (Hansen and Clausen, 1988). The Na^+, K^+-ATPase recovery was very low, at only 0.2 to 8.9%, which may be not representative of the whole population of the respective sarcolemmal components (Hansen and Clausen, 1988). Such low plasma membrane recoveries may raise the possibility that the measured Na^+, K^+-ATPase enzyme is not from the sarcolemma, but rather from contaminating nervous and vascular tissue, adipocytes or fibrocytes (Hansen and Clausen, 1996). The final yield of membrane protein per gram tissue and the specific activity in several studies is shown in Table 2.1. Therefore our laboratory has measured protein in crude muscle homogenates, to ensure complete recovery of all Na^+, K^+-ATPase.

Fortunately, techniques, such as use of immunoelectron microscopy and monoclonal antibodies specific to the enzyme, were used in the studies of Na^+,K^+-ATPase isoform and protein expression and localization, that minimised the limitation of this weakness (Marette *et al.*, 1993, Hundal *et al.*, 1994).

2.2.4 Exercise induce isoform translocation

Many studies have suggested Na^+,K^+-ATPase isoforms may be translocated from an undefined intracellular site to the sarcolemma, which enhances muscle Na^+/K^+ transport capacity (Tsakiridis *et al.*, 1996, Juel *et al.*, 2000a, Juel *et al.*, 2001). Five min of fatiguing one-legged knee extensor exercise increased the sarcolemmal protein

abundance of the α_2 and β_1 isoforms by 70% and 26%, respectively (Juel *et al.*, 2000a). In rats, one hour of treadmill running exercise increased expression of Na^+,K^+-ATPase α_1 and α_2 isoforms in plasma membranes in red, oxidative muscles by 64% and 43%, respectively, and also elevated α_1 and α_2 isoforms in white, glycolytic muscles by 55% and 94%, respectively (Tsakiridis *et al.*, 1996). Therefore, acute exercise appears to induce isoform-specific increases in sarcolemmal Na^+,K^+-ATPase protein abundance. However, these studies were performed using isolated sarcolemmal and intracellular membrane fractions, which show very poor recovery of Na^+,K^+-ATPase (Hansen and Clausen, 1988). One of these studies reported the recovery of only 0.3% of the total number Na^+,K^+-ATPase (Juel *et al.*, 2001). Hence, these techniques may not be representative of the whole muscle membranes, which could lead to potential error.

Additionally, corresponding changes in the proposed intracellular membrane fraction were not detected in one study (Tsakiridis *et al.*, 1996). Furthermore, a recent study reported that ^{3}H-ouabain binding, which quantified Na^+,K^+-ATPase, was not increased in rat muscle by stimulated contraction or insulin (McKenna *et al.*, 2003). These findings suggested that the Na^+,K^+-ATPase transport capacity was not increased by isoform translocation with muscle contraction (McKenna *et al.*, 2003). Thus whether these increases reflect translocation of Na^+,K^+-ATPase isoforms from intracellular to sarcolemmal membrane to form functional Na^+,K^+-ATPase heterodimers still remains unknown.

Table 2.1 Estimated recovery of Na^+,K^+-ATPase in various purified enzyme preparations obtained from skeletal muscle or heart ventricles.

Tissue and Reference	Yield of Membrane Protein, mg/g wet wt	Na^+-K^+-ATPase Activity, μmol·g wet $wt^{-1}\cdot h^{-1}$	Estimated Recovery, %
Skeletal muscle			
Hamster (77)	0.14	2.6	1.7
Rabbit (2)	0.12	0.6	0.4
Rabbit (48)	0.05	0.3	0.2
Rabbit (72)	0.02	1.1	0.8
Rat (70)	5.00	20.0	7.0
Rat (67)	0.17	0.3	0.2
Rat (5)	0.20	5.2	3.5
Rat (38)	0.16	2.4	1.6
Rat (71)	0.43	2.6	1.7
Rat (6)	1.91	25.5	8.9
Rat (26)	2.50	23.0	8.2
Frog (58)	0.15	2.0	1.2*
Heart			
Calf (54)			7.8*
Hamster (66)	1.42	24.0	6.5*
Sheep (78)	0.071	1.1	
Dog (36)	0.065	6.4	

Recovery was calculated as product of membrane protein yield per g tissue wet wt and Na^+-K^+-ATPase activity per mg protein. This was then expressed as percent of total concentration of Na^+-K^+-ATPase measured as enzyme activity or [^{3}H]ouabain binding capacity determined on crude homogenates or specimens of same tissue. For hamster and rabbit skeletal muscle, it was assumed that concentration of [^{3}H]-ouabain binding sites was in same range as found for other mature rodents (300 pmol/g wet wt). * For these values, recovery directly determined within same study is given.

Reference: Hansen and Clausen, 1988.

2.3 Muscle ion regulation and fatigue

2.3.1 Definition of fatigue

Fatigue is a common experience in our daily life and in many clinical settings, but still the processes involved are far from understood. In addition, different investigators have used different definitions for fatigue. It is thus necessary to clarify the various terms that are used. This is particularly important when discussing methods for assessment of fatigue, since different tools and procedures may provide information about specific processes involved in fatigue.

In general, fatigue may arise during muscular contractions due to failure at one or more sites along the pathway of force production from the central nervous system to the contractile apparatus (Edwards, 1981). Muscle fatigue has been described as a failure to maintain force output (Edwards, 1981), as a fall in the maximum force-generating capacity of the muscle (Kent-Braun *et al.*, 2002), or as failure to maintain the required or expected power output (Edwards, 1983). The ability to sustain a given work capacity without decrement requires the maintenance of both force and velocity (Fitts, 1994).

2.3.2 Overview of mechanisms of fatigue

The concept of muscle fatigue as a factor limiting muscle performance has evolved into one in which fatigue is also considered important in order to protect the muscle from injury induced by over-stimulation. No one mechanism accounts for all the changes in muscle performance occurring during fatigue, since fatigue is a complex, multi-faceted phenomenon (Fitts, 1994). Excessive K^+ disturbances during exercise and Na^+,K^+-ATPase abnormalities have been implicated in fatigue in many major diseases, including chronic heart failure (Massie *et al.*, 1996b, Green *et al.*, 2001) and chronic renal failure (Sangkabutra *et al.*, 2003). Research on factors limiting force production or shortening capacity during the fatigue process included

electrophysiologic, metabolic and molecular aspects. This literature review will focus on muscle fatigue due to ionic imbalances that are related Na^+,K^+-ATPase function. Although muscle fatigue affected by intracellular and extracellular ion exchanges was investigated in this thesis, it is recognised that there are multiple causative factors in muscle fatigue.

2.3.3 Metabolism effects on fatigue

In the muscle cell, ATP utilization is dramatically accelerated in an attempt to satisfy the energy requirements of the major processes involved in excitation and contraction, namely sarcolemmal and t-tubular Na^+/K^+ exchange, sarcoplasmic reticulum Ca^{2+} sequestration and actomyosin cycling. To maintain ATP levels, high-energy phosphate transfer, glycolysis and oxidative phosphorylation are all recruited. With intense activity, ATP production rates are unable to match ATP utilisation rates, and reductions in ATP occur accompanied by accumulation of a range of metabolic by-products such as hydrogen ions, inorganic phosphate, AMP, ADP and IMP (Fitts, 1994). Repeated bouts of high-intensity activity or prolonged contractions can also result in depletion of the intracellular substrate, glycogen. Since glycogen is the fundamental fuel used to sustain both glycolysis and oxidative phosphorylation, fatigue is apparent as cellular production of ATP fails to match ATP requirement (Fitts, 1994).

2.3.4 Overview of muscle ion regulation

The action potential is generated by an influx of Na^+, which is then followed by an efflux of K^+ (Sejersted and Sjøgaard, 2000). During muscle contraction, the muscle cell loses K^+ and gains Na^+ with each action potential (Everts *et al.*, 1993). In skeletal muscle, an intramembranous tranverse-tubular protein, which is known as the dihydropyridine receptor (DHPR), senses the voltage change associated with the action potential and mechanically induces Ca^{2+} release from the sarcoplasmic

reticulum (SR) via the ryanodine receptors (RyR), which are the Ca^{2+} release channels (Stephenson et al., 1998). Thus, Ca^{2+} is released into the cytoplasm increasing the cytosolic [Ca^{2+}] and thereby enabling the generation of muscle force. Thus membrane excitation is essential for force development.

2.3.5 Exercise effects on intracellular [K^+] regulation

In human resting skeletal muscle, intracellular [K^+] ($[K^+]_i$) was caculated at around 161-165 mM (Sjøgaard, 1983, Sjøgaard *et al.*, 1985). During exhaustive one-legged dynamic knee-extension exercise, muscle $[K^+]_i$ fell from rest to 129-140 mM (Sjøgaard, 1983, Sjøgaard *et al.*, 1985). Numerous studies have investigated the K^+ loss in animal models (Juel, 1986, Balog and Fitts, 1996). In mouse soleus and EDL muscles, the $[K^+]_i$ fell from 168 mM and 182 mM at rest, to 32 mM and 48 mM, respectively (Juel, 1986). In frog semitendinosus muscle, the muscle $[K^+]_i$ fell from 142 mM to 97 mM with electrical stimulation (Balog and Fitts, 1996).

2.3.6 Exercise effects on interstitial [K^+] regulation

During exercise, potassium (K^+) is released from contracting muscle predominately through K^+ channels associated with the repolarization phase of the action potential. In isolated soleus muscles from the rat, $[K^+]_e$ increased from 4 mM to 8-14 mM during tetanic contractions (Cairns *et al.*, 1995). In earlier studies, investigators using ion-selective microelectrodes inserted into the brachioradialis muscle, reported interstitial [K^+] values of 4-5 mM at rest and a peak value of 9.5 mM during 20 s of maximal, static forearm exercise, although they also reported a higher value of up to 15 mM (Vyskocil *et al.*, 1983). More recent studies using microdialysis probes to measure the muscle interstitial [K^+] have shown that the muscle interstitial [K^+] may increase from ~4.5 mM at rest, to approximately 7-8 mM during intermittent, static calf exercise (Green *et al.*, 1999), to 6-9 mM during knee extensor exercise, and to as much as ~11-12 mM during incremental exercise (Nielsen *et al.*, 2004a, Green *et al.*,

2000, Nordsborg *et al.*, 2003b). Thus, the interstitial [K^+] may double during exercise and clearly indicates that Na^+,K^+-ATPase cannot completely counteract the loss of K^+ from muscle during exercise.

2.3.7 Role of muscle K^+ fluxes in fatigue

Several studies have investigated K^+ fluxes in relation to fatigue. During exercise K^+ is released across the muscle membrane from the intracellular to the extracellular space in the contracting muscle (Juel, 1986, Lindinger and Heigenhauser, 1988, SjØgaard, 1996). Increased $[K^+]_e$ can reduce excitability and force in skeletal muscle (Overgaard and Nielsen, 2001). In human studies, continuous muscle K^+ loss was associated with fatigue (Verburg *et al.*, 1999). During 60 min of fatiguing two-legged knee extensor exercise, the decrease in intracellular K^+ content was linearly related to the fall of maximal force (Verburg *et al.*, 1999). Recently, another study reported that extracellular [K^+] at >10 mM reduced peak tetanic force development by 25–75% during graded dynamic exercise in human skeletal muscle (Juel *et al.*, 2000c). In isolated rat soleus muscle, the $[K^+]_e$ increased from 4 mM to 8-14 mM during tetanic contractions (Cairns *et al.*, 1995, Cairns *et al.*, 1997). There was little depression of force at a $[K^+]_e$ of 8-9 mM, but a large reduction of force occurred at a $[K^+]_e$ of 11-14 mM (Cairns *et al.*, 1995). Increasing $[K^+]_e$ also induced membrane depolarization (Cairns *et al.*, 1995, Cairns *et al.*, 1997). Another study has demonstrated the isometric twitch and tetanic force of intact muscles decreased by 40-69% by elevated $[K^+]_e$ (Clausen *et al.*, 1993). In rat EDL muscle, a reduction in $[K^+]_i$ to below 60 mM reduced twitch force (Nielsen *et al.*, 2004b, Pedersen *et al.*, 2003) and tetanic force (Pedersen *et al.*, 2003). In isolated frog sartorius muscle, high $[K^+]_e$ also reduced twitch and tetanic force (Bouclin *et al.*, 1995).

2.3.8 Exercise effect on Na^+ regulation

In resting human skeletal muscle, the intracellular [Na^+] ($[Na^+]_i$) was approximately 6-13 mM (Sjøgaard, 1983, Sjøgaard *et al.*, 1985) and intense exercise increased the calculated $[Na^+]_i$ to around 24 mM (Sjøgaard, 1983, Sjøgaard *et al.*, 1985). The muscle extracellular [Na^+] ($[Na^+]_e$) was about 130-135 mM at rest (Sjøgaard, 1983, Sjøgaard *et al.*, 1985) and after intense exercise was increased very little because the concentration of the extracellular and interstitial Na^+ were both very high (Sjøgaard *et al.*, 1985).

2.3.9 Role of Na^+ in muscle fatigue

Several studies reported that increasing $[Na^+]_i$ or reducing $[Na^+]_e$ may contribute to muscle fatigue during exercise. In isolated rat muscle, $[Na^+]_e$ reduction from 147 to 25 mM induced a 30% reduction in tetanic force (Overgaard *et al.*, 1997). Similarly, a decline in $[Na^+]_e$ fell from 147 to 30 mM, resulted in a rapid depression in both twitch and tetanic force (Cairns *et al.*, 2003). In isolated frog sartorius muscle, a reduction in $[Na^+]_e$ was shown to reduce excitability and force development (Bouclin *et al.*, 1995). Similarly, a 50% depression in $[Na^+]_e$ decreased both twitch and tetanic force (Nakajima *et al.*, 1975).

However, these results from isolated muscle cannot directly apply to the whole muscle where the $[Na^+]_e$ was increased little because the concentration of the extracellular and interstitial Na^+ were both high after intense exercise (Sjogaard *et al.*, 1985). However, it is possible that the Na^+ flux from the lumen of the t-tubules into the myoplasm is not matched by diffusion from the interstitial space, which would lead to a decrease in t-tubular [Na^+] (Duty and Allen, 1994, Overgaard *et al.*, 1997). Thus increasing $[Na^+]_i$ and decreasing t-tubular [Na^+] may result in a rundown of the trans-sarcolemmal [Na^+] gradient, which has been suggested to cause fatigue (Cairns *et al.*, 2003).

2.3.10 Role of Na^+,K^+-ATPase in muscle fatigue

2.3.10.1 Depressive effects of acute high intensity exercise

Sustained repetitive contractions appear to cause inhibition of muscle Na^+,K^+-ATPase activity. Researchers have reported decreases in maximal Na^+,K^+-ATPase activity, as assessed in vitro by K^+-stimulated 3-O-methylfluorescein phosphatase (3-O-MFPase) activity, after exercise (Fowles *et al.*, 2002a, Fowles *et al.*, 2002b, Sandiford *et al.*, 2004, Leppik *et al.*, 2004, Aughey *et al.*, 2005, Fraser *et al.*, 2002). As a consequence, it is possible that the Na^+,K^+ -membrane transport and membrane excitability could be compromised in tasks in which large force levels are required with high K^+ efflux expected. Moreover, if a similar task is performed under conditions where the muscle metabolic stress is exaggerated as during hypoxia (Connett *et al.*, 1990, Sahlin and Katz, 1989), the decrease in maximal Na^+,K^+ATPase activity and the loss of membrane excitability may be even more pronounced (Sandiford *et al.*, 2004). It is possible that a failure in membrane excitability secondary to reductions in maximal Na^+,K^+ATPase activity may explain the lower mechanical power output and lower peak aerobic power observed in hypoxia compared with normoxia (Robergs *et al.*, 1998).

2.3.10.2 Possible mechanisms for depressed Na^+,K^+-ATPase activity

Several factors are implicated in the exercise-induced depression of maximal in-vitro Na^+,K^+-ATPase activity, including increased free radical production, reduced muscle glycogen content, phosphorylation of α subunits and elevated cytosolic [Ca^{2+}] (Cheng *et al.*, 1997, Kourie, 1998, Matsuda *et al.*, 1991b, Fraser *et al.*, 2002).

2.3.10.3 Relationships between Na^+,K^+-ATPase and exercise performance

The content of Na^+,K^+-ATPase in vastus lateralis showed a weak, but significant, correlation to maximum isometric strength (Klitgaard and Clausen, 1989). Sprint training led to improved K^+ regulation and increased content of ^{3}H-ouabain binding

sites in vastus lateralis, but there was no correlation with sprint exercise performance (McKenna *et al.*, 1993). Intensified running training in 39 endurance-trained men increased the muscle content of ^{3}H-ouabain binding sites, but there was no correlation to indices of endurance performance (Madsen *et al.*, 1994). There was only a weak correlation between ^{3}H-ouabain binding site content and VO_{2max} measured before training (Madsen *et al.*, 1994). However, more detailed studies showed significant correlation between the content of ^{3}H-ouabain binding sites in vastus lateralis and VO_{2max}, performance during a 20-min treadmill test, and performance during cross-country skiing (Evertsen *et al.*, 1997).

Although the individual improvement in an endurance test correlated with the individual changes in Na^{+},K^{+}-ATPase content, there was no significant correlation between pump content and muscle strength or endurance (Medbo *et al.*, 2001). In another study, peak O_2 consumption was significantly correlated to both ^{3}H-ouabain binding site content and 3-*O*-MFPase activity in human vastus lateralis muscle. The ^{3}H-ouabain binding site content also showed a significant inverse correlation to fatigue index (Fraser *et al.*, 2002). In rats, decreases in masseter muscle endurance with age was associated with a 22% reduction in the content of ^{3}H-ouabain binding sites (Norton *et al.*, 2001). A recent analysis of the human Na^{+},K^{+}-ATPase α2- gene suggested that DNA sequence variation at the locus of this gene or a locus in close proximity is associated with the responsiveness of VO_{2max} max and maximum work to a 20-wk endurance training program (Rankinen *et al.*, 2000).

In conclusion, although it is well-documented that various types of training increase physical performance as well as the content of Na^{+},K^{+}-ATPase in skeletal muscle, these two parameters have not been strongly correlated.

2.4 Digoxin

2.4.1 Background

Digoxin is a specific inhibitor of the Na^+,K^+-ATPase and is used to treat patients with severe heart failure (Rose and Valdes, 1994). Digoxin is one of the cardiac (or digitalis) glycosides, a closely related group of drugs having in common specific effects on the myocardium, and which are found in a number of plants (Ruegg, 1992). Digoxin is derived from the leaves of the foxglove plant (*Digitalis purpurea,* Figure 2.1) and has been used medicinally since the days of the ancient Egyptians and the Roman Empire (Rose and Valdes, 1994). It is also effective for controlling certain types of abnormal heart rhythms (Hauptman *et al.*, 1999). The molecular formula of digoxin is $C_{14}H_{64}O_{14}$ and its molecular weight is 780.95.

2.4.2 Digoxin binding in human skeletal muscle

Digoxin inhibits the Na^+,K^+-ATPase activity by binding to a specific receptor on the α subunit (Lichtstein, 1995). This results in increased $[Na^+]_i$ and then via the Na^+/Ca^{2+} exchanger, also increased $[Ca^{2+}]$ within heart cells, which then potentiated the force of contraction of the heart (Fozzard and Sheets, 1985). Digitalis glycosides inhibit Na^+,K^+-ATPase in the sarcolemma of all tissues, but skeletal muscle is one of the most important organs for the pharmacokinetics of digoxin (Joreteg, 1986). About 50% of the total amount of digoxin in the body is bound to skeletal muscle and only 3-4% to the heart muscle (Steiness, 1978). Furthermore, the number of Na^+, K^+-ATPase sites in the total mass of skeletal muscle is around 100 times larger than in the heart (Norgaard *et al.*, 1984). In skeletal muscle, digoxin binds to and blocks ~13% of Na^+,K^+-ATPase in animal models and leads to early muscle fatigue (Schmidt *et al.*, 1993a, Sherman *et al.*, 1988). In CHF patients, the rate of Na^+, K^+-ATPase occupancy in skeletal muscle was 35% with digoxin (Green *et al.*, 2001), which would profoundly alter muscle Na^+/K^+ regulation, excitability and performance.

Figure 2.1 Foxglove plant

Digitalisation does not appear to induce compensatory upregulation of Na^+,K^+-ATPase content in myocardium (Schmidt *et al.*, 1993b) or skeletal muscle in CHF patients (Green *et al.*, 2001). However, the effects of digoxin on Na^+, K^+-ATPase isoform gene and protein expression in skeletal muscle have never previously been studied nor has the possibility of compensatory upregulation in healthy muscle. These were investigated in this thesis.

2.4.3 Digoxin effects on muscle and plasma [K^+] in humans

Digoxin, by binding to the Na^+, K^+-ATPase, inhibits Na^+ and K^+ ion transport and has marked adverse effects on K^+ homeostasis in humans (Schmidt *et al.*, 1995). Skeletal muscle Na^+,K^+-ATPase plays an important role in the active reuptake of potassium into muscle cells.

With digoxin, the serum potassium concentration increased by 0.19 $mmol.l^{-1}$ during rest (Edner *et al.*, 1993). In healthy subjects and in patients with heart failure, a significant decrease in total body K^+ and muscle K^+ was demonstrated with digitalisation (Ericsson *et al.*, 1981). After taking digoxin, muscle K^+ content decreased by 5% and total body K^+ decreased by 9% in healthy subjects (Ericsson *et al.*, 1981). Similarly, in patients with heart failure, the muscle K^+ and total body K^+ decreased by 6% and 8%, respectively (Ericsson *et al.*, 1981). However, no change was noted for the serum potassium (Ericsson *et al.*, 1981).

In cardiac patients, digoxin increased venous [K^+] by 0.2-0.3 $mmol.l^{-1}$ during cycling exercise. Following digitalisation, femoral veno-arterial difference in plasma potassium increased by 50-100% during exercise and total loss of potassium from the leg increased by 138% (Schmidt *et al.*, 1995). Another study reported that serum digoxin was associated with up to a 20% rise in plasma [K^+] during incremental cycle exercise (Norgaard *et al.*, 1991). The effects of digoxin on plasma [K^+] and on muscle

Na^+,K^+-ATPase, which affects muscle K^+ homeostasis, were investigated in this thesis.

2.4.4 Digoxin effects on muscle strength and endurance in humans

Few studies have reported the effect of digoxin on muscle strength. No significant effects of digoxin on muscle isometric endurance were reported in an early study (Bruce *et al.*, 1968), however there were only 4 subjects and each of them appeared to have a performance decrease of 4%. Similarly, the maximal isokinetic muscle strength was not significantly change by digoxin (Sundqvist *et al.*, 1983). However, another study reported that high doses of cardiac glycosides increased skeletal muscle strength in man (Smulyan and Eich, 1976), which indicated that digitalis may improve the maximal dynamic short-time work performance in healthy subjects.

Digoxin leads to early muscle fatigue in animal models (van der Ent *et al.*, 1998, Waller, 1991). Several studies reported muscular endurance and fatiguability were impaired in CHF, where the majority of the patients were taking digoxin (Magnusson *et al.*, 1994, Minotti *et al.*, 1991, Yamani *et al.*, 1995). However, the exact mechanism of digoxin effect on muscle fatigue was still not clear. Therefore the effects of digoxin on muscle strength and fatiguability were investigated in this thesis.

