The Effect of Therapeutic Exercise

and Metabolic Acidosis on Skeletal Muscle

Wasting in Chronic Kidney Disease

By

Emma Louise Clapp

A Doctoral Thesis

Submitted in partial fulfillment of the requirements for the award

of Doctor of Philosophy of Loughborough University 2010

# Abstract

Muscle wasting and increased proteolysis is a major problem in chronic kidney disease (CKD). Exercise is potentially beneficial, but has been under-investigated in pre-dialysis CKD and could theoretically worsen acidosis through exercise-induced lactic acid generation. We therefore investigated effects of 6 months walking exercise with and without additional alkali therapy. 40 patients were recruited (23 male and 17 female, median age 58, range 20-83, mean eGFR±SEM $25.7 \pm 1.2 ml/min/1.73m^2$). 20 undertook walking exercise at a Borg Rating of Perceived Exertion Rate (RPE) of 12-14 for at least 30 minutes, 5 times a week. The other 20 continued with normal physical activity (non-exercising controls). In addition to standard oral bicarbonate therapy (STD), 10 patients in each group were randomised to receive additional bicarbonate (XS). Blood and vastus lateralis muscle biopsies were drawn at baseline, one and six months. 18 exercisers (including 8 in XS group) and 14 controls (6 in XS group) completed the 6 month study.

Exercise tolerance increased after 1 and 6 months in the exercisers, but not the controls, accompanied by a reduced acute lactate response in the XS, but not the STD exercising group. After 6 months of exercise, 9 intramuscular free amino acids showed striking depletion in the STD, but not XS bicarbonate group. This suggests an inhibition of active amino acid transporters, possibly the SNAT2 transporters that are inhibited by acidosis. Studies with cultured myotubes identified glucocorticoid as a possible mediator of acid's inhibitory effect on SNAT2.

The preservation of amino acid concentrations in the XS exercising group was accompanied by strong suppression of ubiquitin E3-ligases MuRF-1 and MAFbx which activate proteolysis through the ubiquitin-proteasome pathway. However, other anabolic indicators (Protein Kinase B activation and suppression of the 14kDa actin fragment) were unaffected in the exercising XS group. Possibly because of this, overall suppression of myofibrillar proteolysis (3-methyl histidine excretion) and increased lean body mass (DEXA) were not observed in the exercising patients. As XS alkali had no effect in non-exercisers, it is concluded that alkali effects in the exercisers arose by countering exercise-induced acidosis.

Sulphuric acid produced from the catabolism of sulphur-containing amino acids ingested in the diet is the main contributor to the daily titratable acid load and hence acidosis in CKD. In these patients the amount of sulphate excreted in urine over 24h varied widely between individuals. This directly correlated with 3-methyl histidine excretion suggesting that sulphate excretion may be a better clinical indicator of acidotic patients at long-term risk of cachexia than conventional measures such as venous bicarbonate. Studies with cultured myotubes confirmed that skeletal muscle is a source of sulphuric acid and showed that production of this acid is partly suppressed by L-Glutamine – a potential novel way to control acidosis in CKD.

**Keywords**: Chronic Kidney Disease, acidosis, exercise, bicarbonate, cachexia, SNAT2.

# Publications, Presentations and Posters

## Publications

Kosmadakis, G.C., Bevington, A., Smith, A.C., Clapp, E.L., Viana, J.L., Bishop, N.C. and Feehally, J. (2010). Physical Exercise in Patients with Severe Kidney Disease. *Nephron Clinical practice, 115(1),* c7-c16.

## Oral Presentations

Clapp, E., Kosmadakis, G., Smith, A.C., Viana, J., Shirreffs, S., Maughan, R., Feehally, J., and Bevington, A. Collapse of intramuscular free amino acid pools in patients with CKD4-5 after 6 months of walking exercise is prevented by alkali supplements. Presented at The *International Congress* on *Nutrition and Metabolism* in *Renal* Disease, 2010**.**

Clapp, E., Kosmadakis, G., Pawluczyk, I., Smith, A.C., Viana, J., Owen, P., John, S., McIntyre, C., Lee, V., Shirreffs, S., Maughan, R., Feehally, J., and Bevington, A. Endogenous sulphuric acid production rate is a strong predictor of myofibrillar protein degradation in patients with CKD4-5. Presented at The *International Congress* on *Nutrition and Metabolism* in *Renal* Disease, 2010**.**

Clapp, E., Butler, H., Kosmadakis, G., Brown, J., Nasim, Z., and Bevington. The response of the key anabolic L-Gln transporter SNAT2 to physiological stress signals in skeletal muscle cells. Rank Prize Meeting, Recent Advances in Nutrient Transport. 18th – 21st August, 2008.

## Posters

Clapp, E., Kosmadakis, G., Smith, A.C., Viana, J., Shirreffs, S., Maughan, R., Owen, P., John, S., McIntyre, C., Feehally, J., and Bevington, A. Combined walking exercise and alkali therapy in patients with CKD4-5 generates anabolic signals but no net suppression of muscle protein catabolism. Presented at the Renal Association, 2010.

Clapp, E., Tooley , L., Evans, K., Amin, A., Nasim , Z., Brown, J., Herbert , T., and Bevington, A. The acidosis-sensing amino acid transporter SNAT2 regulates the anabolic kinase mTOR in skeletal muscle cells through SNAT2's substrate L-Methionine. Presented at the Renal Association, 2010.

Clapp, E., Tooley , L., Evans, K., Amin, A., Nasim , Z., Brown, J., Herbert , T., and Bevington, A. The acidosis-sensing amino acid transporter SNAT2 regulates the anabolic kinase mTOR in skeletal muscle cells through SNAT2's substrate L-Methionine. Presented at The *International Congress* on *Nutrition and Metabolism* in *Renal* Disease**,** 2010**.**

Smith, A., Kosmadakis, G., John, S., Clapp, E., Bevington, A. McIntyre, C., and Feehally, J. A six month walking exercise programme for patients with CKD 4-5: effects on cardiovascular parameters, body composition and quality of life. Presented at The *International Congress* on *Nutrition and Metabolism* in *Renal* Disease**, 2010.**

Clapp, E., Kosmadakis, G., Pawluczyk, I., Viana, J., Smith, A., Feehally, J., Seymour, A., Lee, V., and Bevington, A. Endogenous sulphuric acid production rate is a strong predictor of myofibrillar protein degradation in patients with CKD Stage 4. Presented at the World Congress of Nephrology, 2009.

Clapp, E., Butler, H., Kosmadakis, G., Brown, J., Nasim, Z., and Bevington, A. The key anabolic L-Gln transporter SNAT2 is acutely inhibited by glucocorticoid stress in L6 skeletal muscle cells. Presented at the Renal Association, 2008.

Kosmadakis, G., Bevington, A., Smith, A.C., Clapp, E., Viana, J., Bishop, N., and Feehally.J. Effects of a 6 month exercise programme on uraemic symptoms and functional parameters in patients with chronic kidney disease. Presented at the World Congress of Nephrology, 2008

# Acknowledgements

# Table of Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| 3-MH | 3-Methyl Histidine |
| 4E-BP1 | Eukaryotic initiation factor 4E binding protein 1 |
| 5' TOP | 5 tract of pyrimidines |
| ADP | Adenosine diphosphate |
| ADX | Adrenalectomy |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| ANOVA | Analysis of variance |
| APS | *Ammonium Persulphate* |
| ATP | Adenosine triphosphate |
| $BaCl_2$ | Barium chloride |
| BCAA | Branched chain amino acids |
| BMI | Body mass index |
| BSA | Bovine Serum Albumin |
| BSP | beta-sulphinylpyruvate |
| $Ca^{2+}$ | Calcium |
| CA | Carbonic anhydrase |
| CamK | calmodulin-activated kinase |
| CHO | Carbohydrate |
| $Cl^-$ | Chloride |
| CKD | Chronic kidney disease |
| $CO_2$ | Carbon Dioxide |
| CSA | Cysteinesulphinate |
| CSAD | Cysteinesulphinate decarboxylase |
| Da | Dalton |
| DEPC | Diethylpyrocarbonate-treated |
| DEXA | Dual Energy X-ray Absorptiometry |
| DFBS | Dialysed Foetal Bovine Serum |

| | |
|---|---|
| DMEM | Dulbecco's Modified Essential Medium |
| DNA | Deoxyribonucleic acid |
| DdNTP | Dideoxynucleoside triphosphates |
| dNTP | Deoxynucleotide Triphosphates |
| ECL | Electrochemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| eEF | Eukaryotic elongation factor |
| EGTA | Ethylene glycol tetraacetic acid |
| eIF | Eukaryotic initiator factor |
| eRF | Eukaryotic release factor |
| FBS | Foetal bovine serum |
| FKBP38 | FK506-binding protein, 38kDa |
| FMOC | Fluorenylmethyloxycarbonyl chloride |
| FOXO | Forkhead family of transcription factors |
| g | Grams |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GBM | Glomerular basement membrane |
| GFR | Glomerular filtration rate |
| GDP | Guanosine diphosphate |
| GRE | Glucocorticoid response element |
| GTP | Guanosine triphosphate |
| GUSB | beta-glucuronidase |
| $H^+$ | Hydrogen ion |
| $H_2O$ | Water |
| HBS | Hepes Buffered Saline |
| HBSS | Hanks Balanced Salt Solution |
| $HCO_3^-$ | Bicarbonate |
| HPLC | High Performance Liquid Chromatography |
| IL-6 | Interleukin-6 |