2.4.5 Digoxin effects on peripheral skeletal muscle function in chronic heart failure (CHF)

Fatigue and exercise intolerance is the one of main symptoms of chronic heart failure patients. Abnormalities of skeletal muscle appear to play a role in the limitation of exercise capacity in congestive heart failure. Several studies consistently reported that the fatigability of skeletal muscle is greater in CHF than in healthy people (Magnusson *et al.*, 1994, Massie *et al.*, 1996a, Minotti *et al.*, 1993). The maximal strength and the endurance capacity of quadriceps muscle were 15% and 30% lower

in the CHF patients than in healthy people (Magnusson *et al.*, 1996). Peak VO_2 and resistance to fatigue were lower in patients with heart failure than in healthy subjects (Massie *et al.*, 1996a). These results demonstrate that CHF patients have both a lower local and a lower whole-body work capacity than healthy controls. There are many factors attributed to the exercise intolerance, such as reduced oxidative capacity, altered muscle fiber type composition and fibre atrophy or reduced muscle blood flow (Wilson *et al.*, 1993, Massie and Conway, 1987, Wilson *et al.*, 1984, Schaufelberger *et al.*, 1995). In CHF patients, abnormalities of skeletal muscle play a role in their exercise intolerance; these symptoms may reflect a delay in muscle recovery and a resulting limitation in submaximal exercise tolerance (Yamani *et al.*, 1995). In CHF patients, impaired muscular endurance has been reported when undergoing digoxin therapy (Schaufelberger *et al.*, 1997), which may be partially due to effects of digoxin. Dynamic endurance, quantified as the decline in peak torque during 15 successive isokinetic knee extensions, was significantly reduced in CHF patients compared to healthy subjects, during aerobic and during ischemic exercise (Minotti *et al.*, 1991). The maximal quadriceps muscle strength was found to be significantly reduced in patients with CHF, who also exhibited a higher extent of muscular fatigability (Schulze *et al.*, 2004). Whilst digoxin enhances left ventricular function during incremental exercise in CHF, time to exhaustion and VO_{2peak} were not improved (Morisco *et al.*, 1996). This likely reflects the importance of skeletal muscle abnormalities in restricting maximal exercise performance (Morisco *et al.*, 1996). Digoxin is widely used for the treatment of severe CHF patients. These results suggest that digoxin may adversely affect skeletal muscle function in CHF and contribute to their exercise limitation. To avoid the many complicating factors in CHF patients, therefore this thesis investigated digoxin effects on muscle function in healthy people.

Aims and hypothesis

Aims

The first aim of the thesis was to investigate the effects of acute submaximal exercise, comprising 10 min at 33% VO_2 peak, 10 min at 67% VO_2 peak and continued to fatigue at 90% VO_2 peak, on Na^+, K^+-ATPase mRNA expression and protein abundance in skeletal muscle. The second aim was to investigate the effects of digoxin on muscle Na^+,K^+-ATPase mRNA expression and protein abundance at rest, during and following exercise. The third aim was to investigate the effects of digoxin on muscle strength and fatiguability.

Hypothesis

Three hypotheses were tested in this thesis.

1. That acute exhaustive exercise (10 min at 33%, 10 min at 67% and continued to fatigue at 90% VO_2 peak) will increase the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoform mRNA expression following exercise, without any change in isoform protein abundance;
2. That oral digoxin administration for 14 d in healthy, non-trained humans will increase Na^+,K^+-ATPase isoform gene and protein expression;
3. That oral digoxin administration will reduce quadriceps muscle strength, increase fatiguability and impair cycling exercise performance.

Chapter 3. Methods

3.1 Subjects

Ten active, but not well-trained young healthy subjects, comprising nine males and one female (age, 26 ± 5.9 yr; height, 178.4 ± 9.1 cm; body mass, 75.7 ± 11.3 kg; mean ± S.D.) gave written informed consent and participated in the study. Young rather than older adults were used due to ethical considerations including the increased risk of invasive projects with maximal exercise testing in older individuals. All subjects underwent an initial medical examination to ensure that they had no abnormalities in plasma electrolyte concentrations, kidney function, rest and exercise ECG, and had no prior history of adverse cardiovascular events. All protocols and procedures were approved by the Victoria University Human Research Ethics Committee.

3.2 Experimental overview

Each subject performed a series of exercise tests whilst taking the typical clinical oral dose of digoxin (0.25mg daily), 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. Washout time between cessation of the first treatment and commencement of the second treatment was 4 weeks. In the subjects taking digoxin first, there was the actual washout time (no treatment) of 4 weeks, plus an additional 2 weeks washout, whilst they took the second (placebo) treatment. Thus, the effective washout time was 6 weeks between exercise tests, which would ensure sufficient time for digoxin washout (Figure 3.1).

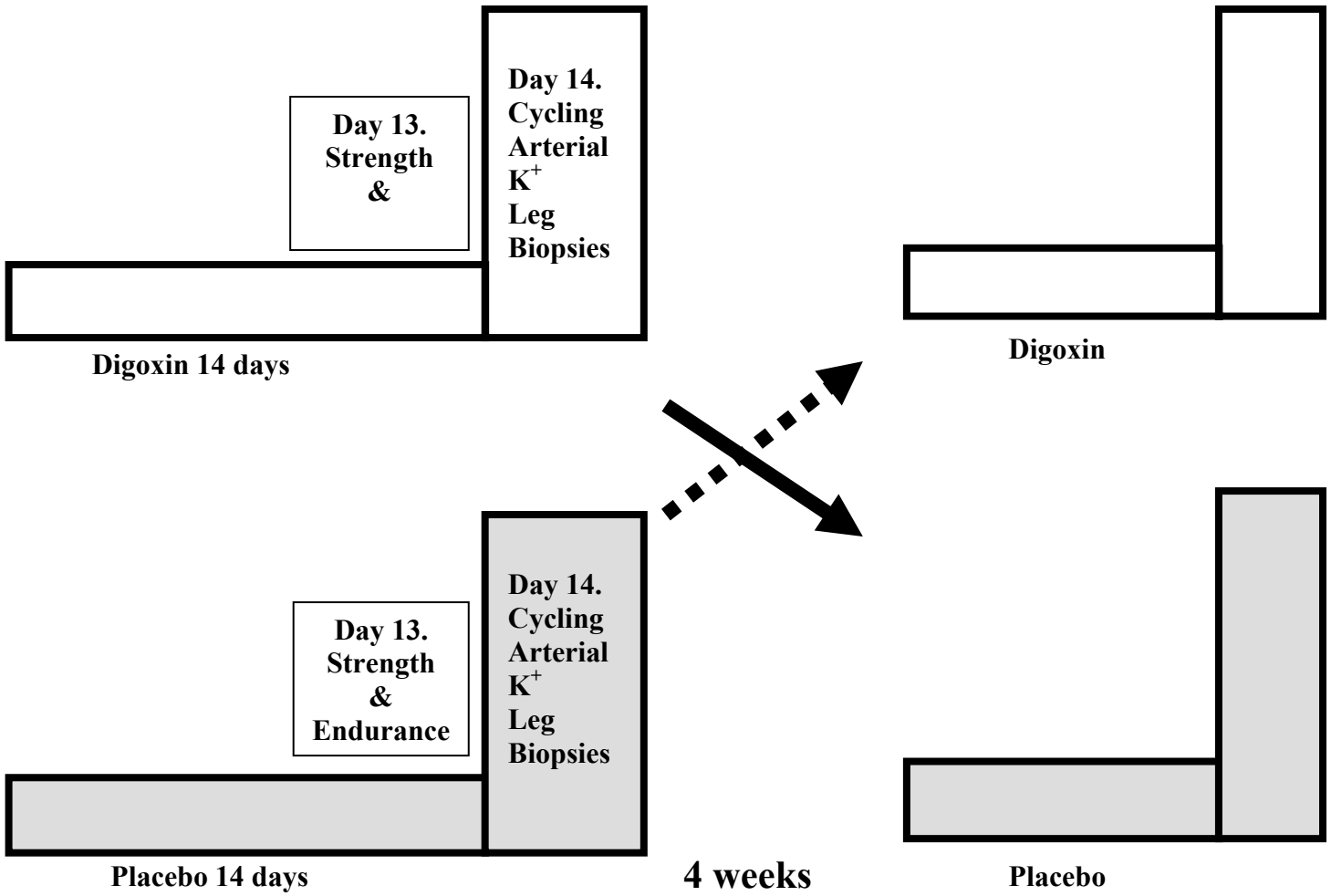

Fig 3.1 Experimental design. Trials were conducted in a crossover, double blind, randomised, counterbalanced design with subjects taking digoxin (0.25 mg per day) or placebo for 14 d. Exercise tests were conducted on d 13 and d 14.

3.3 Exercise Tests

3.3.1 Quadriceps Muscle Strength and Endurance Test

Subjects performed tests of quadriceps muscle strength and endurance (dominant leg) on an isokinetic dynamometer (Cybex Norm 770, Henley Healthcare, USA) on day 13 of digoxin, and of placebo treatments. Three familiarization trials were conducted at least 2 weeks prior to commencement of the trial, minimise any training effect. The dynamometer was calibrated for angle, torque and velocity immediately prior to each test. The subjects were fixed to the dynamometer adjustable chair by belts across the chest and hips to restrict the movement of the upper body, with a belt across the thigh also used to stabilise the active leg. To minimise variability the same positions were recorded for each individual and used in all trials for each subject. Maximal peak torque was measured at 0, 60, 120, 180, 240, 300 and 360 °/s muscle fatiguability was determined from the percent decline in peak torque during 50 repeated maximal contractions, conducted at 180°/s and expressed as a fatigue index: Fatigue Index (%) = ((Peak torque - Final torque) / Peak torque) x 100. Peak torque was defined as the average of the 5 highest peak torque of the first 10 contractions, while final torque was the average of the 5 weakest peak torque of the last 10 contractions. A real-time visual display of torque and work for each contraction was provided to the subjects during the maximal strength and muscle fatigue tests. Verbal support was provided to encourage subjects to exert maximal torque during the muscle strength test, as well as to maintain the appropriate work and kicking frequency during the muscle fatigue test.

3.3.2 $\dot{V}O_2$peak test

Participants initially performed an incremental cycle ergometer (Lode Excalibur, Groningen, the Netherlands) exercise to fatigue, with measurement of peak oxygen

uptake ($\dot{V}O_2$ peak), as a marker of exercise performance and for calculation of workrates corresponding to 33, 67 and 90% $\dot{V}O_2$ peak.

3.3.3 Invasive Leg Cycling Exercise test

This test were conducted on day 14 and comprised cycling at 70rpm for 10 min at 33% $\dot{V}O_2$ peak, 2 min pause, 10 min at 67% $\dot{V}O_2$ peak, 2 min pause, then 90% $\dot{V}O_2$ peak continued to fatigue. Time to fatigue was defined as time until subjects were unable to maintain power output with pedal cadence falling below 55 rpm. Muscle biopsies and radial arterial blood samples were taken before, during and following this test.

3.4 Muscle biopsy sampling

A muscle biopsy was taken at rest, immediately after the cycling exercise periods at 67% and at fatigue, and at 3 hours post-exercise, giving a total of 4 biopsies per trial and 8 in total. Due to the invasive and complex nature of the experiment, it was judged ethically appropriate to take only a single delayed recovery biopsy. This was performed at 3 hr post-exercise to maximise the opportunity of detecting increases in Na^+,K^+-ATPase gene transcripts (Murphy *et al.*, 2004). A local anaesthetic (2% Xylocaine) was injected into the skin and subcutaneous tissue above the vastus lateralis muscle, four small incisions (2 per leg) were made through the skin and fascia, and a muscle sample of approximately 100-120 mg was then excised using a biopsy needle. Samples were immediately frozen in liquid N_2 until assayed later for Na^+,K^+-ATPase isoform mRNA expression and protein abundance.

3.5 Western blotting

Muscle samples (20 – 30 mg) were homogenised for 2 x 10 s at a speed rating of 4-5 (Polytron PT1200; Kinematica, Luzern, Switzerland) on ice in a 1:40 dilution with extraction buffer (25 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate (SDS), 5

mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol, 17.4 μg/ml phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 1 μg/ml aprotinin. A portion of each sample was heated for 10 min at 90º C, and analysed for total protein content (BCA Assay Kit, Pierce, Rockford, Illinois, USA), with bovine serum albumin (BSA) as the standard. The remaining samples were frozen at –80º C for immunoblotting.

SDS-PAGE (10% separating gel, 5% stacking gel) was performed and gels were loaded with 20 (β_1) or 50 (α_1, α_2, α_3, β_2, β_3) μg protein. Following electrophoresis (20 min, 100 V and 90 min, 150 V), the protein was transferred (90 min, 100 V) to 0.45 μm nitrocellulose membrane, and blocked for 2 h with blocking buffer (5% non-fat milk in tris-buffered saline Tween (TBST)). Membranes were incubated overnight at 4 ºC in primary antibodies diluted in blocking buffer containing 0.1% NaN_3. Membranes were washed in 0.05% TBST buffer, and incubated for 1 h in horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins) diluted 1:10,000 in TBST buffer. Following three washes in 0.05% TBST, membranes were dried and treated with chemiluminescent substrate (Pierce SuperSignal West Pico, Illinois, USA). The signal was captured and imaged (Kodak Digital Science Image Station 400_{CF}, Eastman Kodak Company, CT, USA). The linearity of the blot signal versus protein loaded for our experimental conditions was established for each antibody.

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, USA);

α_2: polyclonal anti-HERED (kindly donated by T. Pressley, Texas Tech University); α_3: monoclonal MA3-915 (Affinity Bioreagents, Golden, CO, USA); β_1: monoclonal MA3-930 (Affinity Bioreagents); β_2: polyclonal 610915 (Transduction Laboratories, Lexington, KY, USA); and β_3: polyclonal 610993 (Transduction Laboratories). For comparative purposes, additional polyclonal antibodies used by other researchers who did not detect α_3 or β_2 in human muscle (Hundal *et al.*, 1994, Juel *et al.*, 2000b) were utilised for the α_3 and β_2 isoforms; these antibodies were for α_3: 06-172 (Upstate Biotechnology, Lake Placid, NY, USA); β_2: 06-171 (Upstate Biotechnology); and β_3: 06-817 (Upstate Biotechnology).

3.6 Real-time RT-PCR measurement of mRNA

Total RNA was extracted from 5-10 mg muscle using the FastRNA reagents (BIO 101, Vista, CA, USA) using methods previously employed in our laboratory (Cameron-Smith *et al.*, 2003, Murphy *et al.*, 2003, Murphy *et al.*, 2001). The resulting RNA pellet was dissolved in EDTA-treated water and stored at –80 ºC. Total RNA concentration was determined spectrophotometrically at 260 nm. For each sample, 1 μg of RNA was transcribed into cDNA using the Promega AMV Reverse Transcription Kit (kit A3500; Promega, Madison, Wisconsin, USA), and the resulting cDNA was stored at –20 ºC for subsequent analysis. Real Time-PCR (GeneAmp 7500 Sequence Detection System) was run for 1 cycle (50º C for 2 min, 95º C for 10 min) and 50 cycles (95º C for 15 s, 60º C for 60 s). Fluorescence resulted from incorporation of SYBR Green (SYBR Green Master Mix, Applied Biosystems) to double stranded DNA and this fluorescence was measured after each repetitive cycle. Duplicate wells were run for each sample. Measurements included a no-template control, as well as a human muscle sample endogenous control, cyclophilin (CYC). Primer sequences were designed from published sequences, where possible spanning

exon boundaries to minimise contaminant DNA amplification. Primer sequences were designed from published sequences (Table 3.1), where possible spanning exon boundaries to minimise contaminant DNA amplification. Gene expression was quantified from fluorescence emission using a cycle threshold (C_T) method. 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. Exercise had no significant effect on the mRNA expression of CYC, when expressed in the linear (2^{-C_T}) form. The intra-assay coefficient of variation for each target gene was <15.0% for 2^{-C_T} (Table 3.2), which is within values previously reported (Murphy et al., 2003).

3.7 Statistical analyses

All data are presented as mean±SEM. Muscle data were analysed using a repeated measures two-way ANOVA (treatment, exercise). Post hoc analyses were determined using the Newman-Kuels test. The total alpha or beta subunit mRNA expression at rest compared between digoxin and placebo was analysed using a paired-samples student t-test. Correlations were determined by least squares linear regression. Significance was accepted at $P< 0.05$. Statistical analyses were conducted using the SPSS software package.

Table 3.1 Human Na^+,K^+-ATPase gene α_1-α_3 and β_1-β_3 and CYC primer sequences used for mRNA analyses.

Gene	GenBank Accession	Identity	Sense Primer (5'-3')	Antisense Primer (5'-3')	Exon boundaries
α_1	NM_000701	ATPA1	TGTCCAGAATTGCAGGTCTTTG	TGCCCGCTTAAGAATAGGTAGGT	4
α_2	NM_000702	ATPA2	GAATGAGAGGCTCATCAGCAT	CAAAGTAGGTGAAGAAGCCACC	12-13
α_3	NM_152296	ATPA3	GGTGGCTATGACAGAGCACAA	TGCACACAGTGTGTGTTGTATTT	1-3
β_1	NM_001677	ATPB1	ACCAATCTTACCATGGACACTG	CGGTCTTTCTCACTGTACCCAAT	3-6
β_2	NM_001679	ATPB2	CCAGCATGTTCAGAAGCTCAAC	GCGGCAGACATCATTCTTTTG	4
β_3	BC011835	ATPB3	AGTCTGTCCTGATGGAGCACTT	GCATGCTTGAAGTAATGAAATA	4
CYC	XM_004890	PPIA	CCCACCGTGTTCTTCGACAT	CCAGTGCTCAGAGCACGAAA	

Primer sequences were designed using Primer Express software (Applied Biosystems) from gene sequences obtained from GeneBank. Primer specificity was determined using a BLAST search. CYC, cyclophilin.

Table 3.2 Intra-assay variability of 2^{-C_T} values

Gene	2^{-C_T} CV (%)
α_1	13.8
α_2	10.6
α_3	12.5
β_1	8.93
β_2	12.7
β_3	14
Human CYC	8.58

Each sample was run in duplicate wells in the same Real-Time PCR run. *n* = 80. CV, coefficient of variation; C_T, cycle threshold

Chapter 4. Results

4.1 Serum Digoxin

Serum digoxin at rest was 0.7±0.1 nM at d 13 and 0.8±0.1 nM at d 14 (Mean±SEM) in the digoxin trial, and <0.4 nM (detection limits) for the control trial.

4.2 Exercise Performance-$\dot{V}O_2$peak test

The initial incremental cycling exercise $\dot{V}O_{2peak}$ was 3.67±0.13 L.min^{-1} (Mean±SEM). There were no differences in $\dot{V}O_2$ between digoxin (DIG) and Control (CON) during exercise (Table 4.1). Time to fatigue during leg cycling exercise was not significantly affected by digoxin (CON 254±40 vs DIG 262±49 s).

Table 4.1 Oxygen consumption (L.min^{-1}) during cycle exercise in digoxin (DIG) and control (CON) trials at 33%, 67% and 90% $\dot{V}O_{2peak}$, respectively.

Workrate	DIG	CON
33% $\dot{V}O_{2peak}$	1.21±0.06	1.19±0.07
67% $\dot{V}O_{2peak}$	2.63±0.16	2.68±0.17
90% $\dot{V}O_{2peak}$	3.61±0.09	3.55±0.14

Data expressed as mean ±SEM, n=10.

4.3 Arterial plasma [K^+]

Plasma [K^+] increased above rest at 67% $\dot{V}O_{2peak}$ and increased further at fatigue ($P < 0.05$, Figure 4.1). However, no significant differences were found in plasma [K^+] between DIG and CON.

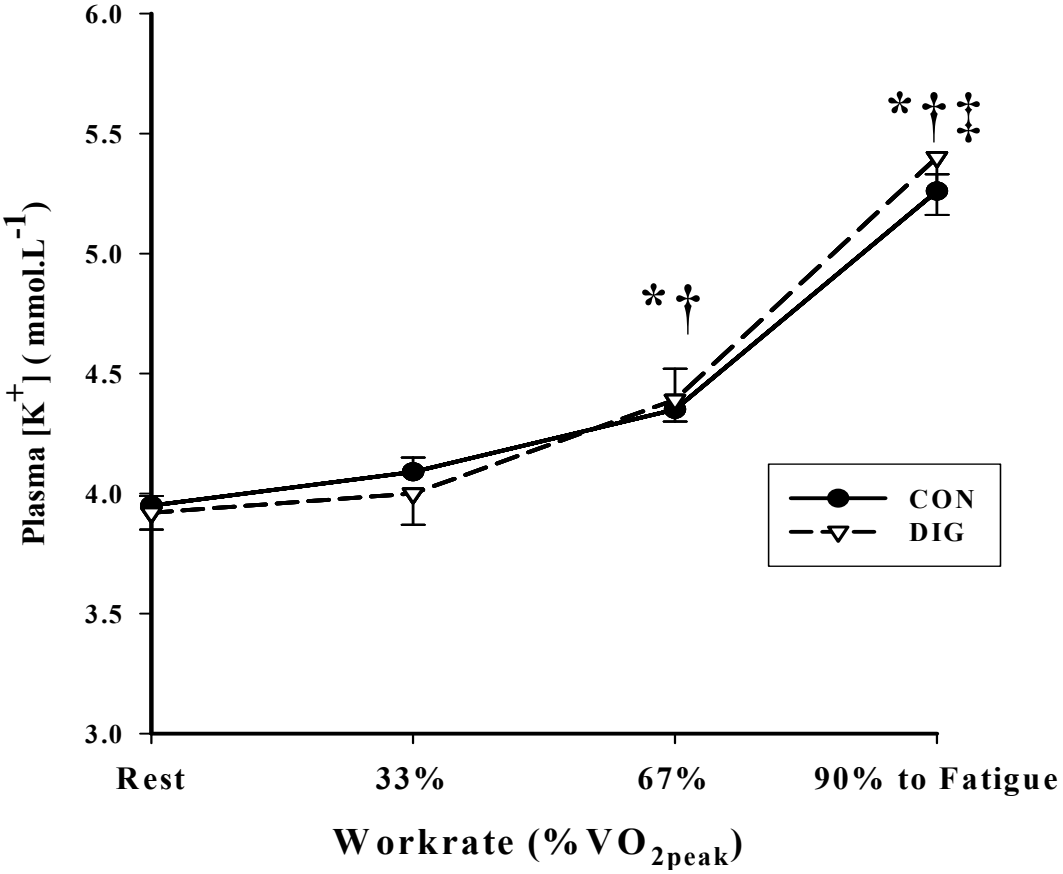

Figure 4.1 Effect of digoxin (DIG; ∇) and control (CON; ●) on plasma potassium concentration ($[K^+]$) during leg cycling exercise at 33%, 67% and 90% $\dot{V}O_{2peak}$ continued to fatigue.

* > rest; † > 33% $\dot{V}O_{2peak}$;

‡ > 67% $\dot{V}O_{2peak}$ ($P < 0.05$, time main effect).

Values are means ± SEM; $n = 10$.

4.4 Muscle Torque

Peak torque during dynamic isokinetic contractions was less at each increasing velocity, as expected ($P < 0.05$), but no significant differences were found between DIG and CON (Figure 4.2). A significant treatment-by-velocity interaction was found ($P < 0.05$), but no significant differences between trials could be detected at any velocity.

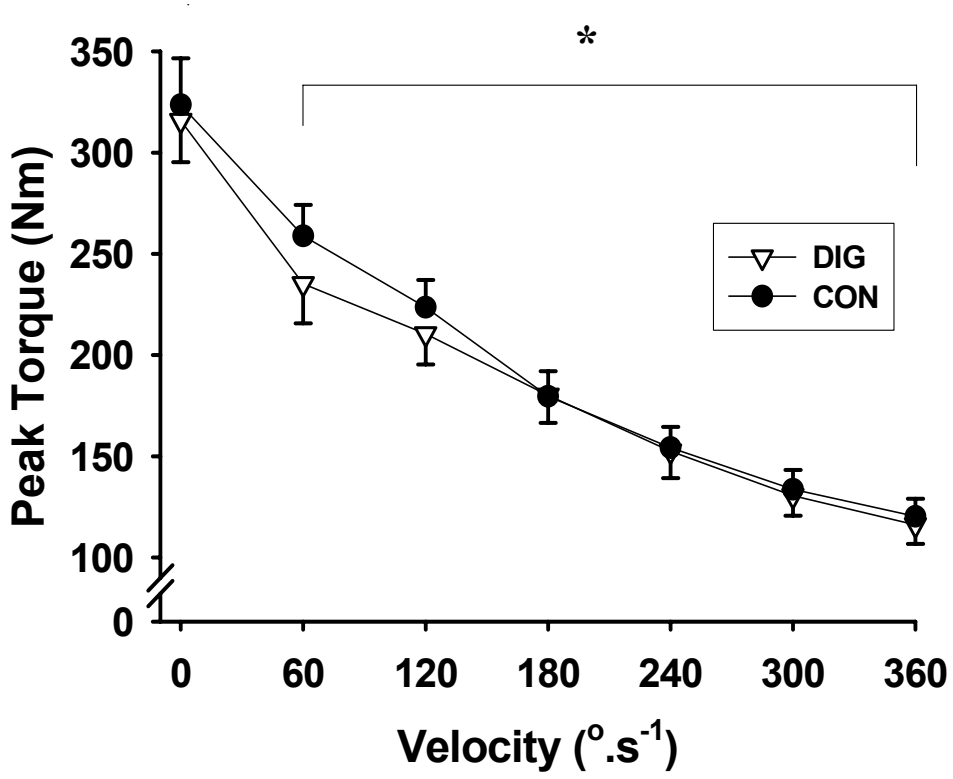

Figure 4.2 Quadriceps muscle torque-velocity relationship during dynamic isokinetic contractions in digoxin (DIG; ▽) and control (CON; ●) trials.

Values are mean±SEM, n=10 (* All values less than 0 $^{o}.s^{-1}$, $P<0.05$, main effect).

4.5 Muscle Fatigue

Peak quadriceps muscle torque declined during 50 repeated contractions ($P < 0.05$, Figure 4.3), but there were no differences in the leg extensor fatigue index between DIG and CON (DIG 56.0±3.0% vs CON 55.0±3.0%).

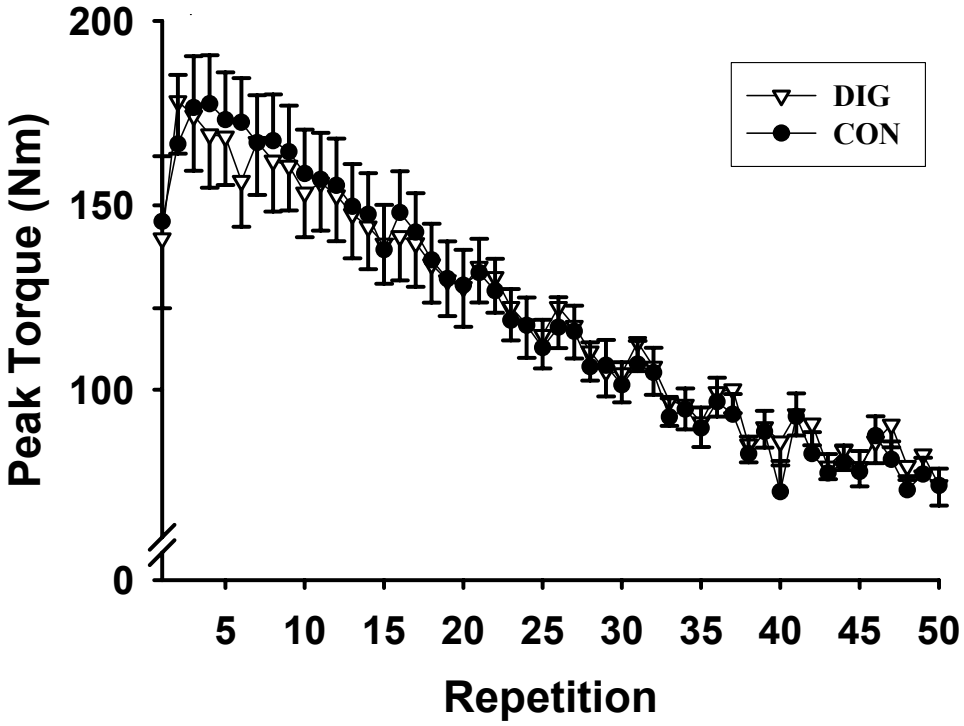

Figure 4.3 Quadriceps muscle peak torque during repeated dynamic isokinetic contractions in digoxin (DIG; ▽) and control (CON; ●) trials.

Values are mean±SEM, n=10.

4.6 Muscle Na^+,K^+-ATPase mRNA and protein expression in crude muscle homogenates

4.6.1 mRNA transcripts

Real-Time RT-PCR analyses demonstrated amplification of each of the primer sets specific to each of the Na^+,K^+-ATPase $\alpha_{1,}$ $\alpha_{2,}$ $\alpha_{3,}$ $\beta_{1,}$ β_2 and β_3 isoforms (Figure 4.4), with no amplification of the no template control samples observed (data not shown). The heat dissociation curve confirmed amplification of only a single gene transcript for each primer set (Figure 4.5). These results indicate the presence of gene transcripts for each of the Na^+,K^+-ATPase $\alpha_{1,}$ $\alpha_{2,}$ $\alpha_{3,}$ $\beta_{1,}$ β_2 and β_3 isoforms in human vastus lateralis muscle.

4.6.2 Protein abundance

Crude muscle homogenates demonstrated protein bands of apparent molecular mass (α_1-α_3,~100-105 kDa; β_1-β_3, ~ 45-52 kDa) specific to each of the $\alpha_{1,}$ $\alpha_{2,}$ $\alpha_{3,}$ $\beta_{1,}$ β_2 and β_3 subunit isoforms. Representative immunoblots of α and β isoforms are shown in Figure 4.6 and Figure 4.7, respectively. These results indicate protein expression of each of the α_1-α_3 and β_1-β_3 isoforms in human skeletal muscle.

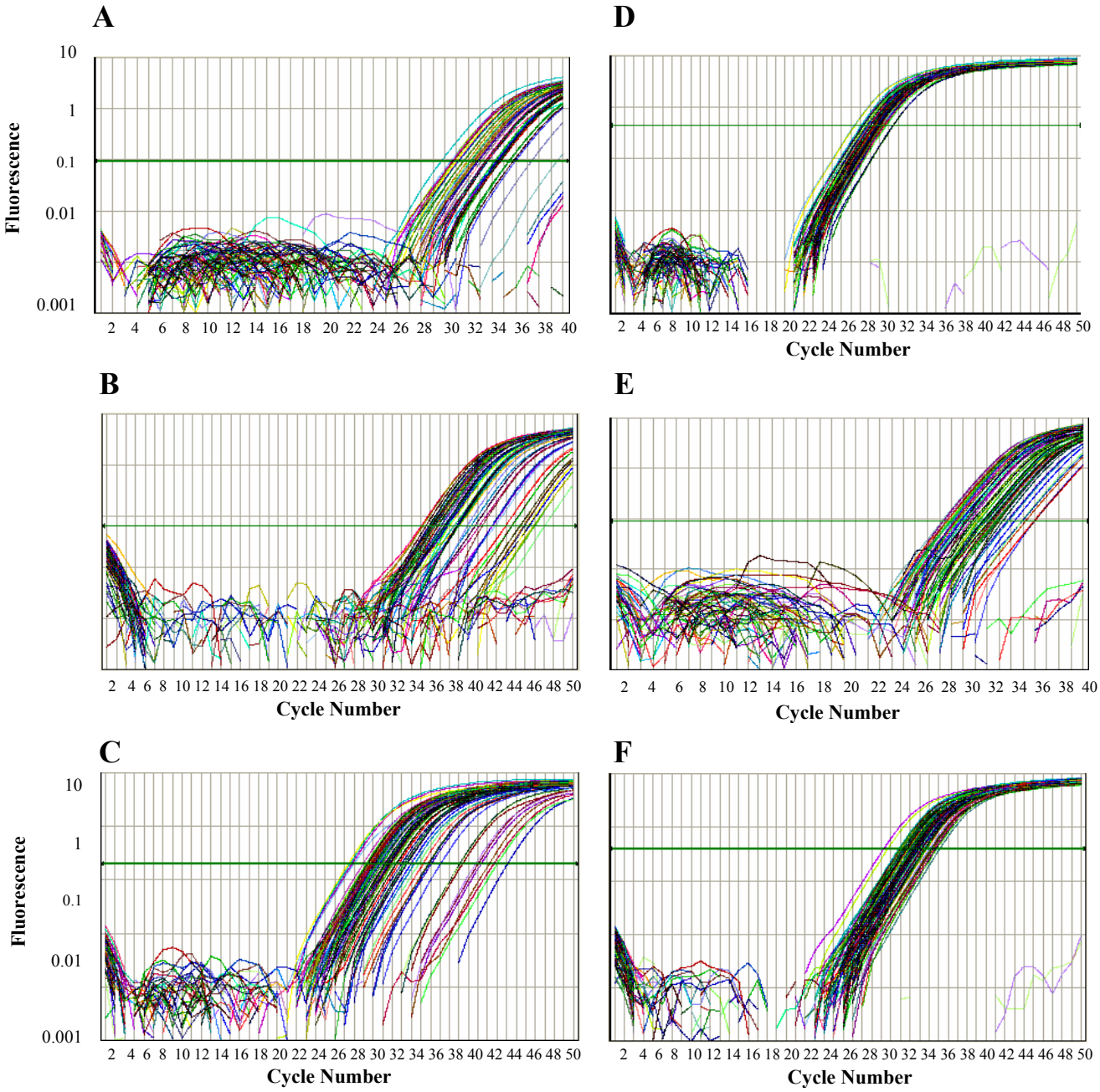

Figure 4.4 Representative amplification plot for Na^+,K^+-ATPase gene transcripts Na^+,K^+-ATPase (A-F) α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in human skeletal muscle.Samples were subjected to 40 or 50 cycles (95°C for 15 s, 60°C for 60 s) with fluorescence measured after each cycle. No template control (NTC) samples did not amplify above threshold.

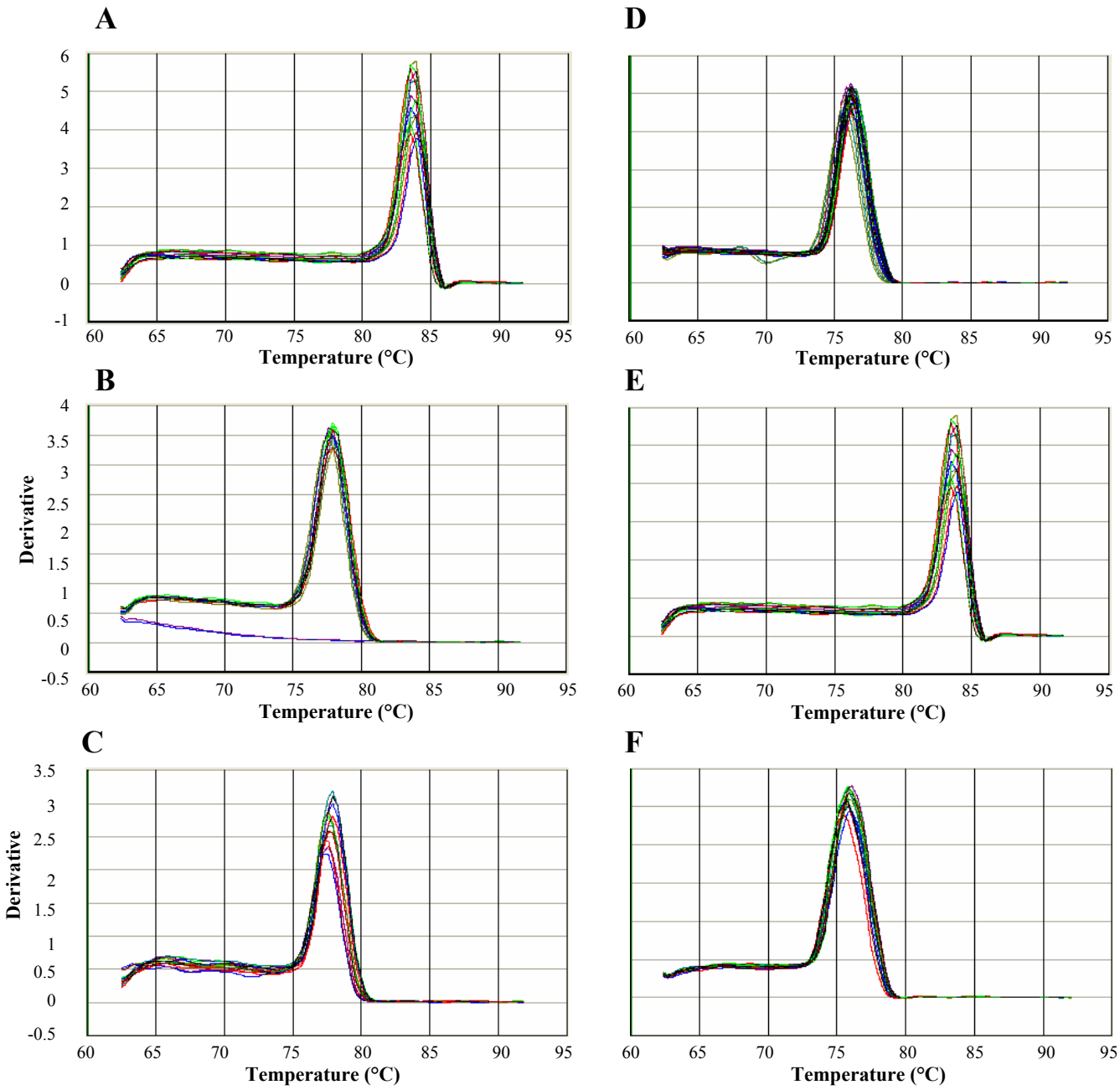

Figure 4.5 Typical heat dissociation curve for Na^+,K^+-ATPase gene transcripts. Na^+,K^+-ATPase (A-F) α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in human skeletal muscle. Following the final PCR cycle, samples and the no template control (NTC) were subjected to a heat dissociation protocol over 60-95°C, with the derivative being the negative of the rate of change in fluorescence as a function of temperature.

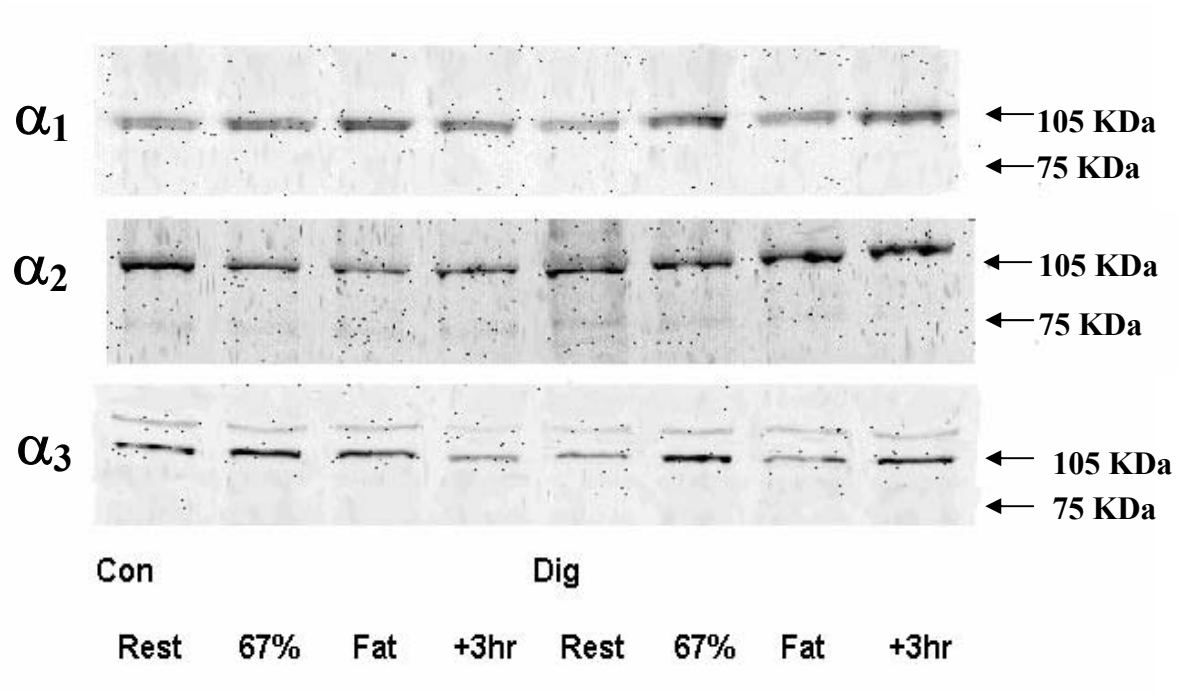

Figure 4.6 Representative immunoblots of Na^+,K^+-ATPase α_1, α_2 and α_3 isoforms in crude muscle homogenates of the human vastus lateralis muscle.

Values at right indicate molecular weight of bands.

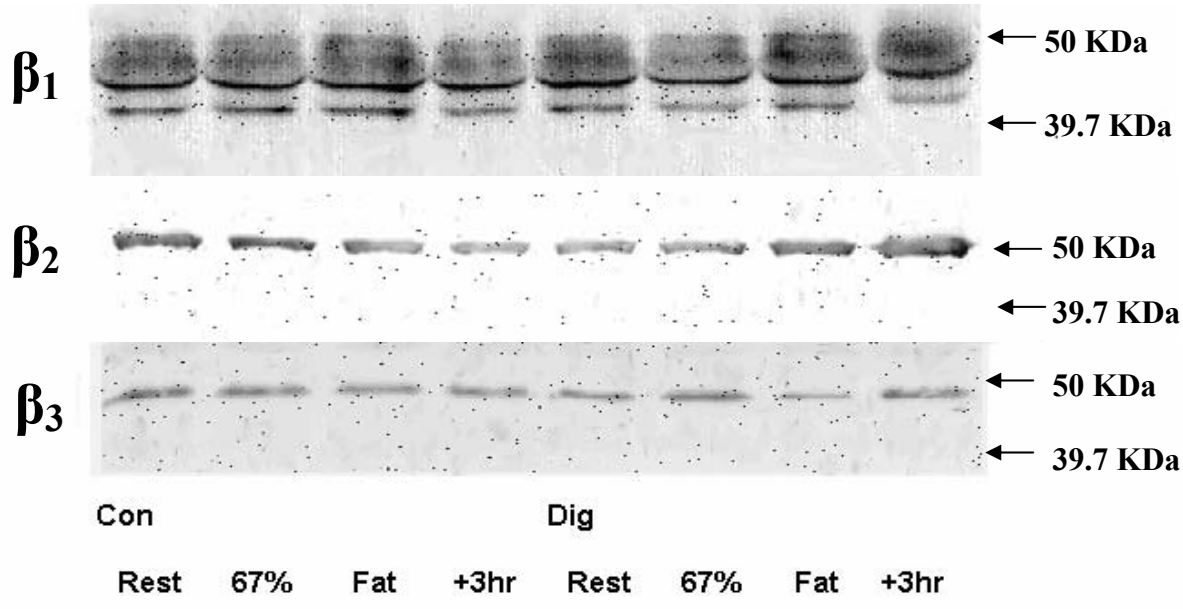

Figure 4.7 Representative immunoblots of Na^+,K^+-ATPase β_1, β_2 and β_3 isoforms in crude muscle homogenates of the human vastus lateralis muscle. Values at right indicate molecular weight of bands.

4.7 Digoxin effects on Na^+,K^+-ATPase mRNA and protein expression in resting muscle

4.7.1 Total α mRNA and total β mRNA expression

Digoxin increased the total α mRNA expression (sum of α_1, α_2 and α_3, $2^{-\Delta\Delta C_T}$) and the total β mRNA expression (sum of β_1, β_2 and β_3, $2^{-\Delta\Delta C_T}$) in resting muscle by 1.9- and 0.6-fold, respectively (P<0.05, Figure 4.8).

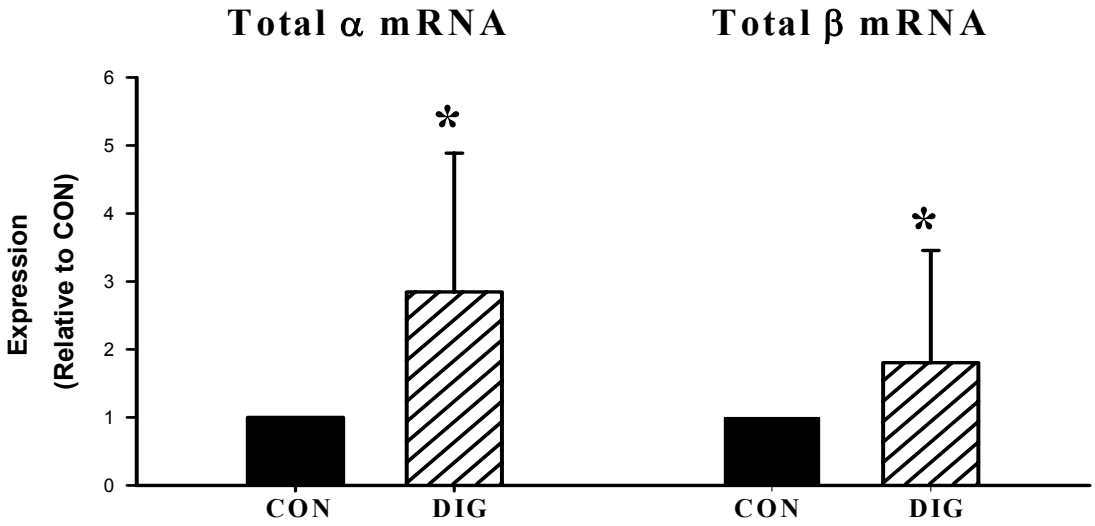

Figure 4.8 Na^+,K^+-ATPase total α (sum of α_1, α_2 and α_3) and total β (sum of β_1, β_2 and β_3) isoform mRNA expression in resting skeletal muscle with digoxin (DIG) and in control (CON).

Data are expressed relative to control resting muscle, means±SEM, n=10.

* DIG > CON, P< 0.05.