| | |
|---|---|
| IRS-1 | Insulin receptor substrate 1 |
| IRS-2 | Insulin receptor substrate 2 |
| $K^+$ | Potassium |
| kg | Kilogram |
| kDa | Kilodalton |
| kPa | kilopascal |
| L | Litre |
| LBM | Lean body mass |
| MAFbx | Muscle Atrophy F-box |
| MAPK | mitogen-activated protein kinase |
| MeAIB | Methylaminoisubyrate acid |
| MEM | Minimum Essential Medium |
| Met-tRNA$_i$ | Methionyl initiator - tRNA |
| min | Minute |
| mg | Milligram |
| mL | Milliliter |
| mmol | Millimole |
| mTOR | Mammalian target of rapamycin |
| mTORC1 | mTOR complex 1 |
| mTORC2 | mTOR complex 2 |
| MuRF-1 | Muscle Ring Finger Protein 1 |
| $Na^+$ | Sodium |
| NaOH | Sodium hydroxide |
| $Na_2SO_4$ | Sodium Sulphate |
| $NH_3$ | Ammonia |
| $NH_4^+$ | Ammonium |
| $NH_4Cl$ | Ammonium chloride |
| NICE | National Institute for Health and Clinical Excellence |
| NMR | ***Nuclear magnetic resonance*** |

| | |
|---|---|
| NRF | Nuclear respiratory factors |
| OPA | Orthophthalaldehyde |
| $P^{70}S6K$ | 70-kDa ribosomal protein S6 kinase |
| PCA | Perchloric acid |
| $PCO_2$ | Carbon dioxide partial pressure |
| PCR | Polymerase chain reaction |
| PCr | Phosphocreatinine |
| PCT | Proximal tubular cell |
| PCT | Proximal convoluted tubule |
| PDK1 | 3-Phosphoinositide-dependent kinase 1 |
| PGC-1α | Proliferator-activated receptor γ coactivator 1α |
| Pi | Inorganic phosphate |
| PI3-K | Phosphatidylinositol 3 kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-trisphosphate |
| $PIP_3$ | Phosphatidylinositol 3,4,5-trisphosphate |
| PKB | Protein kinase B |
| PMSF | Phenylmethanesulphonylfluoride |
| PTH | Parathyroid hormone |
| PVDF | Polyvinylidine Fluoride |
| Raptor | Regulatory associated protein of mTOR |
| REDD-1 | DNA damage responses protein 1 |
| Rheb | Ras homolog enriched in brain |
| Rictor | Rapamycin insensitive companion of mTOR |
| RNA | Ribonucleic acid |
| | (mRNA messenger RNA; tRNA transfer RNA; SiRNA small interfering RNA) |
| RPE | Rating of Perceived Exertion |
| RPL30 | Ribosomal protein L30 |
| rpS6 | Ribosomal protein S6 |
| rpS6K | Ribosomal protein S6 kinase |

| | |
|---|---|
| S6K1 | S6 Kinase 1 |
| S6K2 | S6 Kinase 2 |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SH-2 | Src-homology-2 |
| SNAT | Sodium dependent neutral amino acid transporter |
| $SO_4^{2-}$ | Sulphate |
| TCA | Trichloroacetic Acid |
| TEMED | Tetramethylethylenediamine |
| Tfam | Mitochondrial transcription factor A |
| TNF-α | Tumor necrosis factor - alpha |
| TTBS | Tris-Tween Buffered Saline |
| TSC1 | Tuberous Sclerosis complex 1 |
| TSC2 | Tuberous Sclerosis complex 2 |
| Tris | Tris(hydroxymethyl)aminomethane |
| Ub | Ubiquitin |
| UIC | Universal inhibitor cocktail |
| UPS | Ubiquitin-proteasome system |

Chapter 1


General Introduction

## 1.1 Basic muscle physiology

A skeletal muscle such as rectus femoris, is surrounded by a layer of fascia and contains fascicles (bundles of fibres). Muscle fibres are long, cylindrical, multinucleated cells that lie with their long axes in parallel surrounded by a membrane called the sacrolemma and by a thin sheath of fibrous tissue called the endomysium that divides muscle into individual myofibrils (Figure 1.1). The intracellular structure of a muscle fibre contains sarcoplasm, mitochondria, myoglobin, and the main contractile element of muscle, the myofibrils that vary in length from only a few millimetres to over 30cm laid end to end and are surrounded by an extensive sarcoplasmic reticulum (Maughan et al. 1997a; Silverthorn, 2001a) (Figure 1.2). A muscle can contain a thousand or more myofibrils (Silverthorn, 2001a) that are made up of a continuous series of sarcomeres (contractile units of muscle) and have a striated appearance that can be seen by light microscopy, comprising alternating light and dark bands caused by the arrangement of the myofilaments in each repeating sarcomere (demonstrated in Figure 1.2). The myofilaments overlap each other to a lesser or greater extent depending on whether the muscle is relaxed or contracted. The light band is represented in this diagram by the I band and comprises the thin filament actin and two regulatory proteins troponin and tropomyosin that all extend part way into the A band, the dark band. The A band contains the thick filament myosin and is the only filament to be found in the H zone (Maughan et al. 1997a; Silverthorn, 2001a).
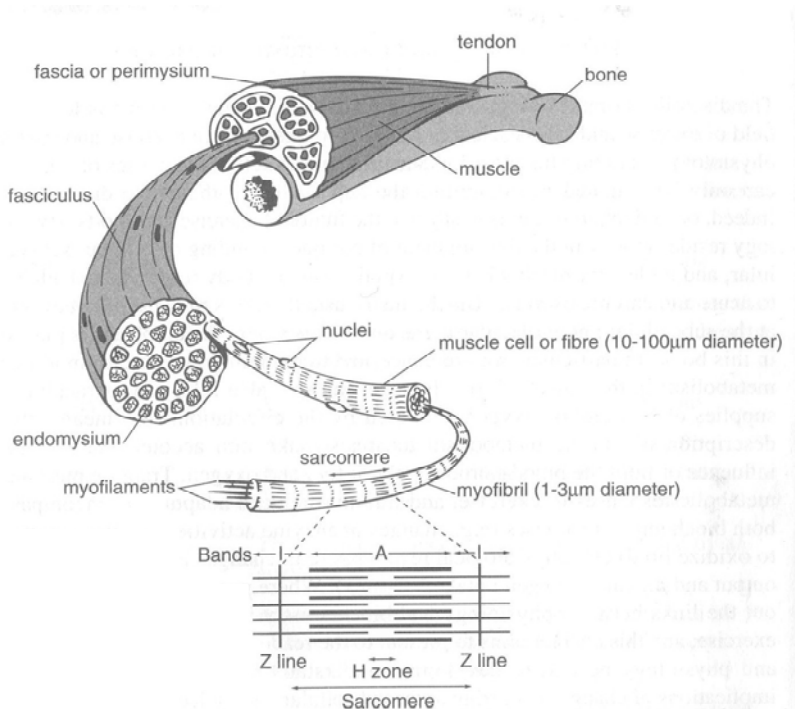
**Figure 1.1. Skeletal muscle anatomy.** Described in the text in Section 1.1.

Diagram copied from Maughan et al. (1997a), Pg 2.
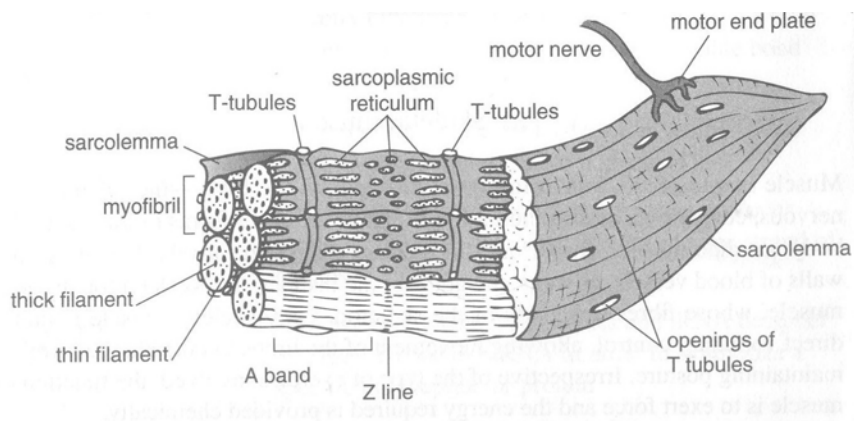


**Figure 1.2. Muscle fibre ultrastructure**

Diagram copied from Maughan et al. (1997a), Pg2.

Actin in its monomeric form is a globular protein (G-actin) and usually polymerizes to form long chains or filaments of fibrous actin (F-actin) and two F-actin molecules twist together to form a helix, creating the thin filaments. The myosin molecule has two globular heads, a neck with an elastic hinge region that allows the head to swivel around the point of attachment, and a tail

region. The globular head has ATPase activity and the ability to bind actin. Each myosin molecule comprises two heavy protein chains that intertwine to form a coiled tail, and a pair of heads protruding in opposite directions. The thick filaments are arranged so that the myosin heads are clustered at the ends and the middle portion (H zone) contains the myosin tails. The myosin molecules are anchored to the Z line by titin, acting to stabilise these filaments (Tortora and Grabowski, 1996a).

## 1.2 Muscle contraction

Individual muscle fibres are usually innervated by just one nerve that excites skeletal muscle through the release of acetylcholine across the motor end plate, ultimately causing the release of $Ca^{2+}$ from the sarcoplasmic reticulum. $Ca^{2+}$ is bound by troponin C, altering the troponin-tropomyosin complex and thereby displacing tropomoysin from the actin binding site on the myosin molecule allowing crossbridge formation to occur. This binding of myosin to the actin molecule results in the myosin head changing from its activated configuration to its 'cocked' shape causing the myosin heads to pull on the thin filament and the filaments to slide past each other. This is known as the power stroke and releases ADP and inorganic phosphate ($P_i$) (from the prior hydrolysis or ATP) from the myosin head. A new ATP molecule can then bind, reducing the affinity of myosin for actin binding and causing detachment of the crossbridge. The ATP is then hydrolysed, returning the myosin head to its activated state in which it is able to bind another actin molecule further along the filament. This cycle repeats many times in the presence of $Ca^{2+}$. In the relaxed state, $Ca^{2+}$ is sequestered from the sarcoplasm back into the sarcoplasmic reticulum via the ATP dependent calcium pump, returning the troponin-tropomyosin complex to its inhibitory position blocking cross bridge formation (Tortora and Grabowski, 1996a).