4.7.2 α subunit and β subunit protein abundance in resting muscle

Digoxin had no significant effect on α subunit (Figure 4.9) or β subunit protein abundance in resting muscle (Figure 4.10). Related to control resting muscle (1.00±0, Mean±SEM), there was no significant change in α_1 (1.14±0.19, *P*=0.50), α_2 (1.44±0.23, *P*=0.096), α_3 (1.12±0.18, *P*=0.52), β_1 (1.19±0.18, *P*=0.32), β_2 (1.86±0.57, *P*=0.17), β_3 (0.85±0.17, *P*=0.39) protein abundance with digoxin. A tendency towards an increased α_2 protein abundance with digoxin was evident (*P*=0.096). Whilst there was a suggestion of a tendency to an increased β_2 protein abundance (*P*=0.17), there was considerable inter-subject variability. The statistical power was insufficient to detect a change in alpha2 and thus an increased sample size may have been beneficial. Statistical power limitations were already discussed in the discussion on page 67-68.

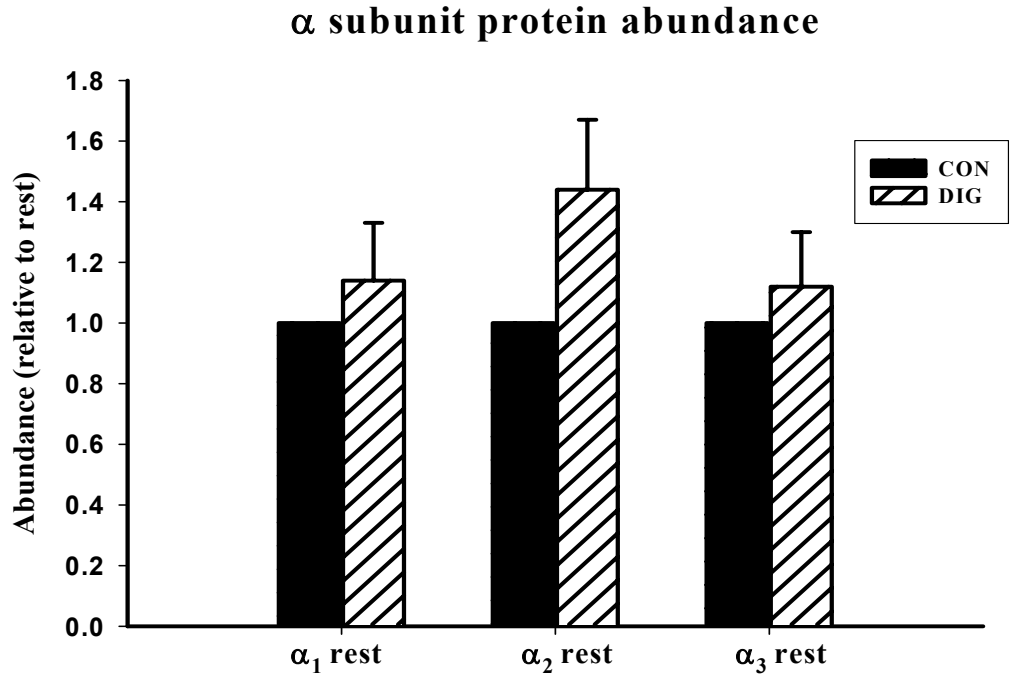

Figure 4.9 Na^+,K^+-ATPase α subunit isoform (α_1, α_2 and α_3) protein abundance in resting muscle with digoxin (DIG) or control (CON).

Data are expressed relative to control resting muscle, means±SEM, n=10.

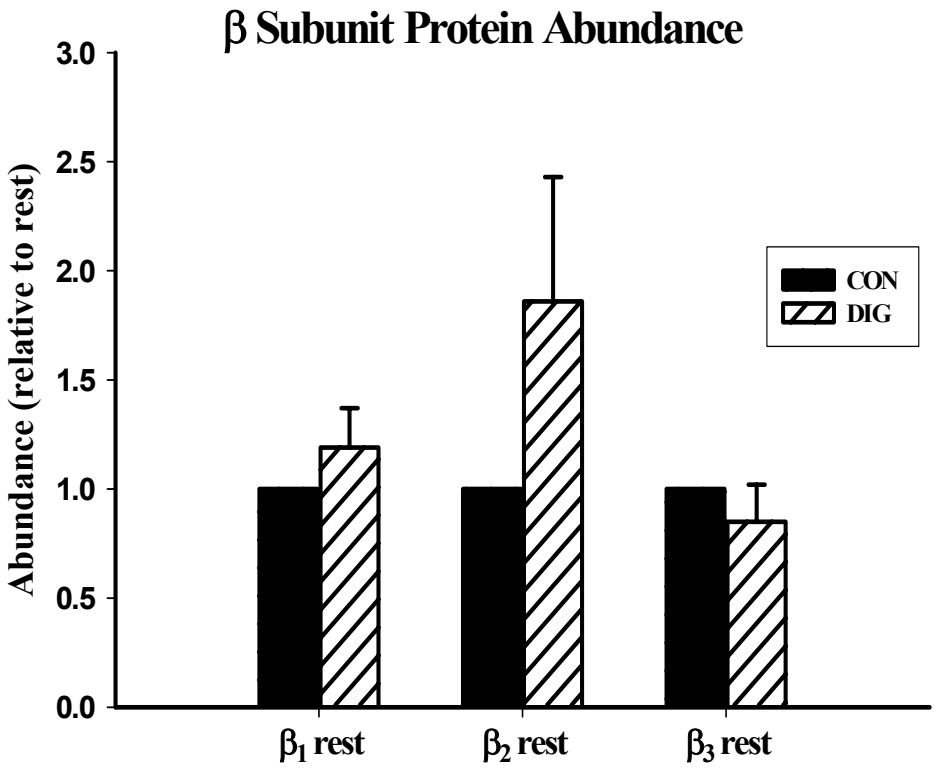

Figure 4.10 Na^+,K^+-ATPase β subunit isoform (β_1, β_2 , β_3) protein abundance in resting muscle with digoxin (DIG) or control (CON).

Data are expressed relative to control resting muscle, means±SEM, n=10.

4.8 Exercise and digoxin effects on Na^+,K^+-ATPase mRNA and protein expression

4.8.1 α_1 isoform

mRNA expression

There were no significant effects of either exercise or digoxin, and no significant treatment-by-time interaction effects for α_1 mRNA expression (Figure 4.11A).

Protein abundance

Similarly, there were no significant effects of either exercise or digoxin, and no significant treatment-by-time interaction effects for crude muscle homogenate α_1 protein abundance (Figure 4.11B).

4.8.2 α_2 isoform

mRNA expression

Exercise did not significantly alter α_2 mRNA expression (Figure 4.12A). Similarly, no significant change occurred with digoxin for α_2 mRNA expression (Figure 4.12A). However, there was a tendency for a treatment-by-time interaction effect for α_2 mRNA expression (P=0.058, Figure 4.12A).

Protein abundance

Neither exercise nor digoxin had an effect on α_2 protein abundance (Figure 4.12B). However, there was a tendency for a treatment-by-time interaction effect for protein abundance (P=0.074, Figure 4.12B).

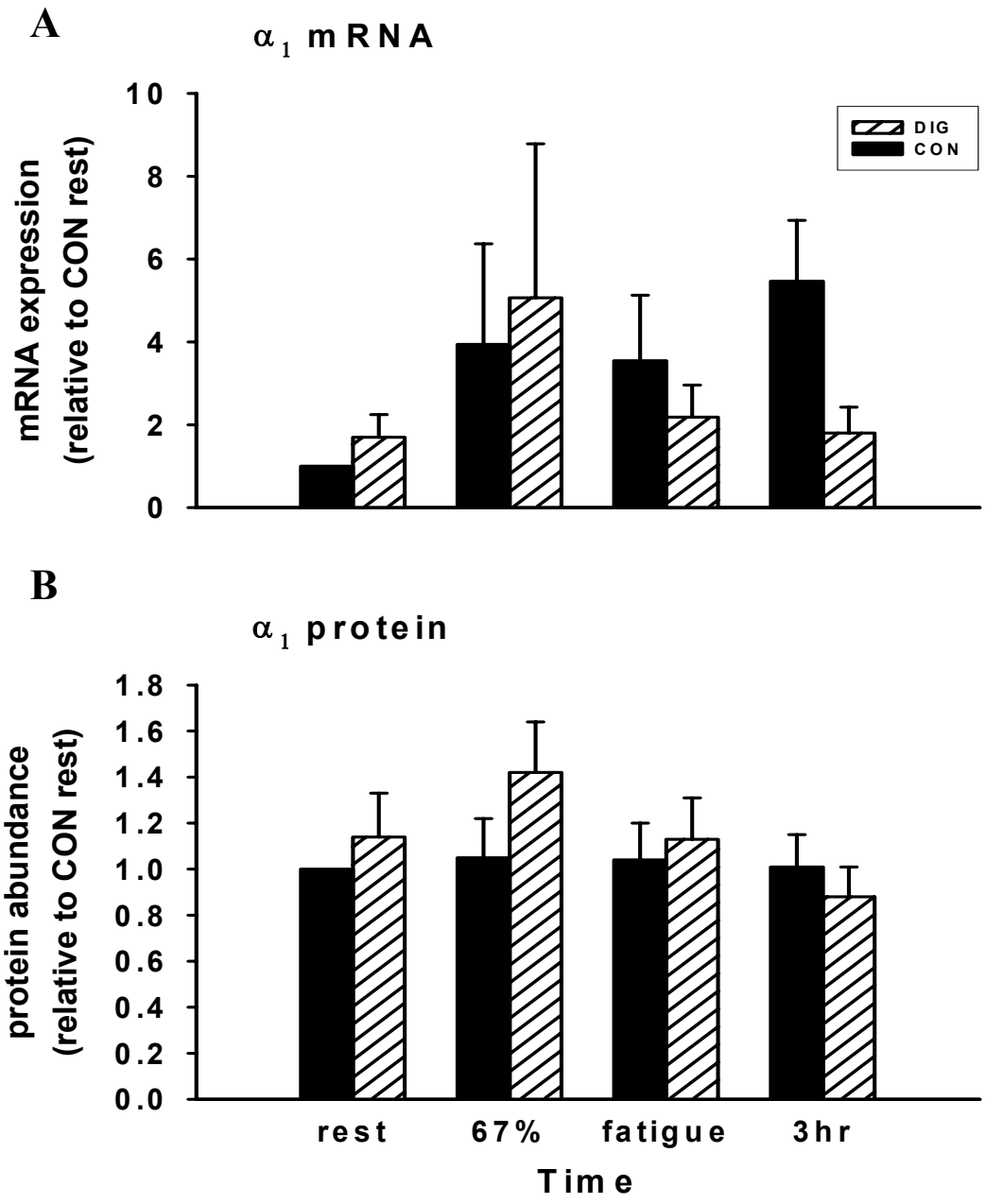

Figure 4.11 Muscle Na^+,K^+-ATPase α_1 mRNA expression and protein abundance before and after cycling exercise.

Samples were collected at rest, after exercise at 67% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and at 3 h post-exercise. All results were normalised against resting CON values. Data are mean±SEM, n=10.

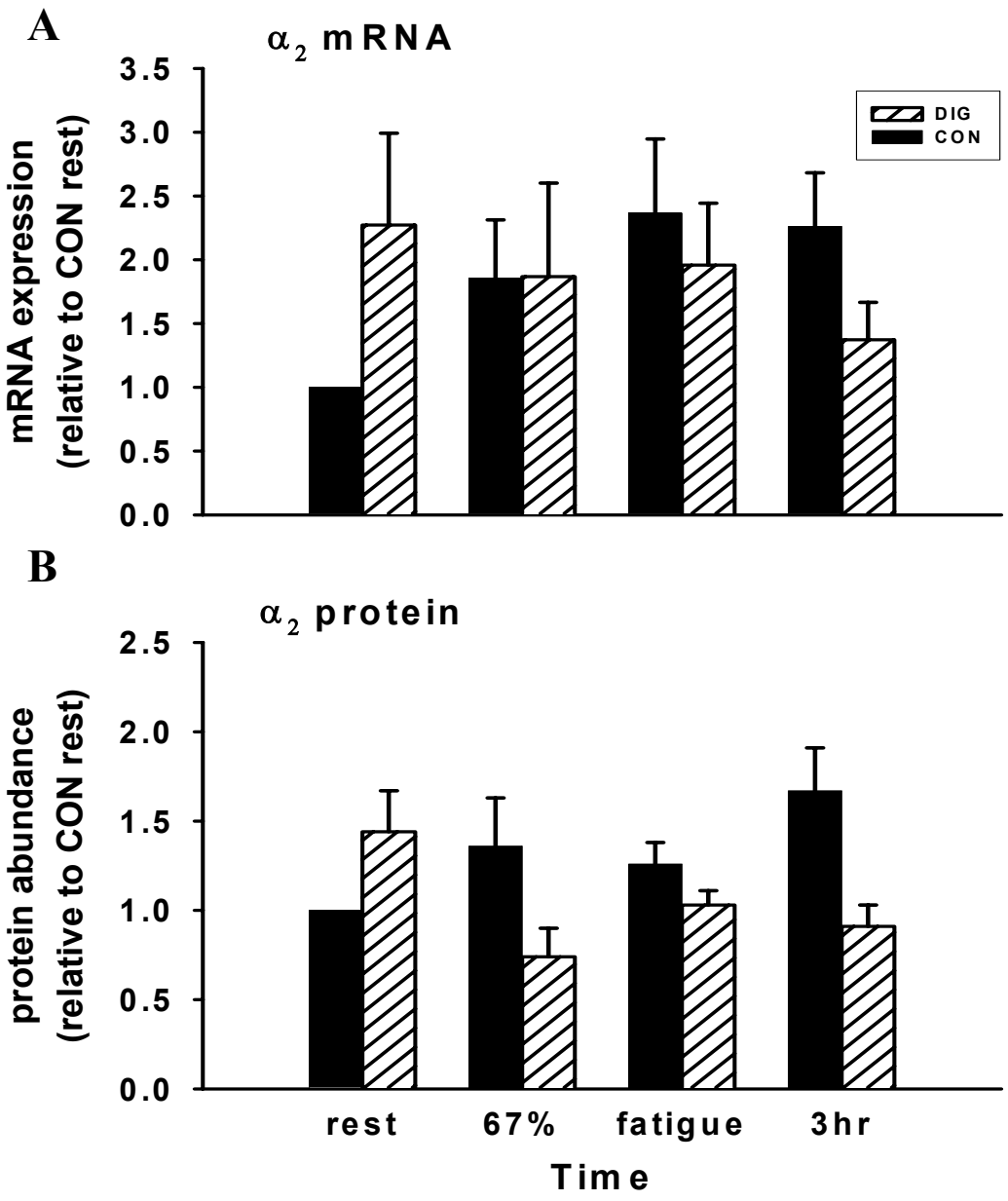

Figure 4.12 Muscle Na^+,K^+-ATPase α_2 mRNA expression and protein abundance before and after cycling exercise.

Samples were collected at rest, after exercise at 67% 90% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and at 3 h post-exercise. All results were normalised against resting CON values. Data are mean±SEM, n=10.

A tendency for a treatment-by-time interaction was observed for α_2 mRNA expression (P= 0.058) and α_2 protein abundance (P= 0.074).

4.8.3 α_3 isoform

mRNA expression

An exercise effect was observed on α_3 mRNA expression, being 2.1-and 2.4-fold higher at 3 h post-exercise than during exercise at 67% $\dot{V}O_{2peak}$ and fatigue, respectively (P<0.05, Figure 4.13A). No significant differences were observed from rest.

There was no significant effect of digoxin on α_3 mRNA expression. However, there was a tendency for a treatment-by-time interaction effect for α_3 mRNA expression (P= 0.069, Figure 4.13A).

Protein abundance

There were no significant effects of either exercise or digoxin, and no significant treatment-by-time interaction effects for α_3 protein abundance (Figure 4.13B).

4.8.4 β_1 isoform

mRNA expression

Exercise had no significant effect on β_1 mRNA expression (Figure 4.14A).

No difference of β_1 mRNA expression was found between digoxin and control trials (Figure 4.14A). There was no significant treatment-by-time interaction effect for β_1 mRNA expression (Figure 4.14A).

Protein abundance

Exercise elevated β_1 protein abundance at 3 h post-exercise by 2.2- and 1.5-fold compared to during exercise at 67% $\dot{V}O_{2peak}$ and fatigue, respectively (P<0.05, Figure 4.14B). However, digoxin had no effect on β_1 protein abundance. Similarly, there was no significant treatment-by-time interaction effect for protein abundance (Figure 4.14B).

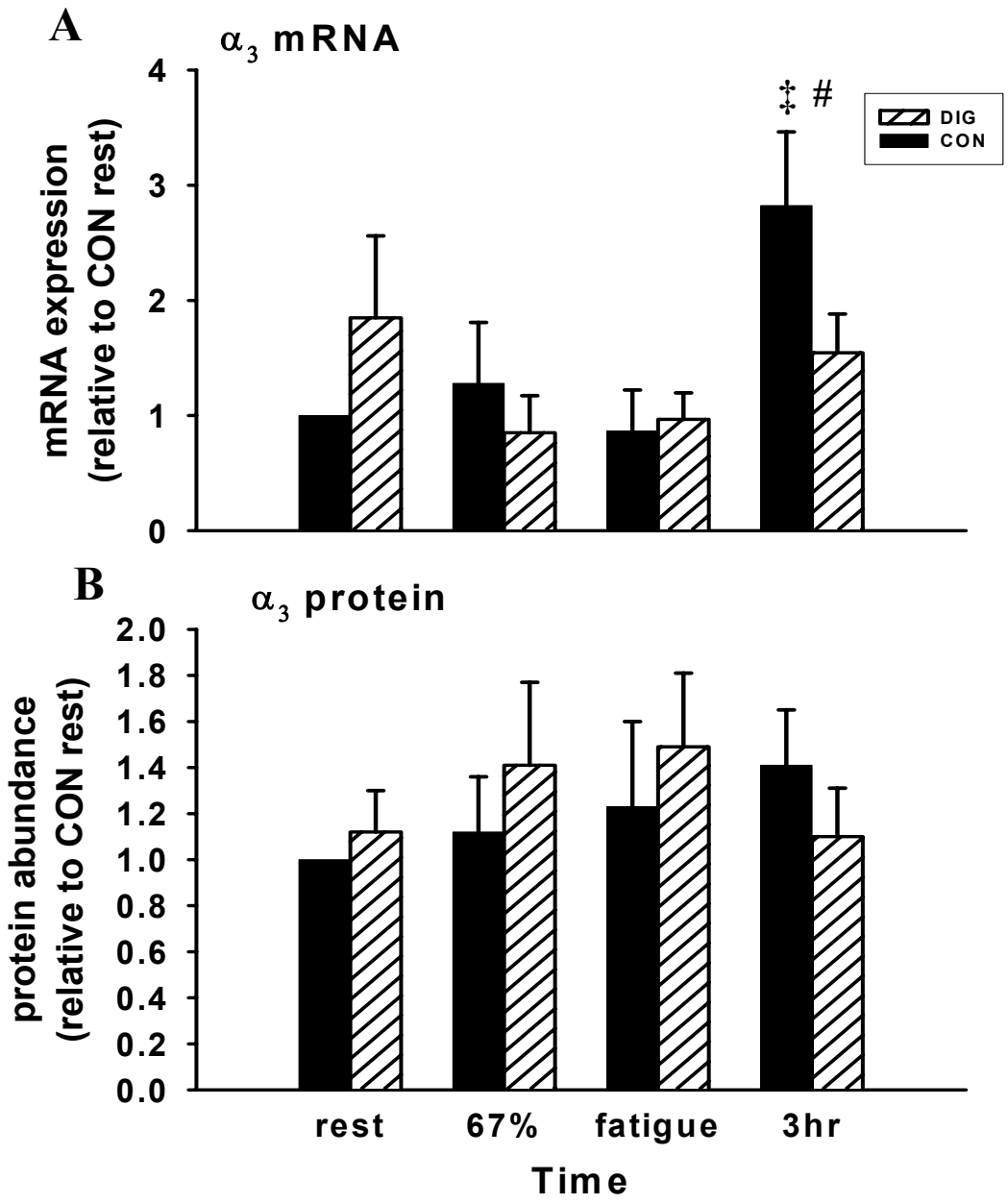

Figure 4.13 Muscle Na^+,K^+-ATPase α_3 mRNA expression and protein abundance before and after leg cycling exercise.

Samples were collected at rest, after exercise at 67% 90% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and 3 h post-exercise. All results were normalised against resting values. Data are mean±SEM, n=10.

‡ $P<0.05$ greater than $\dot{V}O_{2peak}$ 67%,

# $P<0.05$ greater than fatigue.

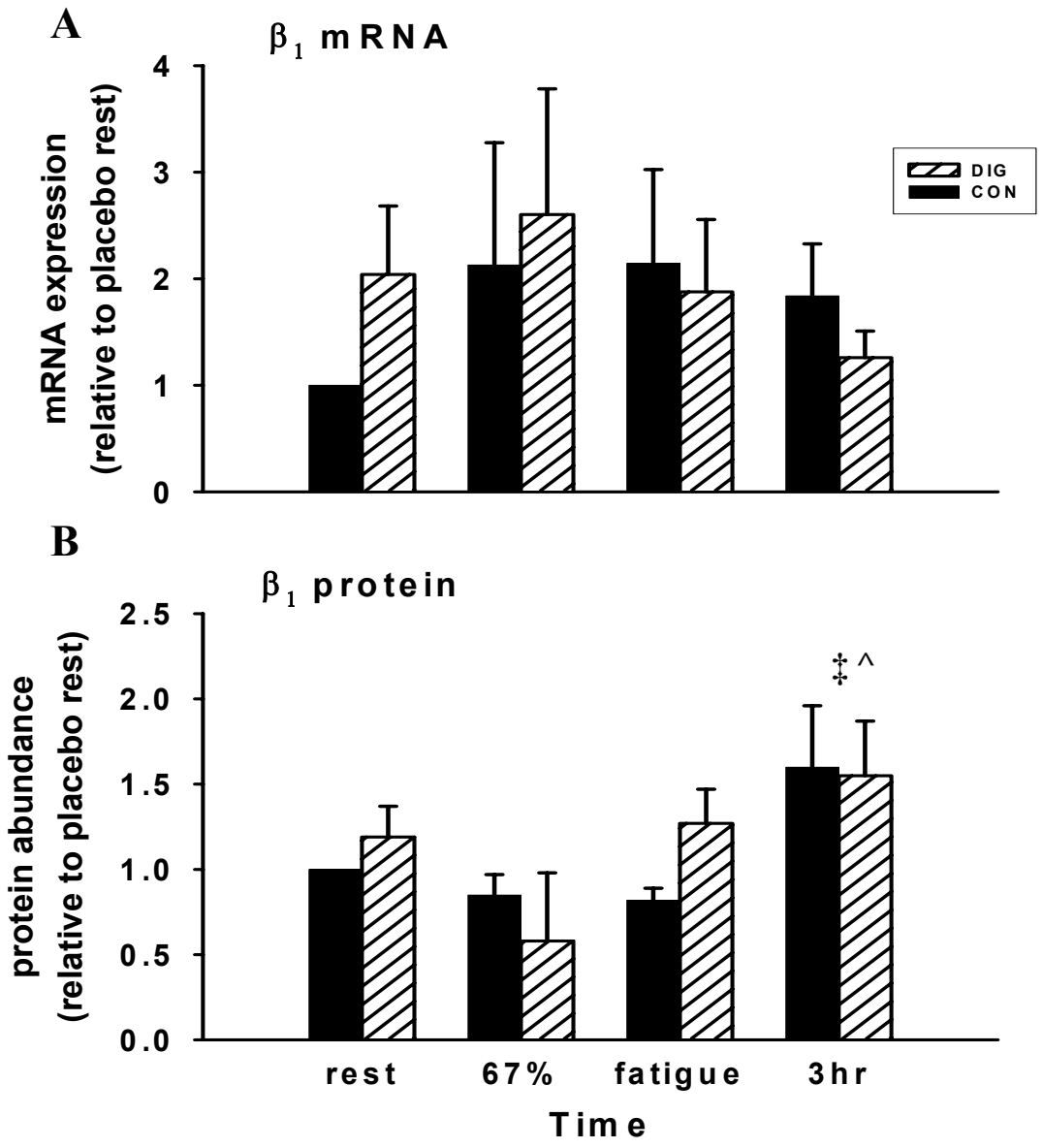

Figure 4.14 Muscle Na^+,K^+-ATPase β_1 mRNA and protein expression before and after invasive leg cycling exercise.