## 1.3 Muscle buffering systems

During high intensity exercise, lactic acid produced by anaerobic glycolysis dissociates to lactate and $H^+$ resulting in a fall in intracellular pH. Skeletal muscle has a large buffering capacity ($\beta m$) that reduces the rate of intracellular pH decrements therefore delaying fatigue. A high $\beta m$ is associated with good sprinting performance and it appears it can be increased with training (Weston et al. 1997).

In humans, the main skeletal muscle buffering systems are; the bicarbonate buffer system , and intramuscular phosphates, protein bound Histidine and Histidine containing dipeptides (carnosine, anserine and balenine) which make up the non-bicarbonate system (Bishop et al. 2009). In humans there is no balenine in skeletal muscle and anserine is only found in very low concentrations and so the most important dipeptide in muscle buffering in humans is carnosine (Abe, 2000).

Skeletal muscle cells have a high concentration of phosphate related compounds, which have a very high capacity of buffering $H^+$ and contribute approximately 20% to the non-bicarbonate buffering system (Hultman and Sahlin, 1980). Proteins containing Histidine residues have been suggested to contribute about 30-70% to the total muscle buffering capacity (Abe, 2000; Hultman and Sahlin, 1980). The majority of protein involved in skeletal muscle buffering is soluble proteins with little involvement from contractile proteins (Abe, 2000). Histidine is the only amino acid that is capable of exerting a buffering function in the physiological pH, owing to the imidazole side ring found in Histidine residues. Imidazole groups are potent buffering constituents as they have a pK value that is close to physiological pH and so one of the two nitrogen molecules of this group can be protonated in the physiological pH range (Abe, 2000).

It has been suggested that free Histidine contributes little to the βm as it is in low concentration in skeletal muscle (Parkhouse and McKenzie, 1984) and Histidine containing dipeptides might be more important due to their larger intramuscular concentration (Bergström et al. 1974). A role for carnosine was established by Smith in 1938 who demonstrated that the imidazole side ring had a pKa of 6.83, which made it a suitable buffer at physiological pH. Carnosine is a dipeptide that is synthesised in skeletal muscle from β-alanine and L-Histidine catalysed by carnosine synthase (Sale et al. 2010). A role for carnosine in intramuscular buffering was first demonstrated by early studies in fish (Abe, 2000) Whilst the intramuscular concentration of carnosine is lower in humans compared to other species, with reported values in the vastus lateralis of 16-20 mmol/kg dw (Harris et al. 1990) these concentrations do vary between fibre types and can be changed with training. Carnosine concentrations have been observed to be twice as high in type II fibres compared to type I (Harris et al. 1998) and Parkhouse and collegues (1985) found the vastus lateralis carnosine content was higher in sprinters and rowers than in marathon runners and untrained individuals and Suzuki et al. (2002) found a significant correlation between carnosine concentration and power output in a wingate test. Furthermore,

Hill et al. (2007) showed that 10 weeks of β-alanine supplementation resulted in an 80% increase in muscle carnosine and a significant improvement in cycling capacity at 110% peak power output. Taken together, this evidence suggests muscle carnosine might be an important factor in determining performance during high intensity exercise and plays an important role in skeletal muscle buffering capacity.

## 1.4 Basic renal physiology

### 1.4.1 Renal anatomy

The two kidneys are located either side of the spine between the posterior wall of the abdominal cavity and the peritoneum at the level of the eleventh and twelfth ribs (Tortora and Grabowski, 1996b). They are about 10cm long and 5cm wide, weigh approximately 150g, are bean shaped and are encased in a fibrous outer capsule called the renal capsule. The interior of the kidney is arranged in two layers, the outer cortex and the inner medulla. The outer cortex contains the renal corpuscle (made up of the glomerulus surrounded by Bowman's capsule), renal tubules, cortical collecting ducts and vasculature. The medulla contains pyramids of straight tubules (medullary pyramids). The apex of each pyramid forms a papilla, which drains urine into the minor calyx then the major calyx (Kriz and Elger, 2007) followed by the renal pelvis and finally the proximal ureter which transports urine to the bladder.
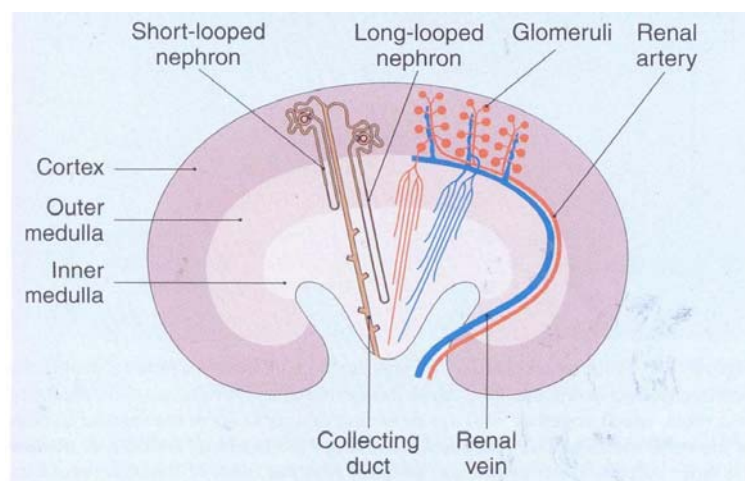


**Figure 1.3. Cross section through a kidney**

Figure copied from Kriz and Elger, (2007), Pg 1.

The nephron is the functional unit of the kidney and regulates salt and water balance through excretion and reabsorption. The nephron consists of the renal corpuscle (glomerulus and Bowman's capsule), the proximal tubule, loop of Henle, distal convoluted tubule and the collecting duct. A healthy kidney usually contains approximately 1 million nephrons (Shirley et al. 2007). There are two types of nephron, which are classified according to the location of the renal corpuscle. Cortical nephrons have their renal corpuscle in the outer renal cortex and have very short loops of Henle. Juxtamedullary nephrons have their renal corpuscle in the medulla and have long loops of Henle that extend deep into the medulla (Lote, 1990).

Renal blood flow is approximately 1200mL/min receiving approximately 20-25% of the cardiac output (Tortra and Grabowski, 1996b) and of this, 125-130mL of plasma is filtered into Bowman's capsule every minute resulting in the production of primary urine. Blood flow to the kidney is usually through a single renal artery entering the kidney at the renal hilus. The renal artery branches to form interlobar arteries which enter the renal cortex and branch further into afferent arterioles. Each nephron receives blood through one afferent arteriole which divides to form the glomerulus (Lote, 1990). Here water and salts are filtered out of the capillary and into the lumen of the tubule, passing along the length of the nephron until, at the end, the remaining filtrate is excreted as urine. The blood leaves the glomerulus and passes into an efferent arteriole and passes into a network of peritubular capillaries and vasa recta which surround the renal tubules. Any filtrate that is reabsorbed from the lumen, returns to the circulation through these capillaries. Eventually these capillaries drain to the renal vein that leaves the kidney through the renal hilus.
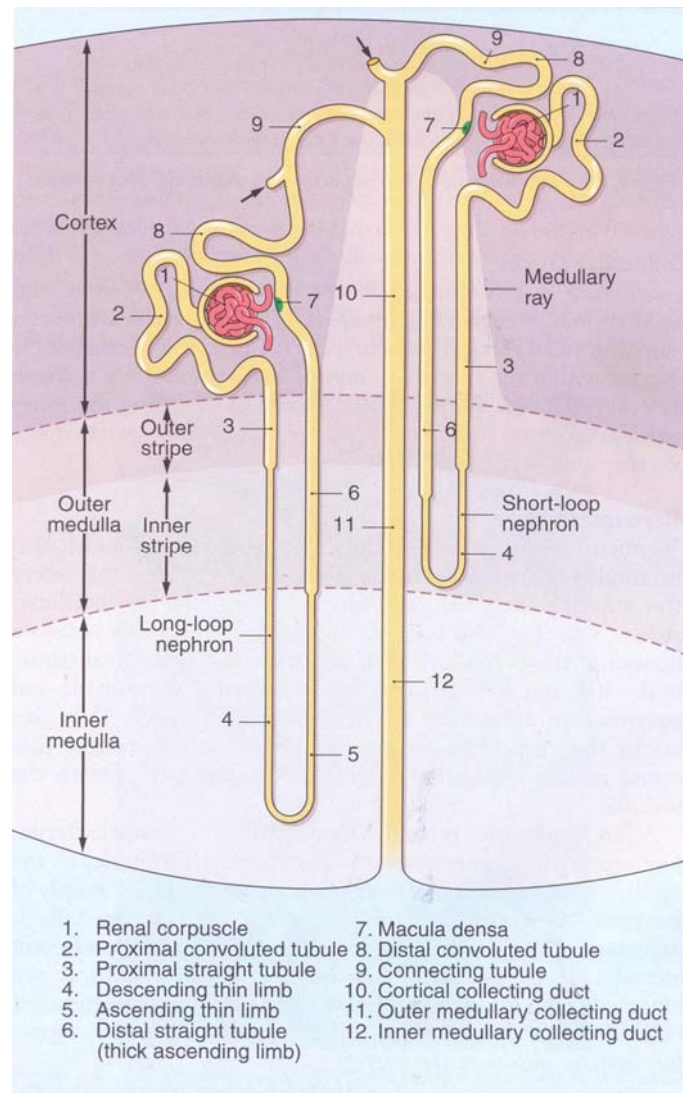
**Figure 1.4. Nephrons and the collecting duct of the kidney**

Figure copied from Kriz and Elger (2007), Pg 1.

**1.4.2 Function of the kidney**

The main function of the kidneys is to maintain homeostatic regulation of the water and ion content of the blood by balancing reabsorption and excretion of solutes to maintain fluid and electrolyte balance. They also serve in the regulation of osmolarity and acid-base balance, excretion of waste and foreign materials, and production and secretion of hormones.

### 1.4.3 Renal processes

The three processes of the kidney are filtration, secretion and reabsorption. Filtration occurs at the renal corpuscle in the cortex of the kidney and the processes of reabsorption and secretion occur in the tubules.

### 1.4.4 Glomerular filtration

Filtration is a relatively non-specific process creating an ultrafiltrate of the plasma containing water and dissolved salts and is almost protein free. A normal glomerular filtration rate is 125ml/min producing 180L/day of primary filtrate. Blood pressure forces water and small solutes out of the capillary through three barriers, the endothelial fenestrations, the basement membrane and the slit diaphragms of the podocytes (glomerular epithelial cells). The resulting solution enters Bowman's capsule and becomes glomerular filtrate. The molecular size, along with shape and charge of the molecule, is the main determinant as to whether a molecule is filtered into Bowman's capsule, or remains in the glomerular capillaries (Lote, 1990).

### 1.4.5 Components of the glomerular filtration barrier

The first of these barriers is the layer of flattened endothelial cells of the fenestrated glomerular capillaries. This fenestrated area covers 20-50% of the endothelial cell surface and allows high permeability to water and small solutes. The filter allows the free movement of molecules up to a molecular weight of 7000Da and molecules up to a weight of 70000Da can be filtered. (Lote, 1990) There is much controversy over the importance of this barrier in the selective filtration process due to uncertainty over the effective size of the fenestrae (Haraldsson et al. 2008). By microscopy these appear large enough to allow the filtration of serum albumin. However, recent evidence indicates that the fenestrae may be an important barrier, due to the cell coat that fills the fenestrae i.e the glycocalyx that contains negatively charged proteoglycans, glycosaminoglycans and plasma proteins that tend to repel plasma proteins (Haraldsson and Sörensson, 2004). The thick basement membrane of the glomerulus (GBM) is the second barrier to filtration and was formerly considered to be the most important barrier (Lote, 1990). It forms a continuous layer allowing filtration of molecules depending upon the molecules' size, shape and charge. It lies in between the glomerular endothelium and the epithelial lining of Bowman's capsule. It contains

collagen like protein and negatively charged proteoglycans (Silverthorn, 2001b). Recent findings suggest the GBM lacks charge selectively and is therefore not such an important contributor to selective filtration (Haraldsson and Sörensson, 2004). The final layer to the filtration barrier is the epithelium of Bowman's capsule containing specialized epithelial cells called podocytes. The podocyte is now regarded as the most important barrier in maintaining selective permeability (Haraldsson and Sörensson, 2004), as failure of the podocyte is associated with proteinuria, initiation of progressive renal disease and the maintenance of progression to end stage renal failure (Pavenstädt et al. 2003). Podocytes have long foot-like processes projecting from the cell body called pedicels. These processes encircle the basement membrane of the glomerulus and processes of adjacent podocytes interdigitate, creating filtration slits that are bridged by the slit diaphragm. The slit diaphragm has recently been discovered to contain many specialised proteins, one of which is nephrin, and mutations in this protein have been found to give rise to severe proteinuria and congenital nephritic syndrome of the Finnish type (Kestila et al. 1998). The slit diaphragm and luminal membrane are covered by sialoglycoproteins giving the podocytes a strong negative charge and preventing the movement of medium sized molecules (Pavenstädt et al. 2003).

**1.4.6 Tubular reabsorption and secretion**

Approximately 180L of plasma are filtered each day and most of this volume is reabsorbed by processes which vary along the length of the nephron. Transport processes within the nephron includes simple and facilitated diffusion and primary or secondary active transport processes. Passive transport is allowed by the concentration gradient that is maintained by the $Na^+$, $K^+$-ATPase pump on the basolateral membrane.

The bulk of the filtered $Na^+$, $Cl^-$, $K^+$ and $HCO_3^-$ are reabsorbed at the proximal tubule facilitated by the microvilli on the apicial surface of the epithelial cells which create a large surface area for reabsorption. The concentration of $Na^+$ in the filtrate is approximately 140mmol/l and is much lower in the cytosol of the epithelial cells favouring movement of $Na^+$ from the tubule into the cell through open leak channels. Approximately 20% enters the peritubular interstitium via $Na^+$ pumps on the basolateral membrane whilst the remaining re-enters the tubule via tight junctions (paracellular pathways) (Shirley et al. 2007). The secretion of $H^+$ into the lumen plays an important role in the reabsorption of $HCO_3^-$ (see Section 1.5 for more detail). The walls of the

proximal tubule are highly permeable to water and approximately 60% of the filtered water is reabsorbed here by diffusion due to the decrease in osmolarity in the tubular lumen following solute reabsorption. There is also near complete reabsorption of glucose, amino acids and low weight molecular proteins that were able to cross the glomerular barrier. This water reabsorption results in a concentration gradient down which $Cl^-$, $K^+$ and urea diffuse via paracellular pathways. Glucose and amino acids are co-transported with $Na^+$ into the epithelial cell against their concentration gradient and diffuse into the peritubular interstitium. Small proteins enter the epithelial cell by endocytosis and are digested inside the cell and released as amino acids (Shirley et al. 2007).

As the filtrate reaches the loop of Henle, the majority of the reabsorption of water and solutes has already taken place. Approximately 25% of the filtered water is reabsorbed in the thin descending limb, which is freely permeable to water, but relatively impermeable to $Na^+$. $Na^+$ is reabsorbed by the $Na^+$-$K^+$-$2Cl^-$ active transporter in the thin ascending limb that is impermeable to water. The U shape of the loop of Henle and the differences in the permeability of the limbs to water and $Na^+$ are responsible for the countercurrent multiplier which generates the medullary osmotic gradient (Shirley et al. 2007).

The distal nephron and collecting duct are responsible for the reabsorption of the remaining $Na^+$ and $K^+$ via active transport mechanisms in the principal cells. These sections of the nephron are permeable to water in the presence of vasopressin that increases permeability by the insertion of channels (aquaporins) into the apical membrane in response to substrate receptor binding, allowing the diffusion of water out of the tubule. $Ca^{2+}$ reabsorption here is regulated by parathyroid hormone (PTH) and vitamin D. The presence of PTH activates $Ca^{2+}$ channels in the apical membrane and a $Ca^{2+}$-ATPase pump on the basolateral membrane. The intercalated cells in the late distal tubule secrete $H^+$ or $HCO_3^-$ into the filtrate that becomes final urine (Shirley et al. 2007).

## 1.5 Acid-base balance

Acid-base balance or pH homeostasis is an essential regulatory process of the body maintaining $H^+$ concentration at an acceptable level, which in a healthy individual, leads to an arterial plasma pH within the range 7.35-7.45 (Tortora and Grabowski, 1996c). A conventional western diet is

high in protein and sulphur amino acids that, when metabolised, produce sulphuric acid (Suliman et al. 1996) and result in the generation of 40-70mEq of $H^+$ per day (Sebastian et al. 2002). This must be buffered, as deviations from the acceptable pH range would denature proteins and disrupt normal body processes (Silverthorn, 2001c). The body has three mechanisms by which to eliminate acid from the body: buffers in the blood that are able to respond very quickly to changes in pH, respiratory responses that deal with 75% of the generated acid, and finally, the renal system. The renal system is much slower that the other two, but is capable of dealing with any remaining disturbance to pH. The main focus here will be the role of the kidneys in maintaining acid-base balance.

Non-volatile acid (acid produced from sources other than carbon dioxide, for example lactic acid and sulphuric acid), is not eliminated by the lungs and is buffered quickly in the blood by bicarbonate ($HCO_3^-$), producing water and carbon dioxide, the concentration of which does not change from the level set and maintained by respiratory control. The function of the kidney is to excrete titratable acid, to regenerate bicarbonate that is lost in this process, and to reabsorb the filtered load of $HCO_3^-$ (Koeppen, 2009).

The reabsorption of $HCO_3^-$ occurs primarily in the proximal tubule, with the remainder being reabsorbed at the thick ascending loop of Henle and a small proportion by the colleting duct (Koeppen, 2009). Filtered bicarbonate is unable to cross the apical membrane of the proximal tubular cell (PTC), so in order to be reabsorbed it combines with $H^+$ which is secreted into the lumen by the apical $Na^+$-$H^+$ antiporter in exchange for a filtered $Na^+$, to form carbonic acid ($H_2CO_3$). Carbonic anhydrase (CA) IV present in the brush border catalyses the dissociation of $H_2CO_3$ to $H_2O$ and $CO_2$. These readily diffuse across the apical membrane into the tubular epithelial cell where, under the influence of CA II, $CO_2$ is hydrated to produce $HCO_3^-$ and $H^+$. The $H^+$ is secreted back into the lumen by the apical $Na^+$-$H^+$ antiporter repeating the cycle. Three molecules of $HCO_3^-$ are transported out of the cell across the basolateral membrane with one molecule of $Na^+$ via the $Na^+$-$HCO_3^-$ symporter. The low intracellular $Na^+$ concentration in the proximal convoluted tubule (PCT) is maintained by the active $Na^+$-$K^+$ ATPase sodium pump on the basolateral membrane that pumps $2K^+$ into the cell for every $3Na^+$ transported out of the cell. This sets up the concentration gradient between the cytosol and the tubular lumen that is utilised by the $Na^+$-$H^+$ antiporter on the apical membrane to bring 1 $Na^+$ into the cell in exchange for 1

$H^+$ secreted into the tubular lumen. The net result is the reabsorption of one $HCO_3^-$ and one $Na^+$ for every $H^+$ secreted into the lumen (Silverthorn, 2001c; Koeppen, 2009).