Samples were collected at rest, after exercise at 67% 90% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and at 3 h post-exercise. All results were normalised against resting CON values. Data are mean±SEM, n=10.

‡ $P< 0.05$ greater than 67% $\dot{V}O_{2peak}$.

^ $P=0.06$ greater than fatigue.

4.8.5 β_2 isoform

mRNA expression

There were no significant effects of exercise or digoxin on β_2 mRNA expression (Figure 4.15A). Similarly, there was no significant treatment-by-time interaction effect for β_2 mRNA expression (Figure 4.15A).

Protein abundance

Neither exercise nor digoxin had a significant effect on β_2 protein abundance (Figure 4.15B). There was no significant treatment-by-time interaction effect for β_2 protein abundance (Figure 4.15B).

4.8.6 β_3 isoform

mRNA expression

Exercise increased β_3 mRNA expression at 3 h post-exercise by 1.8-, 1.4- and 1.6-fold, compared to rest, 67% $\dot{V}O_{2peak}$ exercise and fatigue, respectively (P<0.05, Figure 4.16A).

However, digoxin didn't significantly alter either β_3 mRNA expression, whilst no treatment-by-time interaction effect was found for mRNA expression (Figure 4.16A).

Protein abundance

Similarly, β_3 protein abundance was increased at 67% $\dot{V}O_{2peak}$ and 3 h post-exercise compared to rest, by 1.5-and 1.6-fold, respectively (P<0.05, Figure 4.16B).

Digoxin had no significant effects on β3 protein abundance (Figure 4.16B). However, there was a tendency for a treatment-by-time interaction effect for β_3 protein abundance (P=0.063, Figure 4.16B).

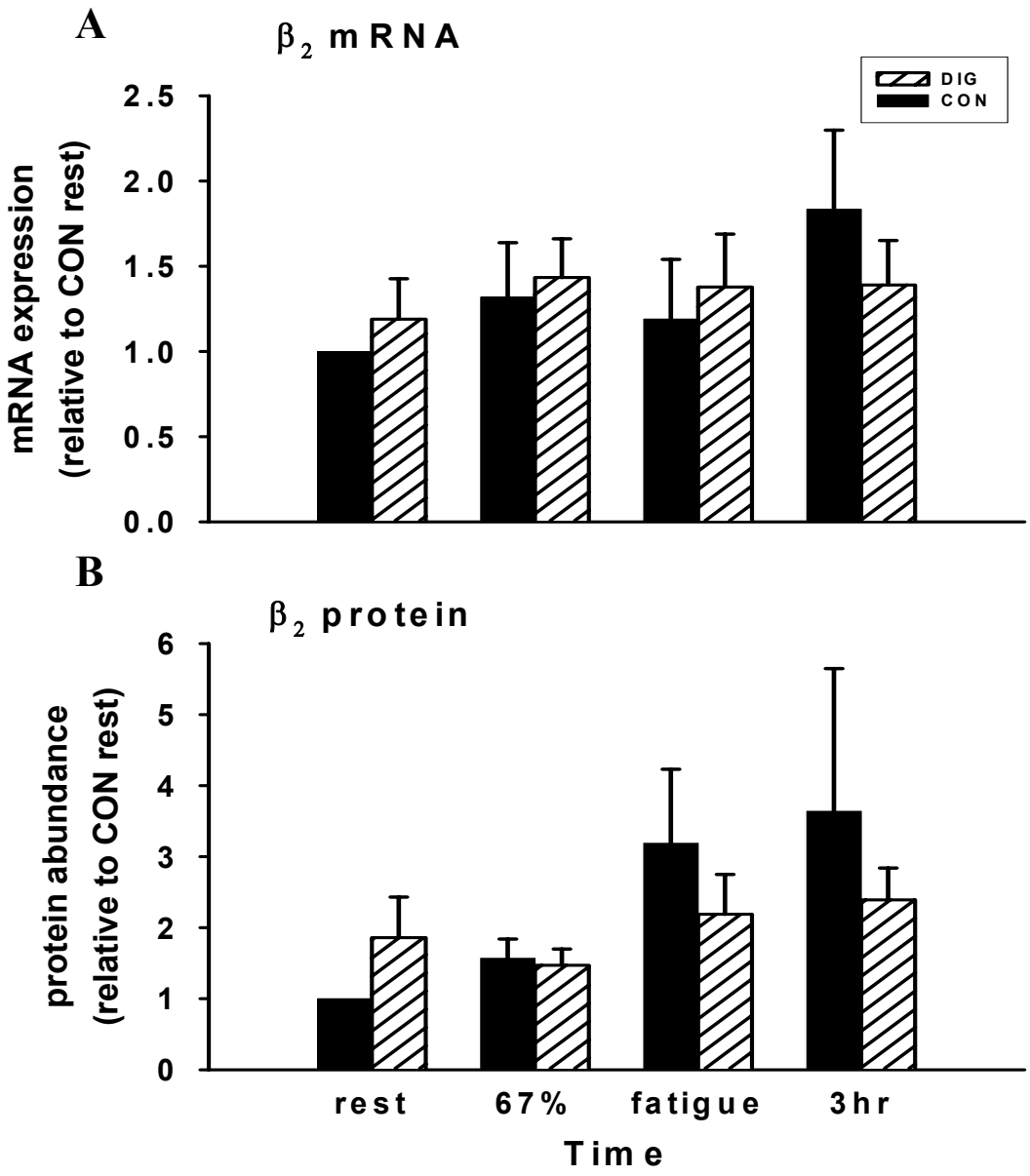

Figure 4.15 Muscle Na^+,K^+-ATPase β_2 mRNA expression and protein abundance before and after cycling exercise.

Samples were collected at rest, after exercise at 67% 90% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and at 3 h post-exercise. All results were normalised against resting CON values. Data are mean±SEM, n=10.

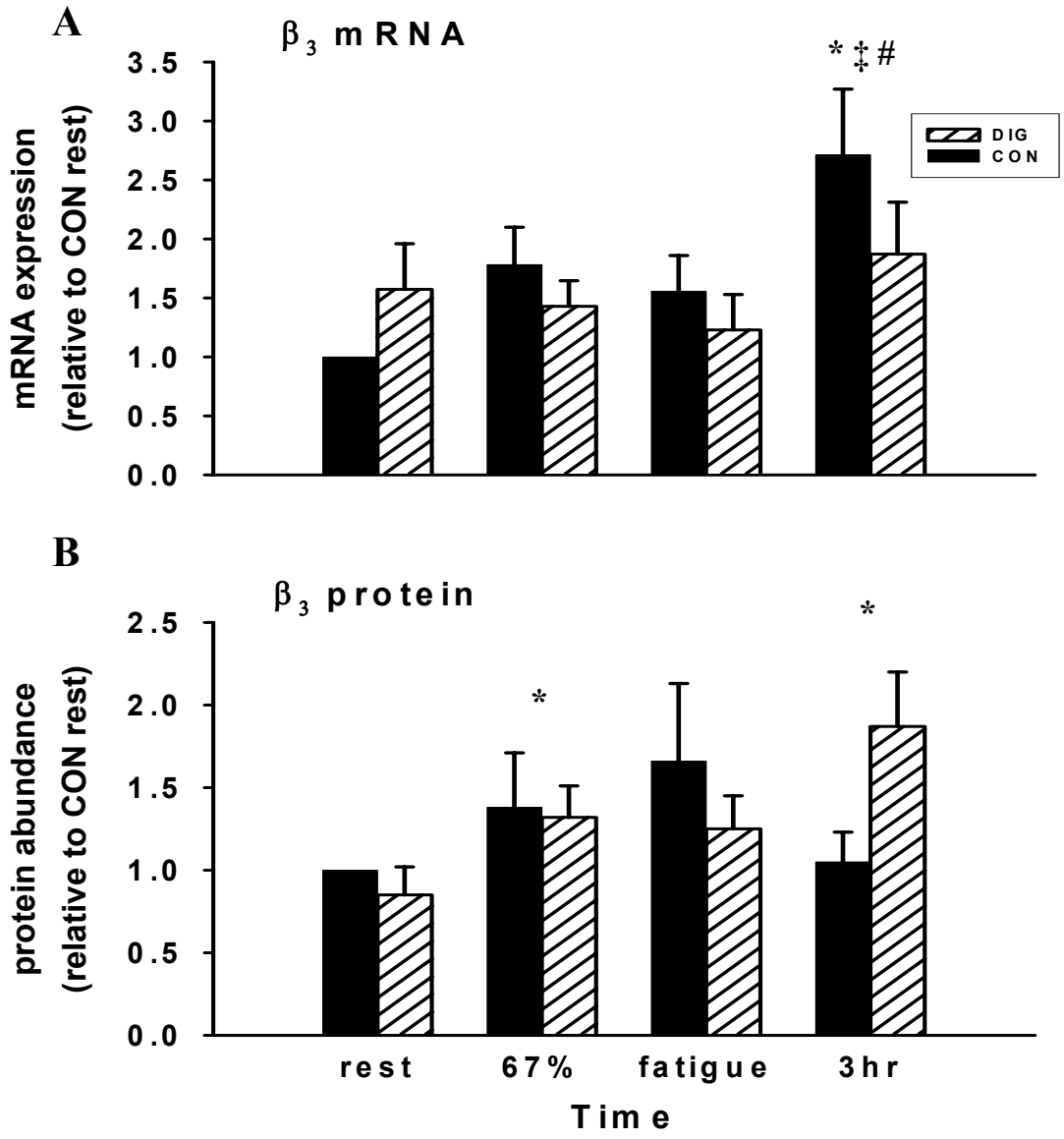

Figure 4.16 Muscle Na^+,K^+-ATPase β_3 mRNA and protein expression before and after invasive leg cycling exercise.

Samples were collected at rest, after exercise at 67% 90% $\dot{V}O_{2peak}$, immediately following 90% $\dot{V}O_{2peak}$ continued fatigue and at 3 h post-exercise. All results were normalised against resting CON values. Data are mean±SEM, n=10.

* $P<0.05$ greater than rest,

‡ $P<0.05$ greater than $\dot{V}O_{2peak}$ 67%,

# $P<0.05$ greater than fatigue.

Chapter 5. Discussion

This thesis investigated the effects of digoxin and exercise on Na^+,K^+-ATPase isoform mRNA and protein expression in human skeletal muscle, with four main findings. The first main finding was that digoxin increased the total α mRNA expression (sum of α_1, α_2 and α_3 mRNA) and the total β mRNA expression (sum of β_1, β_2 and β_3 mRNA) in resting muscle. Second, whilst no significant change with digoxin was detected in protein abundance of any isoform in resting muscle, a tendency towards an increase in α_2 protein abundance was also observed (P=0.096). Third, an exercise main effect for mRNA expression was observed only for α_3 and β_3 isoforms, whilst exercise elevated both β_1 and β_3 isoform protein abundance. The fourth main finding was that the functional measures of quadriceps muscle strength, muscle fatiguability and arterial plasma [K^+] were each unchanged by digoxin, despite the fact that the serum digoxin was elevated to low therapeutic levels.

5.1 Serum digoxin concentration

Digoxin has been used to treat congestive heart failure (CHF) for more than two centuries. Recent findings indicate that the maintenance of serum digoxin concentration (SDC) at the lower end of the clinical reference range, i.e., between 0.5 and 0.8 ng/ml, may reduce mortality rates as well as improve clinical symptoms (Wang and Song, 2005). Another study demonstrated that SDC higher than 1.2 ng/ml was associated with increased mortality and suggested that the effectiveness of digoxin therapy in men with heart failure and a left ventricular ejection fraction of 45% or less may be optimized in the SDC range of 0.5 to 0.8 ng/ml (Rathore *et al.*, 2003). Ahmed *et al.* confirmed that digoxin at SDC 0.5–0.9 ng/ml reduced mortality and hospitalisations in all heart failure patients, including those with

preserved systolic function (Ahmed *et al.*, 2006). Hence, the current preference of the therapeutic digoxin dose is low and between 0.5-0.9 ng/ml. The conversion for digoxin (MW = 780.95 g/mol) from nM to ng/ml units is 1 nM ≈ 0.781 ng/ml. Thus this therapeutic range of approximately 0.5–0.9 ng/ml is equivalent to ≈ 0.64 - 1.15 nM.

A previous study in healthy young adults reported a serum digoxin concentration of 1.0 nM after taking oral digoxin at 0.5 mg per day for 2 weeks (Sundqvist *et al.*, 1983). In the present study, the serum digoxin concentration after taking 0.25 mg per day was 0.7 nM at d 13 and 0.8 nM at d 14, equivalent to ~0.54 and 0.62 ng/ml, respectively. Therefore, after taking digoxin for 2 weeks the serum digoxin concentration reached the low end of the proposed therapeutic level, consistent with current clinical requirements. Furthermore, SDC was also reasonably steady on the two successive test days, making comparisons between results possible.

5.2 Digoxin had no effects on arterial plasma [K^+].

Digoxin, by binding to the Na^+,K^+-ATPase, inhibits Na^+/K^+ transport and has marked adverse effects on K^+ homeostasis. Thus, a digitalis-induced inhibition of Na^+,K^+-ATPase activity would be expected to result in elevated plasma [K^+]. However, in the present study, no significant differences were found in plasma [K^+] between digoxin and control groups. With digoxin, the serum [K^+] increased by 0.19 mmol.l^{-1} during rest in one study (Edner *et al.*, 1993) but was unchanged here (CON 3.95±0.04 vs DIG 3.92±0.04 mM). Few studies have investigated the effects of digoxin treatment on plasma [K^+] during exercise. One study showed that exercise at 2 nM serum digoxin was associated with up to a 20% rise in plasma [K^+] during exercise (Norgaard *et al.*, 1991). Another also reported that plasma [K^+] was increased during exercise with digoxin treatment in CHF patients (Schmidt *et al.*, 1995). However, another study reported no change with digoxin for serum [K^+] (Ericsson *et al.*, 1981). The reason for the lack of effect of digoxin on plasma [K^+] at rest or during

exercise in this study is not clear. This might relate to the low SDC observed. It seems likely that the combination of low serum digoxin concentration achieved in these healthy subjects with a large muscle mass, explains the lack of effect of digoxin on potassium concentration, in comparison to earlier studies in heart failure patients (Norgaard *et al.*, 1991, Schmidt *et al.*, 1995, Ericsson *et al.*, 1981). The only study investigating digoxin in healthy controls that showed an increased potassium concentration utilised a much higher dose(0.05 mg/d) than the present study(0.25 mg/d) (Edner *et al.*, 1993). Alternately, this result, together with suggested changes in mRNA and protein data discussed below, seems to implicate a possible increase in Na^+, K^+-ATPase synthesis with digoxin.

5.3 Digoxin had no adverse functional effects on exercise performance and $\dot{V}O_2$ peak.

This study investigated the possible adverse effects of digoxin on muscle strength and fatiguability during isokinetic contractions and on time to fatigue, and oxygen uptake during incremental exercise. It was hypothesised that oral digoxin administration would reduce quadriceps muscle strength, increase fatiguability and consequently also impair cycling exercise performance. No differences were found between digoxin and control trials in muscle strength, or in the fatigue index during repeated leg extensor contractions. These results confirm that digoxin did not significantly change isokinetic muscle strength in healthy people (Sundqvist *et al.*, 1983). No significant effect of digoxin on muscle isometric endurance was also reported in an early study with only four subjects (Bruce *et al.*, 1968). Alternately, others have shown that serum cardiac glycosides can affect muscle strength and fatiguability, finding that local intra-arterial injection of ouabain, increased skeletal muscle strength (Smulyan and Eich, 1976). They reported however, that skeletal muscle was less sensitive than cardiac muscle to ouabain and that systemic digitalisation had only a minor effect on skeletal muscle (Smulyan and Eich, 1976). Therefore these

findings confirm that digoxin at low therapeutic concentrations did not impair muscle strength or fatiguability in healthy young adults.

There were also no differences in $\dot{V}O_2$ or time to fatigue during incremental exercise between digoxin and control groups. Several previous studies showed that digoxin had no effect on $\dot{V}O_2$peak in heart failure patients (Tanabe *et al.*, 1994, Matsuda *et al.*, 1991a), or in healthy men (Sundqvist *et al.*, 1983). Another study also reported that digoxin didn't change $\dot{V}O_2$ during exercise in patients with chronic isolated atrial fibrillation, which was thought to have been accomplished by an increased oxygen pulse (Matsuda *et al.*, 1991a). However, another study showed that $\dot{V}O_2$ was increased by digoxin therapy (Sullivan *et al.*, 1989). They suggested that this finding was most likely due to an improved matching of ventilation to perfusion.

5.4 Resting muscle: digoxin increased Na^+,K^+-ATPase total α and β mRNA expression.

This is the first study to investigate digoxin effects on Na^+,K^+-ATPase isoform mRNA expression in mammalian skeletal muscle. This thesis hypothesised that oral digoxin administration in healthy, non-trained humans would increase Na^+,K^+-ATPase isoform mRNA expression in resting human skeletal muscle. There was no significant change by digoxin on the mRNA expression of each individual isoform. However, digoxin increased the total α mRNA expression (sum of α_1, α_2 and α_3 mRNA), measured by addition of the $2^{-\Delta\Delta C_T}$ value, and the total β mRNA expression (sum of β_1, β_2 and β_3), suggesting an activation of Na^+,K^+-ATPase isoform gene expression with digoxin.

There is considerable variability in the Na^+,K^+-ATPase mRNA responses to cardiac glycosides in other tissues and species. In rat myocardium, digoxin stimulated expression of the α_3 isoform at both mRNA and protein levels, whereas α_2 was unchanged between

control and digoxin groups (Wang *et al.*, 2000). Similarly, in neonatal rat cardiac myocytes, non-toxic concentrations of ouabain increased β_1 mRNA expression, but decreased α_3 mRNA expression (Kometiani *et al.*, 2000). Partial inhibition of Na^+,K^+-ATPase was found to activate multiple signalling pathways that regulate growth-related genes, in a gene-specific manner (Kometiani *et al.*, 2000). When neonatal rat myocytes were exposed to 5-100 μM ouabain, α_3 mRNA expression was decreased in a dose- and time-dependent manner, whereas α_1 mRNA was not affected (Huang *et al.*, 1997). Exposure of rat cardiocytes to 1 mM ouabain resulted in a three- to fourfold increase in α_1, α_2 and α_3 mRNA expression, and also an approximate two-fold increase in β_1 mRNA expression (Yamamoto *et al.*, 1993). Non-toxic concentrations of ouabain (100 μM) caused the repression of the gene of the α_3 subunit of Na^+,K^+-ATPase in ratcardiac myocytes (Xie *et al.*, 1999, Kometiani *et al.*, 1998). Like ouabain, digoxin also inhibits the Na^+,K^+-ATPase activity (Lichtstein, 1995). On the basis of these animal experiments it is therefore conceivable that oral digoxin therapy could regulate Na^+,K^+-ATPase α and β subunit isoform expression various tissues including in skeletal muscle. The results of the current study strongly suggest that digoxin regulates Na^+,K^+-ATPase isoform expression of both α and β subunits.

5.5 Resting muscle: digoxin effects on muscle Na^+,K^+-ATPase isoform protein abundance.

This study demonstrated that digoxin had no significant effect on the protein abundance of any of the Na^+,K^+-ATPase isoforms present in human skeletal muscle. In resting muscle, relative protein abundance was not significantly increased by digoxin for α_1, α_2, α_3, β_1, β_2, or β_3 isoforms compared to control, although a tendency was observed for an increase in α_2 protein abundance in resting muscle with digoxin (P=0.096). The α_2 isoform is the

dominant α-subunit (~75-80%) in skeletal muscle (Lingrel, 1992, Sweadner, 1989). The α_2-subunit is mainly located in the surface membrane and in the T-tubules but was also present at other undefined intracellular sites (Clausen, 2003, Hundal *et al.*, 1992, Hundal *et al.*, 1993, Hundal *et al.*, 1994). Thus a tendency to an increased α_2 protein with digoxin is consistent with a possible increase in the total Na^+,K^+-ATPase content in muscle. It was not appropriate to sum the protein responses (i.e. sum of α_1, α_2 and α_3) determined by Western Blotting, as the data for each isoform were obtained using different antibodies, each with different affinities for binding.

This study therefore found that digoxin increased in resting muscle the total α mRNA expression (sum of α_1, α_2 and α_3), the total β mRNA expression (sum of β_1, β_2 and β_3) and a tendency was observed for an increase in α_2 protein abundance. A tendency for digoxin-by-exercise interaction effects were also observed for Na^+,K^+-ATPase α_2 isoform for both mRNA expression and protein abundance. Our laboratory has also separately investigated the effects of digoxin on Na^+,K^+-ATPase total content ([^{3}H]-ouabain binding sites) and maximal in-vitro activity (3-O-MFPase activity). Surprisingly, Na^+,K^+-ATPase content was unchanged by digoxin in resting muscle (Digoxin 373±95 vs Placebo 368±75 pmol.g wet weight^{-1}). Similarly, there was no significant change in maximal 3-O-MFPase activity in resting muscle with digoxin (Digoxin 305.5±18.7 vs Placebo 298.1±22.3 nmol.m^{-1}.g^{-1} wet weight) (Petersen A. *et al.*, unpublished results, Appendix). In CHF patients, digoxin therapy did not result in a compensatory upregulation of Na^+,K^+-ATPase in either the myocardium (Schmidt and Kjeldsen, 1991) or in skeletal muscle (Schmidt *et al.*, 1993a). About 50% of the total amount of digoxin in the body is bound to skeletal muscle and only 3-4% to the heart muscle (Steiness, 1978). In CHF patients, the Na^+,K^+-ATPase occupancy with digoxin in myocardium was 34% (Schmidt and Kjeldsen, 1991) and in skeletal muscle was 13% (Schmidt *et al.*, 1993a) and up to 35% (Green *et al.*, 2001). One interpretation of

unchanged [^{3}H]-ouabain binding and 3-O-MFPase activity in this study is that SDC were insufficient to bind enough Na^+,K^+-ATPase to modify these, but this seems unlikely. An alternate explanation is that digoxin induced a compensatory Na^+,K^+-ATPase upregulation. This suggestion is consistent with the increased total α and total β mRNA expression and tendency to elevated α_2 protein abundance. This would mean that a small fraction of pumps was blocked by digoxin and was therefore undetected by [^{3}H]-ouabain binding and 3-O-MFPase measures. In skeletal muscle, digoxin binds to and blocks ~13% of Na^+,K^+-ATPase in chronic heart failure patients (Schmidt *et al.*, 1993a). However, the fractional occupancy is yet to be determined in this study.

5.6 Exercise and digoxin effects on Na^+,K^+-ATPase isoform mRNA expression in human skeletal muscle.

Exercise.

This study investigated acute exercise effects on mRNA expression of all of the six Na^+,K^+-ATPase isoforms expressed in human skeletal muscle. The hypothesis tested was that acute exhaustive exercise would increase Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoform mRNA expression following exercise in healthy, non-trained humans. This was based on an earlier observation that brief fatiguing knee extensor exercise increased each of the Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoform mRNA expression (Murphy *et al.*, 2004, Petersen *et al.*, 2005). In this study, and contrary to expectations, an isoform-specific main effect was observed only for α_3 and β_3 mRNA expression, with each increased only at 3 h post-exercise. In contrast to earlier studies, none of the α_1, α_2, β_1 or β_2 isoform mRNA were increased. There are discrepancies in the literature. A single bout of prolonged submaximal exercise to exhaustion increased α_1, α_3 and β_2 mRNA expression, but not α_2, β_1 or β_3 mRNA (Murphy *et al.*, 2006b). Another human study reported that high-intensity

intermittent one-legged knee extensor exercise increased Na^+,K^+-ATPase α_1 mRNA expression, with no effect of exercise on α_2 and β_1 isoforms; α_3, β_2 and β_3 gene transcripts were not probed (Nordsborg *et al.*, 2003a). The current study differs from consistent findings of these three previous studies that exercise elevated the Na^+,K^+-ATPase α_1 and β_2 mRNA expression (Murphy *et al.*, 2006b, Murphy *et al.*, 2004, Nordsborg *et al.*, 2003a). In rats, 1 h of treadmill running exercise increased the α_1 mRNA in red-type I muscle and the β_2 mRNA in white-type IIb muscles, but the α_2 and β_1 mRNA levels were unaffected by exercise (Tsakiridis *et al.*, 1996). Neither α_3 nor β_3 mRNA expression were detected in that study (Tsakiridis *et al.*, 1996). Recently it was also shown that electrical stimulation of rat EDL muscles induced increased mRNA of only the α_1, α_2 and α_3 isoforms (Murphy *et al.*, 2006a). Collectively these studies indicate that exercise can induce upregulation of Na^+,K^+-ATPase mRNA of some isoforms, although the responses are clearly quite variable. However, after incremental exercise comprising 10 min at 33%, 10 min at 67%, to fatigue at 90% $\dot{V}O_2$ peak, only α_3 and β_3 mRNA transcriptions were upregulated.

Digoxin.

In the present study, it was hypothesised that oral digoxin administration for 14 d in healthy, non-trained adults would increase Na^+,K^+-ATPase isoform mRNA expression. An important finding was that the total α (sum of α_1, α_2 and α_3) and total β (sum of β_1, β_2 and β_3) mRNA expression in resting muscle was increased by digoxin. This points strongly towards an effect of digoxin on Na^+,K^+-ATPase gene transcription. However, it is important to acknowledge that there was no significant effect of digoxin on Na^+,K^+-ATPase isoform mRNA expression, at any time, including rest, during or after exercise at 67% and 90% $\dot{V}O_{2peak}$ or at 3h in recovery after exercise. It is likely that the number of subjects was insufficient to detect exercise induced changes, in the context of considerable

inter-subject and intra-subject variability in mRNA measures. Whilst there was no significant digoxin-by-exercise interaction effect for Na^+,K^+-ATPase isoform mRNA expression, a strong tendency was found for an interaction effect for α_2 (P=0.058), and α_3 (P=0.069) and β_1 (P=0.089) mRNA expression.

A recent study investigated the effects of ouabain and digoxin on Na^+,K^+-ATPase α subunit isoform expression in rat myocardium (Wang *et al.*, 2000). They reported that both ouabain and digoxin stimulated increased mRNA expression of the α_3 isoform, whereas α_2 isoform mRNA expression was unchanged. Further, they reported that whilst α_1 isoform mRNA expression was decreased in ouabain, this was unchanged in digoxin (Wang *et al.*, 2000). It was suggested that both ouabain and digoxin could regulate Na^+,K^+-ATPase α-subunit isoform expression, which might be related to the physiological roles of endogenous ouabain and which might be responsible for the difference between the pharmacological and toxicological effects of ouabain and digoxin. Ouabain and digoxin have different effects on expression of Na^+,K^+-ATPase α subunit isoforms in different tissues in rats (Wang *et al.*, 2000). In the kidney, α_1 mRNA expression was increased by digoxin and ouabain, but α_2 and α_3 mRNA remained unchanged. In the adrenal gland, α_2 and α_3 mRNA expression were both increased, with α_1 mRNA decreased by ouabain. Digoxin increased α_1 and α_3 mRNA expression but had no effect on α_2 mRNA expression. In aortic smooth muscle, both ouabain and digoxin increased α_1 and α_3 mRNA expression, but α_2 mRNA expression was decreased by digoxin but unchanged by ouabain. In hypothalamus, both ouabain and digoxin stimulated α_1 mRNA expression, while α_2 and α_3 mRNA levels remained unchanged. These findings suggested that both ouabain and digoxin could regulate sodium pump α mRNA expression, but had different results in different tissues.

5.7 Exercise and digoxin effects on Na^+,K^+-ATPase isoform protein abundance in human skeletal muscle.

Exercise.

This thesis hypothesised that acute exhaustive exercise would not effect Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoform protein abundance, as demonstrated previously with brief, exhaustive exercise (Murphy *et al.*, 2004). However, increases in both β_1 and β_3 isoform protein abundance were detected with an exercise main effect in this study. The protein abundance at 3 h post-exercise for β_1 was 2.2- and 1.5-fold higher than exercise at 67% $\dot{V}O_2$ peak and at fatigue; whilst protein abundance for β_3 at 67% $\dot{V}O_2$ peak and 3 h post-exercise compared to rest was increased by 1.5-and 1.6-fold, respectively. A recent study from this laboratory reported that prolonged submaximal exercise increased α_3 protein abundance, with a tendency (P=0.057) towards an increased β_1 protein abundance also observed (Murphy *et al.*, 2006b). The lack of effect of acute exercise on α_1, α_2 or β_2 protein abundance in crude homogenates in the present study is therefore consistent with the findings in these studies (Murphy *et al.*, 2004, Murphy *et al.*, 2006b). Numerous studies have investigated the effects of muscle contraction on Na^+,K^+-ATPase content by measuring [^{3}H]-ouabain binding. In human muscle, [^{3}H]-ouabain binding site content was not affected by a brief exercise bout (Petersen *et al.*, 2005). In endurance athletes, there was also no effect of acute incremental exercise on muscle [^{3}H]-ouabain binding content (Aughey *et al.*, 2005). Similarly, no significant differences in the [^{3}H]ouabain binding site content were found during prolonged exercise (Leppik *et al.*, 2004). In rats, [^{3}H]-ouabain binding was unaltered by electrically-induced muscle contraction as well (McKenna *et al.*, 2003). These findings demonstrated that exercise has no effect on [^{3}H]-ouabain binding content.

Several studies have also investigated the effects of exercise on protein abundance in isolated membrane fractions derived from skeletal muscle. They reported that intense one-legged knee extensor exercise in humans elevated the sarcolemmal α_2 and β_1 isoform protein abundance by 70% and 26%, respectively; but the α_3, β_2 and β_3 isoforms were not probed (Juel *et al.*, 2000a). The increased β_1 isoform protein abundance with exercise in this study might appear to be consistent with these previous findings (Juel *et al.*, 2000a). However, findings from crude homogenates cannot be compared to findings in isolated membrane fractions, as it is possible that Na^+,K^+-ATPase may be trafficked from one site to another within the muscle, such that changes would be observed in some cellular membrane fractions, but not in whole muscle. These studies performed using isolated membrane preparations exhibit poor recovery of Na^+,K^+-ATPase (Hansen and Clausen, 1988). The small recoveries reported of 0.2 to 8.9% raise the possibility of large potential errors in Na^+,K^+-ATPase interpretations. Thus use of membrane fractionation technique has limitations in investigating exercise effects on Na^+,K^+-ATPase protein abundance. Additionally, these studies used these techniques primarily to investigate Na^+,K^+-ATPase isoform translocation, so should not be cited as evidence for changes in whole muscle protein abundance of Na^+,K^+-ATPase with exercise. In contrast, this thesis used whole muscle homogenates to measure the Na^+,K^+-ATPase total protein abundance, to ensure maximal recovery of Na^+,K^+-ATPase proteins. The finding of the present study is that there was no effect on Na^+,K^+-ATPase isoform protein abundance after exercise at 67% and 90% $\dot{V}O_{2peak}$ and at 3h-post exercise.

Digoxin.

No significant main effects on Na^+,K^+-ATPase isoform protein abundance were observed for digoxin. However, there was a tendency for treatment-by-time interaction effect for α_2 (P=0.074) and β_3 isoform (P=0.063) protein abundance in human skeletal muscle. The

tendency for a treatment-by-time interaction effect for Na^+,K^+-ATPase α_2 isoform protein abundance is potentially important. It is of interest that tendencies were observed at rest for increased in protein abundance with digoxin of α_1, α_2 and α_3 isoforms. These internally consistent findings point towards a impossible upregulatory effect of digoxin on Na^+,K^+-ATPase α subunits. The failure to detect significant changes here most likely is due to the sample size being too small, relative to typical variability of western blotting.

An apparent decrease in α_2 protein abundance was observed (NS) during cycling exercise at 67% $\dot{V}O_{2peak}$ with digoxin. This apparent decline also appeared to persist at fatigue and at 3h-post exercise. This may reflect variability in the method, but might also be influenced by increased digoxin binding in contracting muscle. At rest, in skeletal muscle, digoxin binds to and blocks ~13% of Na^+,K^+-ATPase in humans (Schmidt *et al.*, 1993a). Digoxin binding to muscle is increased during exercise (Joreteg and Jogestrand, 1983), similar to the increased rate of ouabain binding to stimulated vs resting muscle in the rat (McKenna *et al.*, 2003). This increased binding most likely reflects elevated Na^+,K^+-ATPase activity with muscle contraction. It therefore seems probable that exercise increased binding of digoxin to muscles. More studies need to be conducted to clarify this.

5.8 Study limitations

This study was conducted on young healthy adults, due to ethical considerations, particularly reflecting the lesser risks of conducting invasive projects with maximal exercise testing in young adults. Understanding the effects of digoxin in healthy young adults has direct implications for muscle fatigue in young people. Furthermore, this may also have important implications for older patients with chronic heart failure that receive digoxin therapy. Nine male and one female subjects participated in this study, but there is not likely to be a gender difference in Na^+,K^+-ATPase abundance or activity.

The study design used trials conducted under crossover, double-blind, placebo-controlled conditions. Nonetheless, many results did not quite achieve statistical significance, with several strong tendencies noted. Two important limitations are acknowledged, a small sample size (n=10) and measurement variability. The latter was typical for the measures undertaken. The intra-assay coefficient of variation for each target gene was <14.0% for 2^{-C_T}, consistent with our other studies (Murphy *et al.*, 2004, Murphy *et al.*, 2006b). The variability for oxygen consumption during exercise at 33%, 67% and 90% $\dot{V}O_{2peak}$, calculated from preliminary trials was 2.2%, 1.9% and 4.4%, respectively. The variability of muscle strength and fatigue index, calculated from preliminary trials was also modest, at 5.9% and 6.8%, respectively. No reproducibility data was collected for Na^+,K^+-ATPase isoform protein abundance measuring by western blotting. Thus, the reason for lack of significance in the variables is probably not due to excessive measurement variability, but may reflect a low statistical power due to other forms of variability. The lack of statistical significance for the changes in Na^+,K^+-ATPase isoform mRNA expression and also protein abundance with digoxin and exercise, may reflect the considerable inter- and intra-individual variability, which is compounded by the relatively small number of participants (n= 10). The original intention was to perform these studies in a greater number of participants. However, there were significant practical constraints that prevented this. This was a very long study, with two 2 week treatments, a 4 week washout, 4 full days for experimental measurements and a minimum of 3 days for the pre-tests. This study also required an extensive collaboration with researchers from Deakin University, RMIT University, Austin Hospital and Alfred Hospital. Considering all of these, it was very hard to get additional participants, which placed considerable constraints on the investigation. Since this is the first study to have investigated the effects of digoxin and exercise on

skeletal muscle Na^+,K^+-ATPase isoform mRNA expression or protein abundance, it is also not possible to find related studies to confirm these findings.

5.9 Future perspectives

This thesis investigated the effects of digoxin and acute exercise on the Na^+,K^+-ATPase, which is a key protein involved in potassium regulation in human skeletal muscle. It determined whether digoxin affects Na^+,K^+-ATPase isoforms in muscle and whether this adversely affects performance in healthy individuals. The findings in this thesis strongly implicated that digoxin may induce Na^+,K^+-ATPase compensatory upregulation in skeletal muscle in healthy young adults. This possible rise is important for other researchers to continue to investigate.

This study was conducted on healthy young people, due to ethical considerations, but digoxin is a drug prescribed for patients with severe chronic heart failure, especially in older age groups. It is important to investigate possible digoxin effects on exercise performance, Na^+,K^+-ATPase gene and protein expression in CHF patients, under close clinical supervision.

5.10 Conclusions

In conclusion, despite elevation of serum digoxin to low therapeutic levels (~0.8 nM), functional measurements, comprising quadriceps muscle strength and fatiguability peak oxygen uptake and arterial plasma $[K^+]$ were each unchanged by digoxin. These findings confirm that digoxin at this serum concentration was insufficient to impair physical performance, when taken over a 14 day period, in healthy young adults. Furthermore, digoxin treatment had only minor effects on skeletal muscle Na^+,K^+-ATPase isoform mRNA expression in healthy individuals, with no significant change observed for individual isoforms. However, Na^+,K^+-ATPase total mRNA expression for α subunit and β subunit in resting muscle were increased with digoxin, suggesting an effect of digoxin on

activating Na^+,K^+-ATPase gene expression. A tendency of treatment-by-time interaction effect for α_2 isoform mRNA expression (P=0.058) was also observed. Similarly, digoxin treatment had only minor effects on skeletal muscle Na^+,K^+-ATPase isoform protein abundance in healthy individuals. Tendencies towards an increase in α_2 protein abundance by digoxin (P=0.096), and for a digoxin-by-time interaction effect for α_2 isoform protein abundance (P=0.074) were observed. Exercise increased the mRNA expression of the α_3 and β_3 isoforms, and also elevated the protein abundance of β_1 and β_3 isoforms.

Together these suggest a possible compensatory upregulation in muscle Na^+,K^+-ATPase with digoxin and also indicate that acute exercise can modulate both Na^+,K^+-ATPase mRNA expression and protein abundance in these healthy individuals.

Appendices

List of Appendices

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Victoria University of Technology

Sample Consent Form for Participants Involved in Research

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study investigating the effects of high intensity exercise on skeletal muscle fatigue

CERTIFICATION BY PARTICIPANT

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: **"The effects of a standard clinical dose of digoxin on potassium regulation in muscle and blood, and muscle fatigability during exercise in healthy young volunteers"**
being conducted at Victoria University of Technology by:
Associate Professor Michael McKenna
Associate Professor Henry Krum
Mr Simon Sostaric

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by: **Assoc Prof Michael McKenna, Assoc Prof Henry Krum and Simon Sostaric**
and that I freely consent to participation involving the use on me of these procedures.

Procedures:

1. Preliminary participant screening (blood sample plus ECG Analysis)
2. Forearm anthropometry (length and size measurements)
3. Maximal incremental exercise test of the finger flexor muscles
4. Maximal incremental test and submaximal test on a cycle ergometer
5. Blood flow measurements by method of venous occlusion plethysmography
6. Maximal muscle function test on a Cybex isokinetic dynamometer
7. Digoxin and placebo administration under experimental conditions
8. Arterial catheterisation and blood sampling during rest, exercise and recovery
9. Antecubital venous catheterisation and blood sampling during rest, exercise and recovery
10. Muscle biopsies at rest and during maximal incremental exercise

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: .....**

.....}

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PARTICIPANT INFORMATION

Title:

The effects of a standard clinical dose of digoxin on potassium regulation in muscle and blood, and muscle fatigability during exercise in healthy young volunteers

Investigators.

Associate Professor Michael McKenna, Mr Simon Sostaric,
School of Human Movement, Recreation & Performance, Victoria University
Associate Professor Henry Krum
Department of Epidemiology and Preventive Medicine Monash University, Alfred Hospital

Aims of study.

Digoxin is a drug taken by many patients with heart failure, to increase the performance of their heart. We anticipate that it might make limb muscles weaker and more easily fatigued. This project therefore investigates the effects of digoxin on muscle strength, fatigue and the regulation of potassium in muscle and blood during exercise, in healthy individuals. The knowledge gained from this study may have important implications for the clinical use of digoxin.

Participant Involvement and Overview of Testing.

Visit 1. Participants in the study will be asked to attend the Alfred Medical Centre on one occasion for initial screening purposes to ensure that only healthy individuals can enter the study. This will require 30 minutes.

Visits 2 to 9. Participants will then be requested to attend the Human Performance Laboratory at Victoria University of Technology, Footscray Campus (Room L305, building L) on eight separate occasions for exercise testing trials, over approximately 11 weeks. Whilst each test is tiring, you will recover very quickly. Please refrain from eating for 2 hours before all exercise trials. Visits 2 to 5 will be over about 3-4 weeks, will require about 2 hours on each occasion. Visits 6 to 9 will be over about 6 weeks. Visits 6 and 8 will require 45 minutes on each occasion for measurement of your muscle strength and fatigue. Visits 7 and 9 will require approximately 5 hours each and will involve (i) a forearm exercise test with blood sampling, (ii) a 2 hour rest, (iii) followed by a cycling exercise test with blood and muscle sampling. In the 24 hours prior to each of Visits 6 to 9, participants will be asked to avoid any intense exercise and substances such as caffeine, alcohol, or other drugs and to record all exercise, fluid and food intake.

Visit 2 will involve measures of forearm size, an incremental forearm exercise test and an incremental cycling test; and measures of leg maximal strength and endurance. Details of all tests are given in the next section.

Visit 3 will involve familiarisation trials for forearm, cycling, strength and fatigue testing procedures used for later visits.

Visits 4 and 5. The above tests will be repeated twice to determine how much your exercise test results vary between test sessions.
Visits 6 and 8. Participants will be asked to complete the leg muscle strength and fatigue tests on the Cybex. One visit will be after taking the drug digoxin for 12 days, whilst the other visit will be after taking a sugar placebo for 12 days and will be conducted one month apart to ensure digoxin is completely cleared from your body.
Visits 7 and 9. Participants will be asked to complete the forearm test and leg cycle tests two days after the leg muscle strength and fatigue test. These visits will therefore be after taking the drug digoxin or placebo for 14 days and will also be conducted one month apart.

Exercise Testing Procedures:

Safety Procedures.

Each exercise 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. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety. The most common event associated with maximal exercise testing is fainting. This will be prevented using our standard laboratory procedures. In the unlikely event of emergency situations, a medical practitioner will be in attendance, two members of the research team have current CPR (cardio pulmonary resuscitation) qualifications and the Western Hospital is minutes away by ambulance.

Forearm exercise tests.

Participants will be asked to undertake forearm exercise over several laboratory visits. This uses a specially made handgrip device for flexing your fingers against a resistance. On Visit 2 you will be asked to perform an incremental forearm test, in which the workrate is progressively increased until your muscles fatigue. On Visits 3 to 5, 7 and 9 you will be asked to perform a forearm test comprising three one minute bouts of exercise, followed by a fourth bout continued until fatigue. Exercise will be performed at the peak workrate attained during the incremental forearm test. As with all unaccustomed exercise, you may experience some muscle soreness after the first forearm exercise test, but this should disappear within a few days.

Forearm Blood Flow Measures.

During and after the forearm exercise tests, forearm blood flow will be measured. This involves placing a blood pressure cuff at the wrist and above the elbow. The wrist cuff is inflated to a similar pressure as when your blood pressure is measured. This pressure is maintained for one minute. The elbow cuff is repeatedly inflated to ¼ of this pressure, for 2 to 30 seconds. When inflated briefly, these cuffs restrict blood from leaving the forearm and flowing into the hand. There is no undue risk associated with this procedure and should participants experience anything more than discomfort, the pressure in the cuffs will be immediately released.

Cycling Exercise Tests Procedures.

Participants will be asked to undertake cycling exercise tests over several laboratory visits. On Visit 2 you will be asked to perform an incremental cycling test, in which the workrate is progressively increased until your muscles fatigue. This test is used to determine your aerobic fitness, by measurement of the peak oxygen consumption (VO_2 peak). On Visits 3 to 5, 7 and 9 you will be asked to perform a cycling test comprising 10 min exercise at workrates corresponding to 33% and 67% of that attained during the incremental cycling test. This will be followed by cycling to fatigue at 90% of your peak workrate. Visits 7 and 9 will include blood sampling and muscle biopsies. The total volume of blood taken in each

of these two visits will be ~130ml, less than one quarter of that taken in a blood bank donation.

Digoxin Treatment.

Screening Phase

Participants will be requested to undergo a complete medical history and physical examination. A blood sample will be taken to check for normal electrolytes and kidney function. Your resting ECG will also be assessed to ensure that you have normal heart rate and rhythm.

Treatment and Evaluation

Participants will be given either a sugar tablet (a placebo), or standard-dose digoxin (0.25 mg per day) for 2 weeks, followed by 4 weeks without drug or placebo, and then by taking the alternate treatment (drug or placebo) for a further 2 weeks. The 4 week period is to allow your body to rid itself of digoxin. You take the drug or placebo in random order and will not be informed of this order until the completion of the study. After 7, 12 and 14 days of taking digoxin (or placebo), a blood sample will be taken and digoxin levels in the blood measured, to minimise the risk of accidental overdose and ensure the correct procedures have been followed.

Side Effects

Short-term treatment with digoxin using the standard dose of 0.25mg per day is usually free of side effects. Short-term side effects of digoxin are principally associated with overdose, and may include nausea, vomiting, diarrhoea, lack of appetite. These should respond simply to stopping the drug. Occasionally, too much digoxin may also lead to irregularities of the heart's rhythm. Again this usually responds to stopping the drug. It is possible, although unlikely, that digoxin treatment may adversely affect your ability to undertake heavy exercise, but is unlikely to impact on any lower level day-to-day activity. Although very unlikely, should you experience any adverse effects you should immediately contact the Principal Investigators to review the reported problem and recommend appropriate action. Participants will not be charged for any hospital visits, or for any laboratory measurements.

Muscle Biopsies and Muscle Fatigue Testing:

On Visits 7 and 9, a muscle biopsy will be taken from the thigh muscle of participants, at rest, and following each of the three workrates of the cycling test. Thus four biopsies will be taken on each visit, two from each leg, giving an overall total of eight biopsies. Muscle biopsies are routinely carried out in our laboratory, with no serious 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. 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

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 symptoms disappear within a week. A medical practitioner will perform the whole procedure under sterile conditions. 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 to review the reported problems and recommend appropriate act.