Although the reabsorption of the filtered load of $HCO_3^-$ is an important process, it does not fully replace the $HCO_3^-$ lost in the titration of acid, so new $HCO_3^-$ must be generated through ammoniagenesis. This is the main mechanism by which the kidney regulates acid base balance, accounting for 90% for the acid excreted into the urine (Karim et al. 2005) and subsequent generation of $HCO_3^-$, a process that occurs in the PCT. Glutamine from the peritubular capillaries is deaminated to glutamate via glutaminase and then converted to α-ketoglutarate by glutamate dehydrogenase. Further catabolism of α-ketoglutarate results in the generation of two $NH_4^+$ and two $HCO_3^-$ per molecule of glutamine. The new $HCO_3^-$ exits the cell via the $Na^+$- $HCO_3^-$ symporter and enters the blood and $NH_4^+$ is secreted from the PTC via a $Na^+$-$H^+$ antiporter taking the place of $H^+$ (Karim et al. 2005). Between 40-80% of the $NH_4^+$ is reabsorbed at the thick ascending loop of Henle via the $Na^+$, $K^+(NH_4^+)2Cl^-$ cotransporter, on which $NH_4^+$ is substituted for $K^+$. $NH_4^+$ is also reabsorbed through movement along the paracellular pathway resulting in an accumulation of total ammonia ($NH_4^+$ and $NH_3$) in the medullary interstitium. In order to successfully buffer $H^+$, $NH_4^+$ must be excreted in the urine: if it returns to the blood, it will be metabolised by the liver to urea, generating $H^+$. $NH_4^+$ is secreted back into the tubule at the collecting duct via $NH_4^+$-$H^+$ antiporters and is excreted into the urine. The process of ammoniagenesis is up-regulated in metabolic acidosis resulting in a much larger ammonia excretion. This up-regulation is due to increased expression stimulated by acidosis of the mitochondrial enzymes glutaminase, glutamate dehydrogenase and cytosolic phosphoenolpyruvate carboxykinase (Karim et al. 2005).

As the filtrate passes down the nephron, the reabsorption of water results in an increased concentration of the remaining $HCO_3^-$, which was not removed at the proximal tubule. The majority of this is reabsorbed at the thick ascending loop of Henle, by a similar process to that in the proximal tubule (Koeppen, 2009). The type A intercalated cells of the distal tubule reabsorb any remaining $HCO_3^-$ in the form of $CO_2$ and $H_2O$ as the result of $H^+$ secretion into the tubule. These cells have a high concentration of CA converting $CO_2$ into $H^+$ and $HCO_3^-$, which leaves the cell via a $HCO_3$-$Cl^-$ antiport exchanger on the basolateral membrane back into the blood and the remaining proton is secreted back into the lumen (Koeppen, 2009). Net acid excreted is equal

to the sum of titratable acid plus $NH_4^+$ excretion minus the loss of $HCO_3^-$ in the urine. Free $H^+$ accounts for very little of the excreted acid and is not included in this equation.

## 1.6 Chronic Kidney Disease

Normal kidney function is defined by a glomerular filtration rate (GFR) of approximately 130ml/min for men and 120ml/min for women (Gul et al. 2007). Chronic Kidney Disease (CKD) is defined as kidney damage, or a reduction in GFR to <60ml/min for three months or more, irrespective of the initial cause (Kossi and Nahas, 2007) and is generally due to a reduction in the nephron number (Gul et al. 2007). CKD has been classified into five stages determined by GFR (Table 1.1)

**Table 1.1 Classification of Chronic Kidney Disease.** Adapted from Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines (Kossi and Nahas, 2007).

| Stage | Description | Estimated GFR (ml/minute) | Action |
|---|---|---|---|
| 1 | Kidney damage , normal or increased GFR | ≥90 | Diagnose and treat, slow progression |
| 2 | Kidney damage with mild decrease in GFR | 60-89 | Estimate Progression |
| 3 | Moderate decrease in GFR | 30-59 | Evaluate and treat complications |
| 4 | Severe decline in GFR | 15-29 | Prepare for renal replacement therapy |
| 5 | Kidney Failure or End Stage | <15 | Initiate renal replacement therapy |

The prevalence of CKD is increasing and it is now estimated that approximately 26 million Americans have been diagnosed with CKD, 13% of the population (Bronas, 2009). The prevalence of CKD is lower in the UK with an estimated 8.8% of the population suffering with symptomatic CKD (The Association of Public Health Observatories, 2009), but the number of patients reaching end stage renal failure increased by 4.4% from 2007-2008 (Farrington et al.

2008). The rise in prevalence is most likely due to an aging population and the type II diabetes mellitus epidemic that is occurring. The early stages of CKD are usually asymptomatic and for this reason it is not unusual that it remains undetected until progression occurs. The glomerular barrier prevents the filtration of high molecular weight proteins and any small proteins that are filtered are reabsorbed by the proximal tubule resulting in very small amounts of protein appearing in the urine. Detection of protein in the urine (proteinuria) is usually the first sign of CKD (D'Amico and Bazzi, 2003) indicating either damage to the glomerular barrier allowing filtration of larger protein molecules, or to the proximal tubules preventing the reabsorption of the smaller protein molecules.

Hypertension, obesity, diabetes, hyperlipidaemia and smoking are all risk factors for development of CKD in the general population (Kossi and Nahas, 2007). Hypertension is one the main causes of progression of CKD, by the transference of systemic pressure to the glomerular capillary beds resulting in glomerular hypertension which causes glomerulosclerosis. Therefore blood pressure in these patients is kept under tight control to help slow this progression. The appearance of proteinuria (>500mg/day) is also associated with a faster progression to end stage renal failure which can be controlled by diet and angiotensin receptor blockers (Kossi and Nahas, 2007).

## 1.7 Metabolic acidosis

Metabolic acidosis is reported in the majority of CKD patients when GFR declines to less than 20-25% of normal i.e. CKD Stage 4 onwards. The degree of acidosis usually correlates with the severity of the renal failure (Kraut and Kurtz, 2005) and is the result of a reduced ability to excrete ammonia and titratable acids in the face of a reduced reabsorption and synthesis of bicarbonate (Cibulka and Racek, 2007). Metabolic acidosis is diagnosed when the plasma bicarbonate concentration decreases from the normal range of 24-30mmol/l to 12-22mmol/l. This decrease severely impairs the ability of the blood to buffer $H^+$ quickly. As renal failure progresses to less than 15ml/minute there is an increase in anion gap acidosis due to a decrease in the excretion of non-volatile acid. This acidosis seen in advanced renal failure is termed uraemic metabolic acidosis (Cibulka and Racek, 2007). Metabolic acidosis has been identified as a uraemic toxin because correction results in the amelioration or elimination of many complications of CKD (Mitch, 2006), including a negative nitrogen balance and skeletal muscle wasting (Mitch, 2006; see Section 1.8), anorexia (Kovacic et al. 2003), bone disease (Lefebvre et

al. 1989), negative effects upon cardiac function including suppression of myocardial contractility (Marsiglia et al. 1973) and impaired peripheral vasoconstriction and dilation (Narins and Cohen, 1987). Sodium bicarbonate is a successful treatment of metabolic acidosis (Movilli et al. 2009), but is undesirable due to the associated sodium and water retention and possible exacerbation of hypertension (Kraut and Kurtz, 2005) in patients where blood pressure must be kept under tight control to help reduce progression of the renal disease.

**1.7.1 The source of the titratable acid load - sulphur metabolism**

The dominant contributor to the titratable (non-volatile) acid load, whose excretion fails in uraemic metabolic acidosis, is catabolism of the sulphur amino acids L-cysteine and L-methionine. These are irreversibly metabolised primarily by the liver, and to a lesser extent by the kidneys and brain, to cysteinesulphinate (CSA) via the enzyme cysteine dioxygenase (Suliman et al. 1996; Stipanuk, 2004) (Figure 1.5). CSA can have two fates: it can either be converted to hypotaurine and ultimately taurine by decarboxylation, or by transamination to pyruvate and sulphate. Skeletal muscle also has the ability to convert cysteine to taurine and sulphuric acid (accounting for up to 10% of the whole body sulphate production (Ensuna et al. 1993)), but here the mechanism is less clear because the activity of cysteine dioxygenase is very low in this tissue (Ensuna et al. 1993). The conversion of CSA to sulphate also results in the production of two moles of $H^+$ per mole of cysteine or methionine and is therefore considered to be an acid generating branch of the pathway generating sulphuric acid (Stipanuk et al. 2006; Coloso and Stipanuk, 1989; Bella and Stipanuk, 1995). This branch generates most of the daily titratable acid load of around 40-70mEq of $H^+$ each day in humans (Sebastian et al. 2002), and in individuals with renal impairment is a major contributor to uraemic metabolic acidosis (Cibulka and Racek, 2007). This acidosis is a significant clinical problem in CKD patients and an important contributor to the muscle wasting that is frequently seen in CKD (Mitch, 2006) (see Section 1.8.1). Cysteine is also used in the biosynthesis of glutathione, a key physiological antioxidant compound and so plays a role in both acid base balance and redox status (Métayer et al. 2008).

Methionine

Transsulphuration

Cysteine

$O_2$

Cysteine dioxygenase

Cysteinesulphinate (CSA)

Uraemia ?