Venous catheterisation

Blood samples will be taken during rest, exercise and recovery via a catheter placed in the exercising arm. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle on entering 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 the flow of blood along the tubing can be altered at will. This procedure allows the taking of multiple blood samples without the need for multiple venepuncture (puncturing of the vein). Each time a blood sample is taken, a small volume of fluid will be injected to keep the catheter from clotting. Catheterisation is slightly uncomfortable, with minimal possibility of bruising and infection. The use of sterile, disposable catheters, syringes, single dose vials and aseptic techniques will markedly reduce the possibility of infection. Only staff qualified and experienced in venepuncture will be used in order to prevent complications. Although the possibility of infection, bleeding, local blood clots, local swelling and redness, and bruising are remote, should any one of these conditions eventuate, please inform us immediately and then consult your doctor.

Arterial catheterisation for blood sampling

A similar catheter will be used as above, but will be inserted into the radial artery (wrist) of the non-exercising arm. Arterial puncture and catheterisation is more difficult and may involve more discomfort and bruising formation than with venous punctures. Pain is minimised by use of a local anaesthetic in the skin and near the artery, whilst bleeding and bruising are minimised through use of appropriate pressure techniques for an adequate amount of time after arterial puncture or removal of the catheter. Infection is unlikely as only sterile, unused disposable instruments; single dose vials and aseptic techniques will be used. An experienced medical practitioner, who will remain throughout the entire testing and recovery procedures, will perform all arterial catheterisations.

Timing of blood sampling

During the forearm exercise tests, blood samples will be taken from an artery and a vein before and at the end of each exercise bout, with recovery samples at 1, 2, 5, 10 and 30 min after exercise. Approximately 3 ml of blood will be withdrawn on each occasion (total ~ 60ml). In the leg cycling test, blood samples will be taken from an artery and a vein before and at the end of each exercise bout, with recovery samples at 1, 2, 5, 10 and 30 min after exercise. Approximately 3 ml of blood will be withdrawn on each occasion (total ~ 72ml).

Although the possibility of infection ,significant bruising, bleeding, local blood clot and local swelling and redness, are quite small, you should inform us immediately and consult your doctor if in the 12hrs following the procedure, swelling, temperature, redness and/or pain increases at the site.

If medical treatment is required following any adverse effects associated with participating in this study, any costs will be reimbursed. In recognition of the large time commitment in participating for this study, participants will be reimbursed a total of $200 at the completion of the study.

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, the investigators and yourself accept it that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time without ill repute. Thank you for your co-operation.

Contact Numbers:

Mr Simon Sostaric	**(W) 96884160**	**(mob) 0414 90 7767**
Assoc Prof Michael McKenna	**(W) 9688 4499**	**(H) 5422 6089**
Assoc Prof Henry Krum	**(W) 9909 0042**	**(mob) 0417 325 834**

Any queries about your participation in this project may be directed to the researcher (Name: Assoc. Prof. M.McKenna; ph. 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 MCMC, Melbourne, 8001 (telephone no: 03-9688 4710).

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Footscray Park Campus
Building L, Ballarat Road, Footscray

Please retain this form in your wallet or purse for easy access for the duration of the study

Date:________________________

To whom it may concern, please be advised that Name: ___________________________________ is participating as a volunteer in an experiment conducted at Victoria University of Technology.

The experiment is a placebo-controlled investigation into the effects of 0.25 mg/day oral digoxin on muscle strength, exercise performance and plasma potassium concentration.

S/he will be receiving either 0.25 mg/day digoxin OR placebo orally for a 2 week period, commencing on:

Date:_________________ through to Date:_________________.
S/he will then be receiving the reverse treatment, i.e. either placebo OR 0.25 mg/day digoxin for a 2 week period, commencing on:

Date:_________________ through to Date:_________________.

For further information in the event of an adverse reaction, please contact the Principal Investigators:

Associate Professor Henry Krum (MBBS)	**(W) 9909 0042**	**(mob) 0417 325 834**
Associate Professor Michael McKenna (PhD)	**(W) 9688 4499**	**(H) 5422 6089**
	michael.mckenna@vu.edu.au	
or Mr Simon Sostaric (PhD student)	**(W) 96884160**	**(mob) 0414 90 7767**

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CARDIOVASCULAR AND OTHER RISK FACTORS QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:
" **The effects of a standard clinical dose of digoxin on potassium regulation in muscle and blood, and muscle fatigability during exercise in healthy young volunteers**" 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 Gender: __M_____F____

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 elevated blood pressure? Yes No Don't Know
8. Are you being treated with diuretics? Yes No
9. Are you on any other medications? Yes No
   List all medications? _____
10. 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
    Yes, please elaborate _____
11. Are you currently pregnant or expect to become pregnant during the time in which this experiment is conducted? Yes No

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

Signed: _____ Date: _____

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MUSCLE BIOPSY & ARTERIAL- VENOUS CANNULATION QUESTIONNAIRE:

The effects of a standard clinical dose of digoxin on potassium regulation in muscle and blood, and muscle fatigability during exercise in healthy young volunteers

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 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... _____
   _____

7. Have you ever fainted when you had an injection or blood sample taken? Yes No
   Don't know
   If yes, please elaborate_____

8. Have you previously had heparin infused or injected? Yes No
Don't know
If yes, please elaborate_____

9. Do you or other members of your family have Raynauds disease, or suffer from very poor circulation in the fingers, leading to painful fingers that turn white/blue? Yes No
Don't know

If yes, please elaborate_____

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

Signature: _____ Date: _____

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CASE REPORT FORM

The effects of a standard clinical dose of digoxin on potassium regulation in muscle and blood, and muscle fatigability during exercise in healthy young volunteers

Assoc Prof. Michael McKenna
Assoc Prof. Henry Krum
Mr Simon Sostaric

Victoria University of Technology

STUDY SCHEDULE

DAY -2: _____

* Consent/Inclusion/Exclusion/Physical/ECG/Bloods

DAY 0: _____

* Dispense drug

DAY 1: _____

* Commence drug - phone call to confirm

DAY 7: _____

* Medication compliance /Bloods/Cease drug administration

DAY 12: _____

* Medication compliance /Bloods/Cease drug administration
Muscle strength and fatigue experiments begin

DAY 14: _____

* Medication compliance /Bloods/Cease drug administration
Invasive exercise experiments begin

DAY 36: _____

* Repeat as above from day –2, however, dispensing placebo

Visit 1	**YES**	**NO**	
Informed consent form signed			DATE

.

Participant number.

Participant initials

Date of birth

Height cm

Weight Kg

Male / Female

INCLUSION CRITERIA

	YES	**NO**
Male or female aged 18 to 40		
No clinically significant abnormality on medical history or on physical examination (NO CARDIOVASCULAR DISEASE).		
Laboratory values within the reference ranges for biochemistry		
Normal sinus rhythm ECG at pre-study examination		
Non-smoker		
No previous history or current use of digoxin		
Within 20% ideal body weight (according to GIGA scientific tables)		
If female of child bearing potential, using adequate contraceptive measures		
Written informed consent for study signed		

IF THE ANSWER TO ANY OF THESE QUESTIONS IS **NO**, THE SUBJECT IS EXCLUDED.

For female patients of child bearing potential, a urine pregnancy test must be performed.

negative positive

Result of pregnancy test | | |

If positive, subject is excluded from participation in study

EXCLUSION CRITERIA	**YES**	**NO**
Treatment with antiarrhythmic medications	☐	☐
History of significant cardiovascular disease	☐	☐
Impanted pacemaker, or atrial arrhythmia	☐	☐
History of alcohol or drug abuse.	☐	☐
Participation on a trial with any experimental drug within 30 days of commencement of the study.	☐	☐
Serum creatinine > 30 mmol/l	☐	☐
Concomitant conditions associated with autonomic dysfunction eg diabetes	☐	☐
Current smoker	☐	☐
History or presence of gastrointestinal, hepatic or renal disease or other condition known to interfere with the absorption, distribution, metabolism or excretion of the drug.	☐	☐
Definite or suspected personal history of adverse events or hypersensitivity to the trial drug or to drugs with a similar chemical structure	☐	☐
Participant considered by the investigator to be unwilling, unlikely or unable to comply with the study protocol and restrictions.	☐	☐
Pregnancy	☐	☐
Female participants not taking adequate contraception (the oral contraceptive pill is an exclusion to study participation)	☐	☐

IF THE ANSWER TO ANY OF THESE QUESTIONS IS **YES**, THE PARTICIPANT IS EXCLUDED.

Investigator'sSignature/
Date: _____

MEDICAL HISTORY

Does the participant have any relevant medical history?	YES	NO

If **YES**, please specify;

PHYSICAL EXAMINATION

BP&HR: Average of last 3 of 5 seated Dinamap readings

	Sitting
BP (mmHg)	/
HR (bpm)	

Tick, cross, or provide brief description as required

Pallor ☐ Icterus ☐ Cyanosis ☐ Clubbing ☐

JVP Raised ☐ cm _____

Carotid bruit L ☐ R ☐

Apex beat _____

Heart sounds S1 ☐ S2 ☐ S3 ☐ S4 ☐

Murmurs _____

CHEST:

L: _____

R: _____

ABDOMEN:

Liver _____

Spleen _____

L. Kidney _____

R. Kidney _____

Ascites ☐

Swelling of ankles: L ☐ Severity ☐

R ☐

(1 = mild, 2 = moderate, 3 = severe)

Other abnormalities: _____
_____

ECG Normal ☐ Abnormal ☐

If abnormal please comment;
_____
_____

PATHOLOGY : Date of collection_____

Laboratory Variable	Result	Unit	Normal Range
Creatinine		mmol/L mmol/L	Male 0.07 - 0.13 Female 0.04 - 0.12
Sodium		mmol/L	134 – 146
Potassium		mmol/L	3.5 – 5.0
Urea		mmol/L	2.0 – 8.5

DAY 0.

Date: _____

1. DISPENSE DRUG TREATMENT 1 ☐

DRUG CODE: _____

NUMBER TABLETS DISPENSED= ☐

DAY 1

Date: _____

1. COMMENCE DRUG TREATMENT 1 ☐
   TELEPHONE CALL TO CONFIRM

DAY 14

Date: _____

1 CEASE DRUG TREATMENT ☐
TELEPHONE CALL TO CONFIRM

PATHOLOGY : Date of collection_____

Laboratory Variable	Result	Unit	Normal Range
Creatinine		mmol/L mmol/L	Male 0.07 - 0.13 Female 0.04 - 0.12
Sodium		mmol/L	134 - 146
Potassium		mmol/L	3.5 - 5.0
Urea		mmol/L	2.0 - 8.5

2. PATHOLOGY

BLOOD COLLECTED FOR DIGOXIN ☐
TIME OF LAST DOSE ☐

Laboratory Variable	Result	Unit	Normal Range
Digoxin		**nmol/L**	0.6 - 2.6

MEDICATION COMPLIANCE	
Tablets Dispensed (from Day 1)	
Tablets Returned	
% Compliance	

ADVERSE EVENTS

Treatment Arm □ **1** □ **2** □ **3**

Time period	Time	Date

Has the participant experienced any adverse event YES ☐ NO ☐

If the participant has experienced an adverse event, give details below.

_____

_____

Time of onset (24hr clock)		Date of onset (dd/mm/yy)	
Time resolved (24hr clock)		Date resolved (dd/mm/yy)	

Intensity ☐

1. mild
2. moderate
3. severe

Relationship to study drug ☐

1. unrelated
2. remote
3. possible
4. probable

Outcome ☐

1. complete recovery
2. sequelae
3. still under treatment. Please specify:

_____

Signature/Date _____

Study Termination Form

Termination Date:_____

Has the patient completed the study according to the protocol?

Yes □

No □

If No, select one of the following:

□ the participant did not wish to continue in the study

□ the participant developed a significant concurrent illness

□ other change in inclusion/exclusion criteria (please specify)

□ the participant did not comply with the protocol and/or study staff

□ unacceptable adverse events

□ other_____

Additional comments

_____

_____

_____

Principal Investigator's Signature & Date:

_____

EXPERIMENTAL PROCEDURES TIMETABLE

Participant_____ Date_____

PRE-EXERCISE BLOOD FLOW AND BLOOD SAMPLING TIMETABLE

Participant_____ Date_____

Recovery Time (min:sec)	**Action**	**Action Completed**	**Comments**
-2:00	Arterial Pressure CUFF ON		
-1:45	Take **Rest** Blood Samples		
-1:00	Take Forearm Blood Flows		
-0:30	**RELEASE** Arterial Cuff		
0:00	**BOUT#1** Commence forearm contractions		

Appendix

Estimate of Volunteer Time Requirement

Visit	Week		Screen	Forearm anthropom. & Familirisation	Forearm Incremental	Forearm Fatigue	Cybex Famil'n	Cybex MVC+ fatigue	Vo2 peak	Submax Cycle famil	Submax Cycle + Biopsy	Time (hr)
1	0	**Alfred Hos.**	YES									0.50
2	0.5	**Pre Tests**		YES	YES		YES		YES			2.00
3	1	**Famil trials**				YES		YES		YES		2.00
4	2	**Variability 1**				YES		YES		YES		2.00
5	3	**Variability 2**				YES		YES		YES		2.00
6	5	**Placebo**						YES				0.75
7	5	**Placebo**				YES					YES	5.00
8	11	**Washout Digoxin**						YES				0.75
9	11	**Digoxin**				YES					YES	5.00
												20.00

Appendix 2. Subject physical characteristics.

Subject	Age (years)	Body mass (kg)	Height (cm)
1	28	67.63	174.5
2	21	73.11	180.5
3	20	75.80	179.0
4	21	64.58	173.5
5	40	103.18	189.0
6	30	80.63	179.5
7	23	66.79	173.0
8	24	82.68	188.0
9	26	74.63	159.0
10	27	68.49	188.0
n	10	10	10
Mean	26	75.75	178.4
SD	5.9	11.33	9.1

Appendix 3. Peak O_2 consumption and exercise data from incremental cycling exercise test.

Subject	$\dot{V}O_{2peak}$ (L.min^{-1})	Power output (W)		
		33%	67%	90%
1	3.43	42	145	214
2	3.64	50	185	276
3	3.42	49	170	252
4	3.11	48	159	234
5	3.78	61	205	303
6	4.24	76	251	369
7	3.93	79	213	303
8	4.32	81	212	300
9	3.11	48	159	234
10	3.74	67	181	257
n	10	10	10	10
mean	3.67	60	188	278
SD	0.42	15	32	52
SEM	0.13	5	10	16

Appendix 4. Exercise data from leg cycling test in preliminary (Variability, Var), digoxin (DIG) and control (CON) trials.

Leg exercise time to fatigue				
Subject	**Var 1**	**Var 2**	**DIG**	**CON**
1	573	530	609	438
2	356	285	182	262
3	308	307	290	179
4	307	300	223	210
5	315	289	246	272
6	148	145	121	112
7	225	282	108	78
8	375	381	194	343
9	230	257	197	199
10	579	610	451	445
n	10	10	10	10
mean	341.6	338.6	262.1	253.8
SD	140.6	136.3	155.5	124.9
SEM	44.4	43.1	49.2	39.5

Appendix 5. Serum digoxin concentration at d 7, d 13 and d 14 in digoxin (DIG) and control (CON) trials.

Subject	**DIG**			**CON**		
	d 7	**d 13**	**d 14**	**d 7**	**d 13**	**d 14**
		(nM)			**(nM)**	
1	0.8	0.7	0.7	<0.4	<0.4	<0.4
2	0.7	0.8			<0.4	<0.4
3	<0.4	0.5	0.5	<0.4	<0.4	<0.4
4	0.9	0.9	1.1	<0.4	<0.4	
5	0.7	0.7	0.8	<0.4	<0.4	<0.4
6	0.8	0.6	0.8	<0.4	<0.4	<0.4
7	0.4	0.4	0.6	<0.2	<0.2	<0.2
8	1.0	1.0	0.9	<0.4	<0.4	<0.4
9	0.7	0.8	0.8	<0.2		<0.2
10	0.5	0.5	0.7	<0.2	<0.2	<0.2
n	9	10	9	9	9	9
mean	0.7	0.7	0.8			
SD	0.2	0.2	0.2			
SEM	0.1	0.1	0.1			

***** later trials the Alfred Hospital revised their lower detection limits from "<0.4 nM" to "<0.2 nM".

Appendix 6. Oxygen consumption ($L.min^{-1}$) during cycle exercise at 33%, 67% and 90% VO_{2peak} in digoxin (DIG) and control (CON) trials.

Subject	DIG			CON		
	33%	67%	90%	33%	67%	90%
		($L.min^{-1}$)			($L.min^{-1}$)	
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
SEM	0.06	0.16	0.09	0.07	0.17	0.14

Appendix 7. Arterial plasma [K^+] (mmol.L^{-1}) in digoxin (DIG) and control (CON) trials at rest and during cycle exercise at 33%, 67% and 90% VO_{2peak}.

Subject	DIG				CON			
	Rest	33%	67%	90%	Rest	33%	67%	90%
	(mmol.L^{-1})				(mmol.L^{-1})			
1	3.94	4.01	4.46	5.90	4.16	4.09	4.33	5.90
2	4.05	4.37	4.40	5.55	3.97	4.40	4.17	5.95
3	3.95	4.81	5.00	6.71	3.77	3.87	4.32	5.80
4	4.04	3.60	4.20	5.20	4.00	4.02	4.49	5.36
5	3.90	4.12	4.72	5.53	4.06	4.20	4.51	5.31
6	3.48	3.53	4.30	5.04	3.75	3.83	4.35	5.10
7	3.88	3.98	4.31	4.04	4.08	4.23		4.26
8	4.29	4.13	4.43	5.18	3.93	4.26	4.48	5.07
9	3.71	3.37	4.00		3.94	4.10	4.30	4.66
10	3.94	4.10	4.10	5.44	3.80	3.94	4.24	5.15
n	10	10	10	9	10	10	9	10
mean	3.92	4.00	4.39	5.40	3.95	4.09	4.35	5.26
SD	0.21	0.43	0.29	0.71	0.14	0.18	0.12	0.54
SEM	0.07	0.13	0.09	0.24	0.04	0.06	0.04	0.17

Appendix 8. Muscle peak torque (Nm) at 0, 60, 120, 180, 240, 300 and 360 velocities (0 ^{o}.s^{-1}) in digoxin (DIG) and control (CON) trials.

Subject				**DIG**							**CON**			
	0	**60**	**120**	**180**	**240**	**300**	**360**	**0**	**60**	**120**	**180**	**240**	**300**	**360**
				(Nm)							**(Nm)**			
1	320	241	229	190	155	140	124	273	179	167	154	128	112	96
2	412	319	259	203	170	148	139	418	328	260	214	180	150	128
3	337	238	215	195	158	145	122	309	250	223	127	154	126	101
4	321	250	228	184	149	135	117	308	218	211	184	158	126	110
5	298	234	206	181	152	129	113	343	265	226	186	156	135	122
6	424	334	285	236	207	182	166	394	336	291	245	217	182	171
7	260	210	162	126	108	94	81	275	226	172	140	111	95	84
8	392	303	275	236	199	174	150	385	274	262	233	210	169	156
9	234	147	146	118	86	85	80	235	176	159	133	110	91	78
10	251	188	185	151	128	113	104	205	225	180	161	132	111	122
n	10	10	10	10	10	10	10	10	10	10	10	10	10	10
mean	325	246	219	182	151	135	120	315	248	215	178	156	130	117
SD	67	59	46	40	37	31	28	70	55	46	42	38	30	30
SEM	21	19	15	13	12	10	9	22	17	14	13	12	9	9

Appendix 9. Muscle Fatigue Index (FI) in digoxin (DIG) and control (CON) trials, conducted at 180 velocity (0 $^{o}.s^{-1}$).

Subject	DIG			CON		
	Mean of highest 5	Mean of weakest 5	FI (%)	Mean of highest 5	Mean of Weakest 5	FI (%)
1	155.4	81.0	47.9	190.0	63.2	66.7
2	199.6	93.6	53.1	198.2	84.2	57.5
3	175.2	80.2	54.2	163.8	54.0	67.0
4	181.6	80.6	55.6	183.2	73.0	60.2
5	183.2	73.0	60.2	166.8	74.0	55.6
6	244.8	72.0	70.6	241.4	71.0	70.6
7	124.2	77.4	37.7	127.8	73.4	42.6
8	233.2	98.8	57.6	233.8	101.8	56.5
9	119.4	55.0	53.9	119.6	52.8	55.9
10	142.4	80.4	43.5	149.0	89.8	39.7
n	10	10	10	10	10	10
mean	175.9	79.2	53.4	177.4	73.7	57.2
SD	42.3	11.9	9.1	40.6	15.3	10.0
SEM	13.4	3.8	2.9	12.8	4.8	3.2

Appendix 10. Digoxin effects on Na^+,K^+-ATPase total α mRNA expression (sum of α_1, α_2 and α_3) in resting muscle.

Subject	DIG			CON		
	α_1	α_2	α_3	α_1	α_2	α_3
1	3.43	5.84	2.09	1.00	1.00	1.00
2	0.71	0.16	0.15	1.00	1.00	1.00
3	1.11	5.72	0.13	1.00	1.00	1.00
4	0.90	1.22	0.37	1.00	1.00	1.00
5	0.74	0.60	0.36	1.00	1.00	1.00
6	2.52	1.04	4.33	1.00	1.00	1.00
7	0.31	0.49	0.72	1.00	1.00	1.00
8	5.72	1.75	6.77	1.00	1.00	1.00
9	0.44	4.92	3.13	1.00	1.00	1.00
10	1.12	1.00	0.42	1.00	1.00	1.00
n	10	10	10	10	10	10
mean	1.70	2.27	1.85	1.00	1.00	1.00
SD	1.72	2.27	2.25	0.00	0.00	0.00
SEM	0.54	0.72	0.71	0.00	0.00	0.00

All data expressed relative to CON rest for that isoform.

Appendix 11. Digoxin effects on Na^+,K^+-ATPase total β mRNA expression (sum of β_1, β_2 and β_3) in resting muscle.

Subject	DIG			CON		
	β_1	β_2	β_3	β_1	β_2	β_3
1	1.46	0.90	0.65	1.00	1.00	1.00
2	1.72	2.71	1.82	1.00	1.00	1.00
3	7.70	0.32	1.59	1.00	1.00	1.00
4	0.72	0.97	0.91	1.00	1.00	1.00
5	1.75	1.00	1.47	1.00	1.00	1.00
6	1.73	1.51	0.96	1.00	1.00	1.00
7	1.02	0.83	0.80	1.00	1.00	1.00
8	2.02	0.60	1.31	1.00	1.00	1.00
9	1.97	2.23	4.89	1.00	1.00	1.00
10	1.31	0.83	1.31	1.00	1.00	1.00
n	10	10	10	10	10	10
mean	2.04	1.19	1.57	1.00	1.00	1.00
SD	2.03	0.75	1.22	0.00	0.00	0.00
SEM	0.64	0.24	0.39	0.00	0.00	0.00

All data expressed relative to CON rest for that isoform.

Appendix 12. Digoxin effects on Na^+,K^+-ATPase α_1, α_2 and α_3 protein abundance in resting muscle.

Subject	**DIG**			**CON**		
	α_1	**α_2**	**α_3**	**α_1**	**α_2**	**α_3**
1	0.56	0.54	0.33	1.00	1.00	1.00
2	0.85	2.71	1.30	1.00	1.00	1.00
3	0.87	1.06	1.14	1.00	1.00	1.00
4	1.19	0.98	0.62	1.00	1.00	1.00
5	0.56	0.86	2.23	1.00	1.00	1.00
6	0.77	2.15	1.17	1.00	1.00	1.00
7	2.04	1.17	0.51	1.00	1.00	1.00
8	2.15	1.19	1.39	1.00	1.00	1.00
9	1.70	1.21	1.72	1.00	1.00	1.00
10	0.67	2.49	0.83	1.00	1.00	1.00
n	10	10	10	10	10	10
mean	1.14	1.44	1.12	1.00	1.00	1.00
SD	0.61	0.74	0.58	0.00	0.00	0.00
SEM	0.19	0.23	0.18	0.00	0.00	0.00

All data expressed relative to CON rest for that isoform.

Appendix 13. Digoxin effects on Na^+,K^+-ATPase β_1, β_2 and β_3 protein abundance in resting muscle.

Subject	**DIG**			**CON**		
	β_1	β_2	β_3	β_1	β_2	β_3
1	2.52	5.80	0.78	1.00	1.00	1.00
2	0.69	0.89	0.87	1.00	1.00	1.00
3	0.93	1.92	0.91	1.00	1.00	1.00
4	1.07	0.80	2.19	1.00	1.00	1.00
5	1.12	3.07	0.91	1.00	1.00	1.00
6	1.57	0.22	1.02	1.00	1.00	1.00
7	1.54	0.79	0.45	1.00	1.00	1.00
8	0.54	0.68	0.59	1.00	1.00	1.00
9	1.19	3.78	0.45	1.00	1.00	1.00
10	0.74	0.66	0.34	1.00	1.00	1.00
n	10	10	10	10	10	10
mean	1.19	1.86	0.85	1.00	1.00	1.00
SD	0.58	1.81	0.52	0.00	0.00	0.00
SEM	0.18	0.57	0.17	0.00	0.00	0.00

All data expressed relative to CON rest for that isoform.

Appendix 14. Digoxin and exercise effects on Na^+,K^+-ATPase α_1 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	3.43	38.45	3.31	2.77	1.00	25.11	16.45	13.50
2	0.71	0.28	2.19	3.20	1.00	0.18	0.96	12.95
3	1.11	1.49	0.96	1.16	1.00	0.95	0.75	0.15
4	0.90	2.01	1.05	3.12	1.00	0.31	0.20	5.21
5	0.74	2.43	1.09	0.66	1.00	6.25	0.58	5.47
6	2.52	1.53	1.59	6.14	1.00	3.47	6.44	5.24
7	0.31	0.24	0.74	0.14	1.00	0.96	4.65	0.48
8	5.72	1.84	8.69	0.39	1.00	1.20	1.20	2.27
9	0.44	0.47	1.94	0.24	1.00	0.22	0.57	2.56
10	1.12	1.91	0.29	0.19	1.00	0.79	3.67	6.84
n	10	10	10	10	10	10	10	10
mean	1.70	5.07	2.19	1.80	1.00	3.94	3.55	5.47
SD	1.72	11.76	2.44	1.97	0.00	7.67	5.00	4.65
SEM	0.54	3.72	0.77	0.62	0.00	2.43	1.58	1.47

All data expressed relative to CON rest.

Appendix 15. Digoxin and exercise effects on Na^+,K^+-ATPase α_2 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	5.84	1.32	1.99	1.28	1.00	1.60	2.14	2.30
2	0.16	0.65	0.54	0.53	1.00	0.57	3.15	0.01
3	5.72	8.17	4.89	1.77	1.00	3.67	6.77	1.43
4	1.22	1.47	1.52	1.61	1.00	0.44	0.59	2.49
5	0.60	0.47	0.56	0.34	1.00	2.73	0.56	3.25
6	1.04	2.13	2.95	2.81	1.00	2.58	2.68	3.75
7	0.49	0.25	0.37	0.58	1.00	0.71	3.10	1.49
8	1.75	0.94	2.91	0.65	1.00	1.31	1.02	4.33
9	4.92	2.37	3.33	2.94	1.00	4.50	2.49	2.67
10	1.00	0.91	0.54	1.23	1.00	0.44	1.15	0.89
n	10	10	10	10	10	10	10	10
mean	2.27	1.87	1.96	1.37	1.00	1.85	2.37	2.26
SD	2.27	2.32	1.53	0.92	0.00	1.45	1.84	1.33
SEM	0.72	0.73	0.48	0.29	0.00	0.46	0.58	0.42

All data expressed relative to CON rest.

Appendix 16. Digoxin and exercise effects on Na^+,K^+-ATPase α_3 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	2.09	2.14	0.75	2.10	1.00	2.48	1.16	5.46
2	0.15	0.03	0.78	1.06	1.00	0.03	0.09	5.28
3	0.13	0.38	0.50	0.15	1.00	0.31	0.05	0.08
4	0.37	0.44	0.21	3.46	1.00	0.12	0.06	0.71
5	0.36	0.11	0.14	1.08	1.00	0.26	0.23	1.98
6	4.33	1.53	2.10	0.66	1.00	3.14	2.89	4.20
7	0.72	0.22	1.46	3.08	1.00	0.62	0.31	2.60
8	6.77	0.19	1.69	1.20	1.00	0.62	0.49	2.82
9	3.13	2.98	1.77	1.80	1.00	4.96	2.94	4.66
10	0.42	0.51	0.25	0.85	1.00	0.25	0.43	0.44
n	10	10	10	10	10	10	10	10
mean	1.85	0.85	0.97	1.55	1.00	1.28	0.86	2.82
SD	2.25	1.01	0.73	1.06	0.00	1.67	1.13	2.02
SEM	0.71	0.32	0.23	0.34	0.00	0.53	0.36	0.64

All data expressed relative to CON rest.

Appendix 17. Digoxin and exercise effects on Na^+,K^+-ATPase β_1 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	1.46	0.99	1.33	0.66	1.00	0.05	1.56	0.20
2	1.72	1.94	1.46	1.35	1.00	2.08	2.42	0.60
3	7.70	13.04	7.84	2.79	1.00	12.17	9.75	1.98
4	0.72	2.21	0.96	2.08	1.00	0.32	0.28	0.80
5	1.75	2.58	1.31	2.01	1.00	2.84	2.60	4.63
6	1.73	0.95	0.68	1.07	1.00	1.05	1.23	0.68
7	1.02	0.57	0.63	0.52	1.00	1.07	1.05	1.54
8	2.02	1.55	2.33	0.91	1.00	0.54	0.33	4.07
9	0.97	0.64	1.21	0.80	1.00	0.78	0.72	0.98
10	1.31	1.56	1.03	0.41	1.00	0.39	1.51	2.92
n	10	10	10	10	10	10	10	10
mean	2.04	2.60	1.88	1.26	1.00	2.13	2.14	1.84
SD	2.03	3.73	2.15	0.79	0.00	3.63	2.78	1.54
SEM	0.64	1.18	0.68	0.25	0.00	1.15	0.88	0.49

All data expressed relative to CON rest.

Appendix 18. Digoxin and exercise effects on Na^+,K^+-ATPase β_2 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.90	1.93	3.06	2.18	1.00	2.63	1.79	2.77
2	2.71	0.47	0.57	0.79	1.00	0.68	0.86	2.13
3	0.32	2.13	0.17	0.22	1.00	0.27	0.54	0.56
4	0.97	0.52	1.28	1.79	1.00	0.42	0.47	1.16
5	1.00	1.54	1.09	1.00	1.00	1.33	0.20	1.44
6	1.51	2.70	2.88	2.81	1.00	3.04	3.78	2.65
7	0.83	1.22	1.92	1.64	1.00	1.30	1.23	0.89
8	0.60	1.26	0.99	0.66	1.00	0.91	0.19	5.31
9	2.23	1.76	1.40	2.06	1.00	2.30	2.07	0.98
10	0.83	0.81	0.43	0.73	1.00	0.30	0.76	0.45
n	10	10	10	10	10	10	10	10
mean	1.19	1.43	1.38	1.39	1.00	1.32	1.19	1.83
SD	0.75	0.72	0.98	0.83	0.00	1.01	1.11	1.47
SEM	0.24	0.23	0.31	0.26	0.00	0.32	0.35	0.47

All data expressed relative to CON rest.

Appendix 19. Digoxin and exercise effects on Na^+,K^+-ATPase β_3 mRNA expression in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.65	1.59	1.71	0.59	1.00	1.13	0.76	1.27
2	1.82	1.37	0.04	3.73	1.00	3.72	2.61	4.14
3	1.59	0.98	1.15	0.37	1.00	1.18	1.45	0.99
4	0.91	1.04	1.29	1.34	1.00	0.74	0.72	2.00
5	1.47	1.49	0.53	1.01	1.00	2.68	1.60	2.72
6	0.96	1.52	1.75	2.63	1.00	1.93	2.72	2.26
7	0.80	0.29	0.49	1.22	1.00	0.96	1.48	1.60
8	1.31	0.98	1.18	0.60	1.00	1.00	0.23	3.22
9	4.89	2.67	3.45	4.07	1.00	2.91	0.95	6.99
10	1.31	2.36	0.71	3.15	1.00	1.60	3.05	1.93
n	10	10	10	10	10	10	10	10
mean	1.57	1.43	1.23	1.87	1.00	1.78	1.56	2.71
SD	1.22	0.69	0.95	1.39	0.00	1.00	0.95	1.77
SEM	0.39	0.22	0.30	0.44	0.00	0.32	0.30	0.56

All data expressed relative to CON rest.

Appendix 20. Digoxin and exercise effects on Na^+,K^+-ATPase α_1 protein abundance in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.56	0.86	0.42	0.62	1.00	0.71	0.89	0.44
2	0.85	0.54	0.63	0.68	1.00	0.27	0.36	0.28
3	0.56	1.03	0.83	0.65	1.00	0.90	1.16	1.09
4	0.87	1.76	1.00	1.58	1.00	1.37	1.55	1.20
5	1.19	1.02	1.00	1.28	1.00	1.53	0.83	0.89
6	2.04	1.51	2.09	0.75	1.00	0.78	1.23	1.64
7	2.15	2.46	1.96	1.45	1.00	0.90	2.02	1.45
8	0.77	2.03	1.34	0.78	1.00	1.55	0.88	1.11
9	0.67	0.72	0.58	0.43	1.00	0.51	0.34	0.76
10	1.70	2.25	1.44	0.56	1.00	1.93	1.10	1.23
n	10	10	10	10	10	10	10	10
mean	1.14	1.42	1.13	0.88	1.00	1.05	1.04	1.01
SD	0.61	0.68	0.57	0.40	0.00	0.52	0.51	0.43
SEM	0.19	0.22	0.18	0.13	0.00	0.17	0.16	0.14

All data expressed relative to CON rest.

Appendix 21. Digoxin and exercise effects on Na^+,K^+-ATPase α_2 protein abundance in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.54	1.72	0.97	0.79	1.00	1.26	1.40	1.32
2	2.71	1.05	1.19	0.67	1.00	1.36	1.99	1.67
3	0.86	1.98	0.98	0.64	1.00	0.85	1.14	1.28
4	1.06	0.93	0.81	1.18	1.00	1.78	0.86	1.79
5	0.98	0.71	0.42	0.48	1.00	0.52	0.99	1.42
6	1.17	0.67	1.15	1.20	1.00	0.93	1.08	0.61
7	1.19	0.90	1.09	0.59	1.00	0.95	1.05	1.31
8	2.15	1.21	1.26	1.46	1.00	1.79	1.77	2.26
9	2.49	1.07	1.21	1.49	1.00	3.48	1.36	3.42
10	1.21	1.97	1.25	0.57	1.00	0.64	0.98	1.59
n	10	10	10	10	10	10	10	10
mean	1.44	0.74	1.03	0.91	1.00	1.36	1.26	1.67
SD	0.74	0.49	0.26	0.39	0.00	0.86	0.37	0.75
SEM	0.23	0.16	0.08	0.12	0.00	0.27	0.12	0.24

All data expressed relative to CON rest.

Appendix 22. Digoxin and exercise effects on Na^+,K^+-ATPase α_3 protein abundance in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.33	0.48	0.90	0.92	1.00	0.80	0.63	1.41
2	1.30	0.64	0.70	0.81	1.00	0.66	0.54	0.97
3	2.23	2.78	3.01	2.84	1.00	1.37	4.48	2.99
4	1.14	1.33	0.72	0.93	1.00	0.61	0.98	1.45
5	0.62	0.32	1.49	0.29	1.00	0.30	0.62	1.03
6	0.51	0.52	0.79	1.12	1.00	0.61	0.55	0.29
7	1.39	3.54	3.40	1.46	1.00	1.63	0.82	1.73
8	1.17	1.41	1.62	0.87	1.00	1.12	1.45	1.08
9	0.83	0.64	0.50	0.79	1.00	1.22	1.11	0.96
10	1.72	2.48	1.78	0.94	1.00	2.91	1.13	2.16
n	10	10	10	10	10	10	10	10
mean	1.12	1.41	1.49	1.10	1.00	1.12	1.23	1.41
SD	0.58	1.13	1.00	0.68	0.00	0.75	1.18	0.75
SEM	0.18	0.36	0.32	0.21	0.00	0.24	0.37	0.24

All data expressed relative to CON rest.

Appendix 23. Digoxin and exercise effects on Na^+,K^+-ATPase β_1 protein abundance in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	2.52	1.32	2.13	1.47	1.00	0.68	0.97	1.69
2	0.69	0.40	0.50	0.66	1.00	0.56	0.70	0.75
3	1.12	0.96	0.98	1.14	1.00	1.11	0.75	0.86
4	0.93	0.75	1.27	1.42	1.00	1.75	0.83	1.39
5	1.07	2.29	1.33	3.55	1.00	0.93	0.76	1.05
6	1.54	2.10	1.78	3.21	1.00	0.70	1.04	4.26
7	0.54	4.18	2.01	1.44	1.00	0.45	1.18	1.73
8	1.57	0.58	1.72	1.21	1.00	0.86	0.65	1.77
9	0.74	0.67	0.51	0.57	1.00	0.79	0.37	0.84
10	1.19	0.43	0.45	0.85	1.00	0.72	0.94	2.37
n	10	10	10	10	10	10	10	10
mean	1.19	0.58	1.27	1.55	1.00	0.85	0.82	1.60
SD	0.58	1.19	0.64	1.02	0.00	0.37	0.23	1.07
SEM	0.18	0.40	0.20	0.32	0.00	0.12	0.07	0.36

All data expressed relative to CON rest.

Appendix 24. Digoxin and exercise effects on Na^+,K^+-ATPase β_2 protein abundance in skeletal muscle.

Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	5.80	2.29	0.48	1.10	1.00	3.13	9.68	3.48
2	0.89	0.83	1.36	1.61	1.00	1.27	1.34	0.68
3	3.07	2.08	3.06	3.66	1.00	2.41	4.14	2.72
4	1.92	2.34	2.99	3.26	1.00	2.48	2.53	2.57
5	0.80	1.30	0.97	2.06	1.00	0.66	0.91	1.33
6	0.79	1.09	1.44	2.29	1.00	1.13	1.23	1.13
7	0.68	0.31	0.87	1.24	1.00	0.46	0.79	0.52
8	0.22	1.48	4.37	3.33	1.00	1.58	0.87	1.01
9	0.66	0.85	0.69	0.29	1.00	0.87	1.88	1.44
10	3.78	2.19	5.65	5.07	1.00	1.73	8.54	21.49
n	10	10	10	10	10	10	10	10
mean	1.86	1.47	2.19	2.39	1.00	1.57	3.19	3.64
SD	1.81	0.72	1.76	1.44	0.00	0.87	3.29	6.35
SEM	0.57	0.23	0.56	0.45	0.00	0.27	1.04	2.01

All data expressed relative to CON rest.

Appendix 25. Digoxin and exercise effects on Na^+,K^+-ATPase β_3 protein abundance in skeletal muscle.

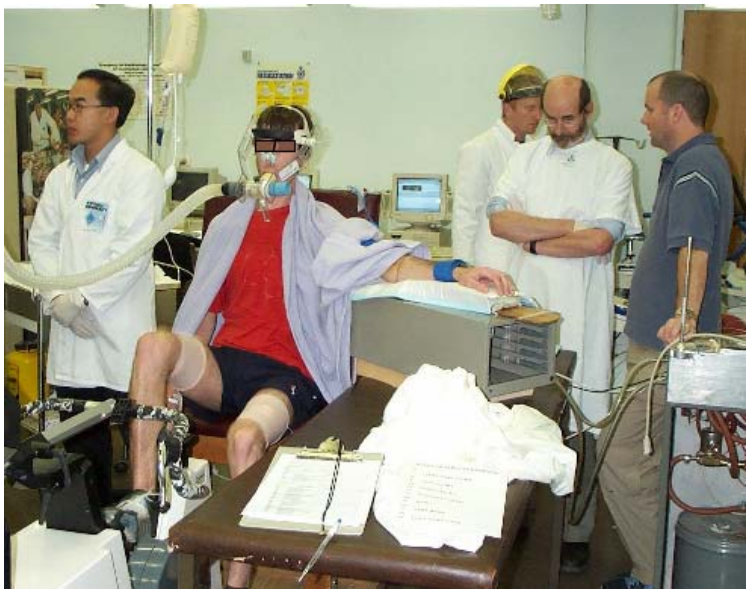
Subject	DIG				CON			
	Rest	**67%**	**FAT**	**+3 h**	**Rest**	**67%**	**FAT**	**+3 h**
1	0.78	1.65	1.29	2.23	1.00	3.01	1.65	1.08
2	0.87	1.66	1.71	3.68	1.00	2.87	5.53	0.91
3	0.91	1.38	1.30	2.03	1.00	1.22	2.30	1.18
4	0.91	0.86	1.92	2.51	1.00	2.22	1.06	2.01
5	2.19	2.36	2.33	2.04	1.00	1.32	1.56	1.56
6	0.45	0.47	0.87	0.17	1.00	0.58	0.73	0.47
7	0.59	1.73	0.59	1.45	1.00	0.69	0.77	0.91
8	1.02	1.39	1.34	2.02	1.00	1.50	1.64	1.65
9	0.34	0.40	0.32	0.23	1.00	0.19	0.35	0.31
10	0.45	1.31	0.82	2.32	1.00	0.20	1.01	0.45
n	10	10	10	10	10	10	10	10
mean	0.85	1.32	1.25	1.87	1.00	1.38	1.66	1.05
SD	0.52	0.60	0.62	1.05	0.00	1.03	1.47	0.56
SEM	0.17	0.19	0.20	0.33	0.00	0.33	0.47	0.18

All data expressed relative to CON rest.

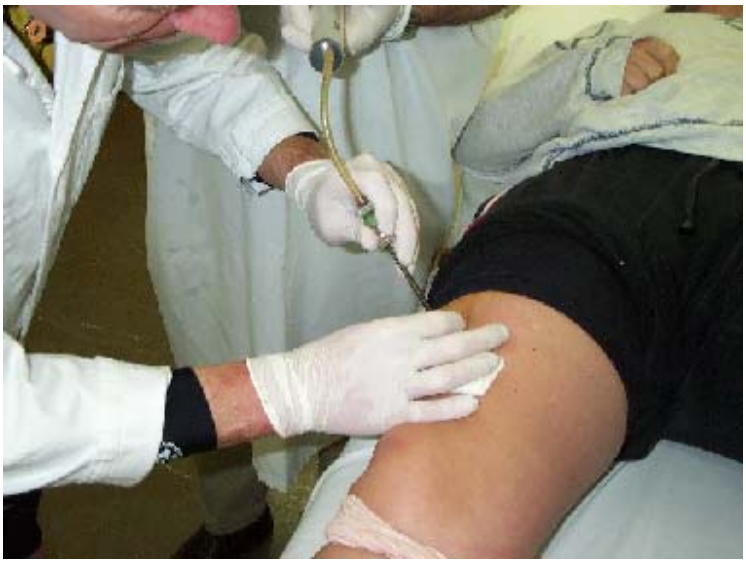
Appendix 26. Statistic Data of mRNA expression and Protein abundance

mRNA		Effect size	Observed Power	Significant Effects	Protein		Effect size	Observed Power	Significant Effects
α_1	treatment	0.03	0.08	0.609	α_1	treatment	0.115	0.162	0.309
	time	0.09	0.21	0.477		time	0.101	0.245	0.401
	treatment * time	0.12	0.29	0.314		treatment * time	0.122	0.295	0.313
α_2	treatment	0.000	0.05	0.994	α_2	treatment	0.238	0.323	0.128
	time	0.060	0.153	0.636		time	0.028	0.093	0.854
	treatment * time	0.238	0.610	0.058		treatment * time	0.276	0.706	0.074
α_3	treatment	0.067	0.112	0.441	α_3	treatment	0.048	0.093	0.515
	time	0.356	0.867	0.007		time	0.081	0.198	0.508
	treatment * time	0.228	0.582	0.069		treatment * time	0.132	0.322	0.274
β_1	treatment	0.058	0.103	0.474	β_1	treatment	0.198	0.266	0.170
	time	0.071	0.177	0.565		time	0.331	0.824	0.012
	treatment * time	0.211	0.537	0.089		treatment * time	0.057	0.147	0.654
β_2	treatment	0.001	0.051	0.923	β_2	treatment	0.062	0.106	0.461
	time	0.132	0.321	0.275		time	0.206	0.524	0.096
	treatment * time	0.076	0.187	0.537		treatment * time	0.080	0.196	0.512
β_3	treatment	0.176	0.237	0.199	β_3	treatment	0.015	0.063	0.718
	time	0.384	0.907	0.004		time	0.221	0.695	0.034
	treatment * time	0.181	0.454	0.140		treatment * time	0.233	0.597	0.063
Total α		2-tailed paried T-test		0.017					
Total β				0.041					

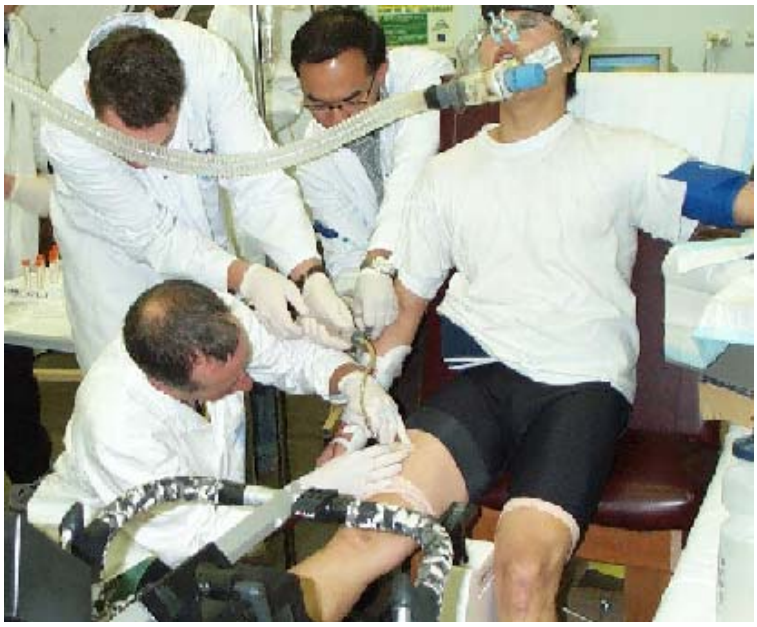
Appendix 27. photos from experiment day.



Incremental cycling test



Muscle biopsy at rest



Muscle biopsy during cycling test

Blood sample processing

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