CSA decarboxylase

Aspartate aminotransferase

β-Sulphinylpyruvate    $NH_2$    $CO_2$    Hypotaurine

Transfer to a keto acid

Hypotaurine dehydrogenase

Pyruvate    $H_2SO_3$

Taurine

Sulphite oxidase

$H_2SO_4$
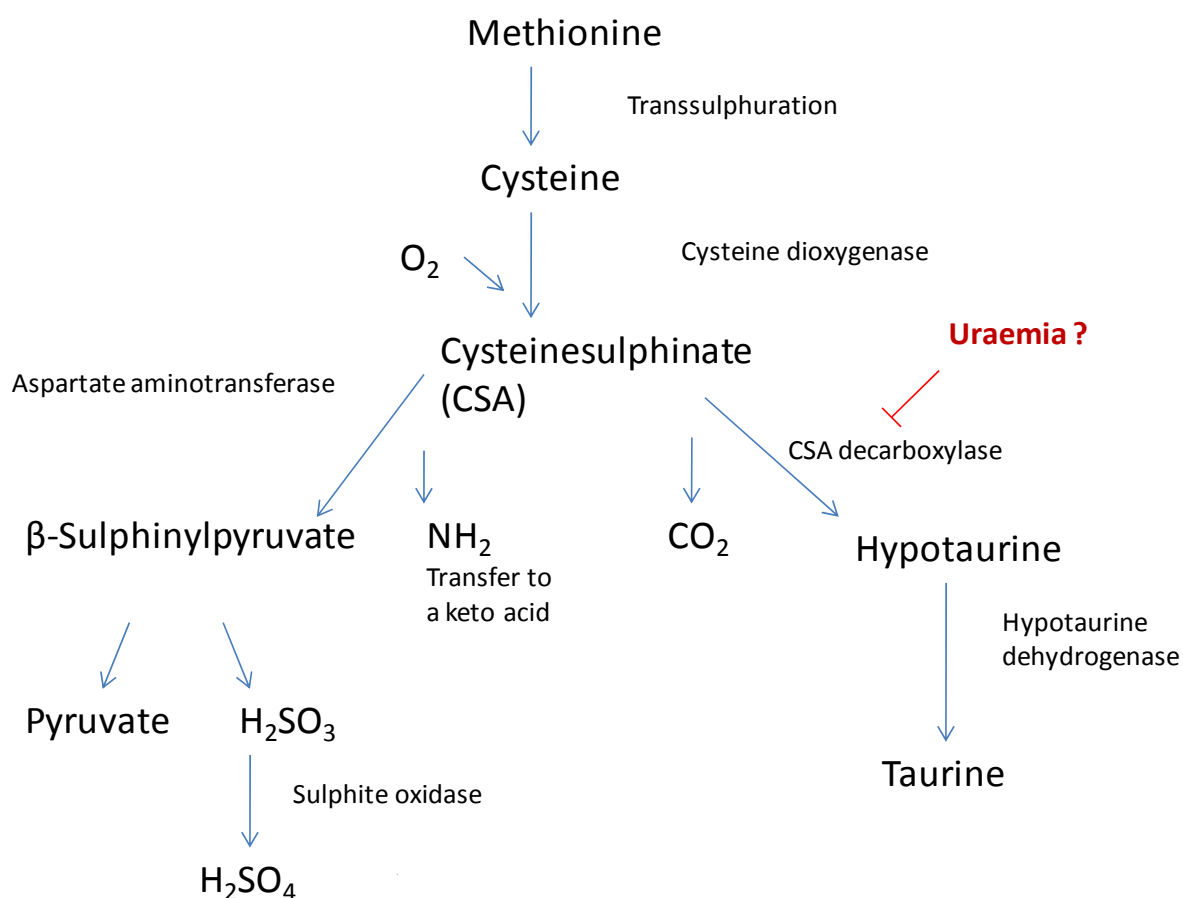
**Figure 1.5. Simplified scheme showing pathways for the catabolism of L-methionine and L-cysteine (adapted from Suliman et al. 1996).**

**1.7.2 Plasma concentrations of sulphur amino acids and metabolites in CKD patients**

The metabolism of sulphate may be abnormal in CKD, because despite normal plasma methionine concentrations (Gulyassy et al. 1970; Condon and Asatoor, 1971), the concentrations of cysteine and homocysteine, a homologue of cysteine, both in the free and bound forms, are frequently seen to be raised in uraemic patients (Flűgel-Link et al. 1983; Robins et al. 1972). Plasma taurine levels, however, in non-dialysed and haemodialysis patients tend to be lower than those seen in healthy individuals (Alvestrand et al. 1982; Qureshi et al. 1989; Suliman et al. 1996) and Bergström and colleagues (1989) reported that in CKD patients the muscle and plasma taurine concentrations were both reduced by the same margin, suggesting that this represents a true depletion of taurine from the cells rather than a decline in the cell/plasma concentration gradient.

The concentrations of CSA in the plasma have also been reported to be high (Suliman et al. 1996) and it was speculated that low plasma taurine accompanied by these high levels of CSA represents a defect in the activity of cysteinesulphinate decarboxylase (CSAD), the rate limiting step in the conversion of CSA to taurine (Suliman et al. 1996). This enzyme requires vitamin B6 as a cofactor, of which CKD patients are commonly deficient (Kopple et al. 1981). However, patients in a number of studies by Suliman and colleagues (1999, 2001, 2002) were administered vitamin B6 supplements, and so the authors concluded that uraemia itself inhibits CSAD. Anorexia and malnutrition are frequently seen in these patients (Suliman et al. 2002) and it is likely that this results in a reduced taurine intake that, when coupled with a reduced synthesis from CSA as proposed by Suliman et al. (1996), causes these reduced plasma taurine levels. Taurine has many important cellular functions including stabilization of the membrane potential, antioxidant properties and a positive inotropic effect on the heart (Suliman et al. 2002; Schaffer et al. 2009) and depletion could possibly result in muscle fatigue and cardiovascular disease (Suliman et al. 2002), common co-morbidities in CKD patients (Nussbaum and Garcia, 2009; Pecoits-Filho et al. 2002). It is possible therefore that in CKD the flux through this pathway is diverted away from taurine and towards the generation of pyruvate and sulphuric acid (Figure 1.5).

## 1.8 Cachexia

Cachexia is defined as the progressive loss of muscle mass and muscle protein associated with chronic disease (Tan and Fearon, 2008) and is frequently reported in many disease states such as uraemia, diabetes, sepsis and burn injury (Workeneh et al. 2006). (The skeletal muscle abnormalities observed in CKD are described in detail in Section 1.16.2). Cachexia is an important clinical problem as it is strongly associated with morbidity and mortality (Griffiths, 1996) and even mild degrees of CKD are capable of stimulating proteolysis leading to cachexia (Wang et al. 2006). For a long time malnutrition was believed to be the primary cause of cachexia (Mitch, 1998), but there is now much evidence to suggest that this is not the case (Mitch, 2002) as nutritional therapy has little impact on nitrogen balance (Mak and Cheung, 2006). The mechanisms causing muscle loss in uraemia are complicated and are likely to be multifactorial including acidosis, neuropathy, oxidative stress, hyperparathyroidism, protein

restriction, inflammation, anorexia and physical inactivity (Mak and Cheung, 2006; Johansen et al. 2003). This thesis will focus on the effects of metabolic acidosis and glucocorticoid.

### 1.8.1 Metabolic acidosis and cachexia

Early reports demonstrated that fasted acidotic rats exhibit an increase in urinary ammonia with no change in urea production (Hannaford et al. 1982), leading to the speculation that the nitrogen required for this increase in ammonia production came from skeletal muscle. May et al. (1986) were the first to demonstrate a direct association between acidosis and an increase in skeletal muscle proteolysis in rats. They rendered rats acidotic through ammonium chloride administration and assessed changes in protein synthesis in *ex vivo* incubated muscle by L-[U-$^{14}$C] phenylalanine incorporation into muscle and changes in protein degradation by the rate of tyrosine released into the media. An increase in the rate of muscle proteolysis was observed in the acidotic rats, with no corresponding changes in the rate of muscle protein synthesis. However, an acute inhibitory effect of acidosis on muscle protein synthesis (measured by [ring-$^{2}$H$_5$] phenylalanine incorporation into muscle) has been demonstrated in humans rendered acidotic by ingestion of ammonium chloride (Kleger at al. 2001) and it has been suggested from *in vitro* cell culture work, that the primary effect of acidosis may be on protein synthesis, followed by a chronic (possibly compensatory or adaptive) effect on protein degradation (Evans et al. 2008). The protein catabolic effect of metabolic acidosis was later confirmed by May and colleagues in 1987 in rats with an induced renal failure and a more physiological degree of acidosis, as a criticism of the 1986 study by these authors was that the rats were subjected to a severe acidosis unlikely to be seen in CKD. Furthermore, this increase in proteolysis (measured by tyrosine release from the hindquarter) in acidotic rats could be prevented by the correction of acidosis using bicarbonate (May et al. 1987). Alkali therapy in the form of sodium bicarbonate is commonly used in CKD patients to correct acidosis and is associated with improvements in nitrogen balance (Reaich et al. 1993; Papadoyannakis et al. 1984), increases in body weight, decreases in morbidity (Szeto et al. 2003) and decreases in protein catabolism (Movilli et al. 2009). Stein and colleagues (1997) administered either a high or low alkali dialysis fluid to peritoneal dialysis patients, achieving plasma bicarbonate concentrations of 23mmol/l in the low group and 27mmol/l in the high group. After one year, those patients who received the additional alkali had significant improvements in body weight and mid arm circumference (a measure of muscle mass) and fewer hospital admissions. Normal adults with an experimental acidosis

induced using oral ammonium chloride had increased whole body protein degradation (determined from the kinetics of infused L-[l-$^{13}$C] Leucine) and amino acid oxidation (Reaich et al. 1992), confirming acidosis as an important catabolic factor. The mechanisms through which this occurs will be discussed in Section 1.13.

Sodium bicarbonate has obvious positive effects on protein balance (Reaich et al. 1993), but how might this be occurring? It is well established that acidosis increases protein degradation through a stimulation of the ubiquitin proteasome system (Lecker et al. 2006). Animal studies have shown that acidosis induced by CKD in conjunction with glucocorticoids reduce insulin signalling (Bailey et al. 2006) resulting in an increase in the abundance of the 14kDa fragment - a marker of myofibrillar degradation. Sodium bicarbonate is unable to cross the muscle sarcolemma, but may act to reduce protein degradation by diffusing out of the plasma into the interstitial fluid, the environment to which the cell membrane is exposed, and thereby increasing interstitial pH and through effects on pH sensors on the skeletal muscle membrane, for example SNAT2, may possibly relieve the inhibition on insulin signalling reducing protein degradation rates. Indeed this has been shown in preliminary results by Bailey and colleagues (2006) in rats, but whether this is the case in humans is unclear.

However, the effects of acidosis do not appear to occur exclusively through low pH. May et al. (1986) found there was no increase in proteolysis rates when the muscles from normal rats were incubated at pH 7.1 and the incubation of muscles from acidotic rats at pH 7.4 was unable to fully reverse the abnormal proteolysis rates. These authors concluded that there must be another signal required for the acceleration of muscle proteolysis.

## 1.8.2 Glucocorticoids and cachexia

Previous studies have demonstrated that glucocorticoids are capable of increasing protein degradation and inhibiting protein synthesis (Goldberg et al. 1980) and May et al. (1986) reported that metabolic acidosis was associated with an increase in glucocorticoid production. This led to the investigation of the possible contribution of glucocorticoids to the muscle wasting process.

As previously described, May et al. (1986) found acidotic rats had increased rates of protein degradation. This observation was then extended to the effects of glucocorticoid on protein

degradation. They found these elevated rats of proteolysis were abolished if acidotic rats underwent an adrenalectomy (ADX) and were increased again following the administration of dexamethasone, suggesting that glucocorticoids are necessary for the increase in proteolysis seen in uraemia (May et al. 1986). This was later supported by the finding that ADX also prevents the increase in mRNA for ubiquitin and proteasome subunits (see Section 1.11.1) that was observed in intact acidotic rats, therefore preventing the activation of protein degradation pathways (Price et al. 1994), a response that was also restored by dexamethasone. Oxidation of branched chain amino acids (BCAA) may be a further mechanism by which acidosis causes a loss of muscle protein mass in CKD (Lofberg et al. 1997) (see Section 1.14) and May et al. (1996) reported that this increase in oxidation of BCAA, in particular Leucine, also requires glucocorticoids. There was no increase in oxidation of BCAA in ADX rats with acidosis, but treatment with dexamethasone significantly increased Leucine oxidation (May et al. 1996). This suggests that glucocorticoids appear to be required for the acidosis-induced muscle wasting, but on their own, are insufficient.

Contrary to the effects following ADX, blockade of the glucocorticoid receptor using the specific antagonist RU38486 failed to block muscle wasting in acid loaded rats with intact kidneys (Pickering et al. 2003) at least partly because this drug impairs acid excretion and worsens acidosis.

It is important to note that in CKD all the evidence above suggesting a role for glucocorticoids in the muscle wasting process have been performed using a rodent model and using pharmacological doses of dexamethasone. Therefore it is not known how applicable this research is to humans.

This evidence for a catabolic role of glucocorticoids, in particular dexamethasone, when incombination with metabolic acidosis, is in contrast to its clinical effects and application in humans. Dexamethasone has anti-inflammatory properties and is used clinically to treat inflammatory and autoimmune conditions (Rhen and Cidlowski, 2005). In support of this, a recent study by Crossland and colleagues (2010) demonstrated that in rats with endotoxaemia, a low dose of dexamethasone reduced muscle protein loss, observed by a prevention of the reduction of the muscle protein:DNA ratio that was seen to fall in rats with endotoxaemia without dexamethasone. This effect of dexamethasone was seen to occur with a reduced mRNA expression of TNF-α and IL-6 in muscle, suggesting the anti-inflammatory properties of

dexamethasone resulted in the maintenance of muscle mass in a condition that is usually considered to be catabolic.

These contrasting results may be explained by dose of dexamethasone that was used, with a much lower dose used by Crossland and colleagues (2010) compared to those described above. However, there is research to show that in catabolic conditions, the involvement of glucocorticoids include physiologically relevant levels of glucocorticoid (Du et al. 2005a). For example, in acidosis, adrenalectomy reduces stimulation of the ubiquitin proteasome system and therefore reduces protein degradation rates (May et al. 1986; Price et al. 1994). It therefore may be more likely that the differences in glucocorticoids effects are due to different disease conditions. Endotoxaemia, unlike CKD, is not usually associated with a chronic metabolic acidosis, so it may be possible that when glucocorticoids, even at a physiological dose, are combined with metabolic acidosis, the results are catabolic and not anabolic in nature as their use in other conditions may suggest.


## 1.9 Protein turnover

Skeletal muscle accounts for 40% of total body weight and 50% of total body protein (Griffiths, 1996). Approximately 3.5 – 4.5 g of protein/kg is degraded and synthesised every day (Mitch and Goldberg, 1996) equating to the amount of protein contained in 1 – 1.5 kg of skeletal muscle. In order for nitrogen balance to be maintained, rates of protein synthesis and degradation must be very tightly controlled, any small discrepancy that occurs could over many weeks lead to a substantial loss of muscle mass; indeed such a discrepancy is thought to be the basis of cachexia.


### 1.9.1 Methods to determine muscle protein synthesis and breakdown *in vivo*

The gold standard method by which to investigate protein synthesis is to measure it directly using the incorporation of a stable isotope into muscle tissue. Fractional protein synthesis rates can be measured using a continuous primed intravenous infusion of an amino acid that is not transaminated or oxidised in muscle, or an alternative approach is to administer the tracer as a flooding dose to quickly equilibriate the tracer in the intracellular and extracellular amino acid pools. Both methods are frequently used and presently there is no evidence to suggest the use of one method over the other. Commonly used tracers are, L- [l-$^{13}$C] Leucine, L-[l-$^{13}$C] α-Ketoisocoporate and L-[$^2$H$_5$] Phenylalanine. Once a steady state is reached in the precursor pool,

muscle biopsies can be taken and analysed using GC-combustion-IRMS methodology (Wagenmakers, 1999; Smith et al. 2007) to determine tracer enrichment and therefore protein synthesis rates. However, this method does not provide any information on protein degradation rates. The Arterio-Venous (A-V) tracer dilution method using a two or three compartmental model, is commonly used and allows simultaneous measurements of protein synthesis and degradation to be made. A tracer amino acid, for example $[^2H_5]$ Phenylalanine is given by primed intravenous infusions and under steady state conditions samples of arterial and venous blood from catheters inserted into the femoral artery and vein are made. Net balance of the tracer amino acid is calculated by the concentration in the arterial blood minus the concentration in the venous blood multiplied by blood flow. Protein degradation rates are commonly calculated from the A-V dilution of the $[^2H_5]$ Phenylalanine tracer. This method is invasive and technically demanding and should only be used in the certainty of the presence of steady state conditions (Kumar et al. 2009). This topic is discussed in detail in two reviews by Wagenmakers (1999) and Rennie (1999).

For ease, several indirect measures of myofibrillar protein degradation are commonly used: 3-Methyl Histidine (3-MH) excretion rates, accumulation of the 14kDa actin fragment and mRNA expression of components of the proteasome and E3 ligases such as MAFbx and MuRF-1. The use of 3-MH excretion as an index of the rate of myofibrillar proteolysis is based upon the presence of N-Methylated Histidine residues in actin and myosin heavy chain proteins (Nissen, 1997). The cleavage of myofibrils releases 3-MH which, as it has no specific tRNA, cannot be re-used for protein synthesis and so is excreted into the urine (Welle, 1999). Thus the excretion rate of 3-MH over a 24h period provides an indication of the rate of myofibrillar degradation. There are a few inherent problems with this method; Actin is present in all cells, not only muscle cells, and although 3-MH excretion will primarily represent myofibrillar breakdown, a proportion will also result from non-myofibrillar proteins such as skin and intestine, which has a much higher rate of turnover than muscle. However, it has been quoted that in healthy individuals, muscle turnover represents the majority of 3-MH appearing in the urine, accounting for approximately 75% of the total 3-MH excretion (Ballard and Tomas, 1983). It is assumed that 3-MH appearing in the urine is from endogenous muscle proteolysis and not from exogenous sources. However, 3-MH derived from dietary protein will be absorbed and ultimately also excreted in the urine. For this reason, to ensure that 3-MH excreted is coming primarily from the degradation of myofibrillar proteins and is not influenced by dietary protein intake, individuals

are placed on meat-free diets for three days prior to the day on which the urine collection is made due to the slow clearance of 3-MH. In order to determine 3-MH excretion rate, the total volume of the 24 hour urine collection is recorded and a sample is taken, deproteinsed and 3-MH concentration measured using High performance liquid chromatography. This method is frequently used to assess protein breakdown rates, particularly in chronic disease states due to the relative ease of collecting a 24h urine sample compared to the more invasive methods such as stable isotopes, and the low cost in the collection and analysis of the sample.

The degradation of the myofibrils results in the production of a small 14kDa actin fragment (this is discussed in more detail in Section 1.11.2) which is present in the insoluble fraction of the muscle lysates and is easily detected by western blotting. This method has been significantly correlated against measured rats of muscle protein degradation using stable isotope techniques (Workeneh et al. 2006).

Degradation of myofibrils is performed primarily by the ubiquitin proteasome system. The mRNA expression of subunits of the proteasome and of E3 liageses which are considered to be the rate limiting step in protein degradation by the proteasome (Lecker et al. 2006) have been used as an indicator of the activity of this system. However, recent evidence from Greenhaff et al. (2008) has shown that in humans the expression of these E3 ligases at the mRNA level do not relate to the protein degradation rate measured using $[^2H_5]$Phenylalanine dilution technique. This suggests that when used in isolation, caution is required interpreting this data.

## 1.10 Protein synthesis

In contrast to protein degradation (Section 1.11 below), there is only one known pathway for protein synthesis. In this process, the genetic information in the chromosomes is first transcribed into RNA which, following processing to messenger RNA (mRNA), is then translated into the protein amino acid sequence in the cytosol. The process of translation can be separated into three stages, initiation, elongation and termination (Proud, 2006). Most of the control of protein synthesis occurs at the initiation step and in the signalling events preceding it. Before the mRNA sequence can be translated, it must first undergo pre-translational modifications. The sequence is capped on the 5' end with a methylated guanosine derivative, 7-methylguanosine and a polyA tail is added at the 3' end (Welle, 1999).

**1.10.1 Translation initiation**

This process involves the assembly of the ribosomal complex at the initiation codon on the mRNA sequence. A methionyl initiator tRNA (Met-tRNA$_i$) is in its peptidyl(P) site with its anticodon base-paired to the start codon (usually AUG) on the mRNA strand (Mitchell and Lorsch, 2008). This process requires the involvement of many non-ribosomal proteins, known as eukaryotic initiator factors (eIFs). The first step involves the delivery of the Met-tRNA$_i$ to the 40S ribosomal subunit by the trimeric GTP-binding protein eIF2. eIF2 can only bind Met-tRNA$_i$ in the GTP-bound state, which is regulated by eIF2B, a guanine nucleotide exchange factor that replaces a GDP molecule on eIF2 with GTP and allows the formation of the eIF2·GTP·Met-tRNA$_i$ ternary complex. This complex then binds to the 40S subunit in the absence of mRNA facilitated by eIF1A and eIF3 forming the 43S pre-initiation complex (Acker and Lorsch, 2008; Kapp and Lorsch, 2004). The GDP-GTP exchange process is an important regulatory step in translation initiation. The phosphorylation of eIF2's α subunit by an eIF2 kinase (which can occur for example during severe amino acid depletion) prevents this exchange and therefore the binding of Met-tRNA$_i$ to the start codon (Proud, 2006).

The next step in translation initiation is the recruitment of mRNA to the ribosome by the eIF4F complex which comprises the 3 eIF's 4A, 4E and 4G (Merrick, 1992) and is regulated by the 4E binding protein 4E-BP1 (Figure 1.6). eIF4E is released from the inactive 4E-BP1·eIF4E complex by the phosphorylation of 4E-BP1 allowing it to bind to the scaffold protein eIF4G that makes additional contacts with eIF4A. Together these three eIFs form the eIF4F complex that is assembled on the 5' end of the mRNA strand (Kimball and Jefferson, 2006). eIF4A comprises a RNA helicase that unwinds secondary structures in the mRNA's 5' untranslated region, promoting ribosomal binding to the mRNA. eIF4E recognises and binds the 5' cap on the mRNA sequence and in conjunction with eIF3 and the poly A binding protein, loads the mRNA onto the 43S initiation complex (Kapp and Lorsch, 2004). This complex then starts scanning in the 5' to 3' direction to find the AUG start codon. Upon the recognition of the start codon, eIF1 is released from the 43S complex resulting in the hydrolysis of GTP by eIF2 releasing Met-tRNA$_i$ into the P site of the 40S subunit. The remaining eIF's dissociate from the complex and the 60S subunit is joined to the 40S, facilitated by eIF5B, resulting in formation of the 80S initiation complex and the elongation phase of translation can begin (Acker and Lorsch, 2008).
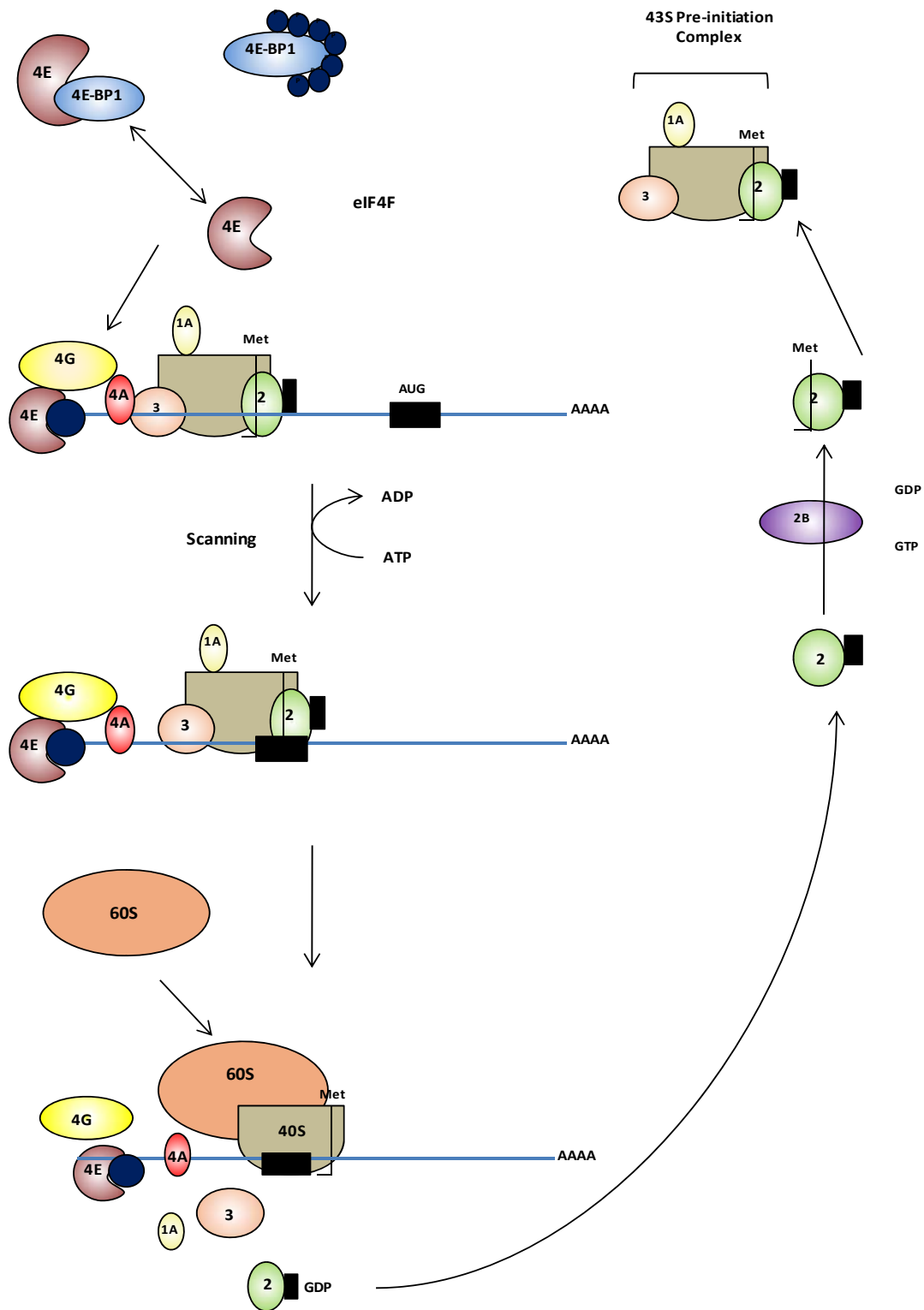
**Figure 1.6. Schematic representation of the events of translation initiation.**
This figure has been adapted from information described in Proud, (2006); Kimball and Jefferson, (2006); Merrick, (1992); Acker and Lorsch, (2008). Initiation factors are shown schematically as individually labelled coloured circles or ellipses.

**1.10.2 Elongation**

Elongation is the addition of amino acids to the growing polypeptide chain in a stepwise manner. The process requires only two elongation factors, eEF1A that delivers the charged tRNA molecules to the ribosome and eEF2 that promotes the translocation of the ribosome along the mRNA strand (Groppo and Richter, 2009). There are three stages to the elongation process that are repeated until a stop codon is encountered. These are the binding of aminoacyl-tRNA, peptidyl transfer and translocation. The ribosome contains three binding sites, the aminoacyl (A site), the peptidyl (P site) and exit (E site) (Noble and Song, 2008). Elongation begins with a peptidyl tRNA in the P site of the ribosome next to an empty A site. The GTP-bound eEF1A delivers an aminoacyl-tRNA to the A site for decoding to take place. When a correct match is made between the mRNA codon and the tRNA anticodon, a conformational change occurs in the small ribosomal subunit catalysing the hydrolysis of GTP by eEF1A releasing the aminoacyl tRNA into the A site. Almost immediately, a peptide bond is formed between the peptidyl tRNA and the incoming amino acid catalysed by peptidyl transferase activity on the large ribosomal subunit. The ribosome is then moved forward three bases facilitated by eEF2 and the now deacylated tRNA is displaced into the E site, the peptidyl tRNA is translocated from the A site to the P site and the A site becomes empty once again. This cycle is repeated until one of the stop codons is encountered, UAA, UAG or UGA (Noble and Song, 2008).

**1.10.3 Termination**

Upon recognition of a stop codon in the A site, elongation is terminated. This process is mediated by two proteins; eukaryotic release factor 1 (eRF1) and eRF3. In the GTP-bound state, eRF1 recognises the stop codon and induces the hydrolysis of the final peptidyl tRNA bond to the polypeptide chain catalysed by peptidyl-transferase at the centre of the ribosome (Janzen and Geballe, 2004). This releases the completed polypeptide chain and disassembles the ribosomal complexes (Kapp and Lorsch, 2004). eRF3 is a GTPase protein whose activity requires both the 80S ribosome and eRF1 and stimulates eRF1 activity in response to GTP hydrolysis (Zhouravleva et al. 1995). Unlike eRF1, the necessity for eRF3 in the termination of protein translation in unclear (Janzen and Geballe, 2004).

## 1.11 Major proteolytic systems in skeletal muscle

Intracellular protein degradation is important as it allows for adaptation to occur in response to changing physiological circumstances and it is an essential quality control mechanism that removes misfolded and damaged proteins (Lecker et al. 1999). Skeletal muscle, like other tissue, contains three major proteolytic systems:

- The lysosomal system: Together with the proteasome system, the lysosomes comprise one of the major intracellular proteolytic systems in mammalian cells (Zhao et al. 2007). Lysosomes are small spherical organelles containing proteases called cathepsins and have an acidic pH ranging from 3.5 – 6.5. This system is primarily involved in the digestion of organelles (especially mitochondria) (Scott and Klionsky, 1998) by autophagy, phagocytosis and endocytosis. The myofibrils are too large to be engulfed by lysosomes and skeletal muscle cells contain very few lysosomes and so it is unlikely that this system has a large role to play in the turnover of myofibrillar proteins (Goll et al. 2008). It has recently been shown that FOXO transcription factors are capable of activating cathepsin L (Mallinson et al. 2009).

- The calcium dependent system that relies on the activity of calpains, a family of $Ca^{2+}$-dependent cysteine proteases. This system controls the disassembly of tropomyosin and therefore plays an important role in muscle contraction and it has been discovered that in muscular dystrophy this system is up-regulated contributing to the loss of muscle mass (Carragher, 2006).

- Finally, there is the cytosolic ATP-dependent pathway(s), of which the best described is the ATP-dependent ubiquitin-proteasome system (UPS), responsible for degrading actin and myosin following prior cleavage by caspase-3. This is quantitatively the most important system in skeletal muscle (Rock et al. 1994). The high rates of proteolysis seen in uraemia and other catabolic states have been attributed to the stimulation of the UPS following the observation that an increased expression of mRNAs encoding certain components of this system occurs (Mitch and Goldberg, 1996), and that blocking the proteasome using MG132 blocks the accelerated protein degradation that is seen in uraemia (Bailey et al. 1996).

### 1.11.1 Ubiquitin-proteasome pathway

The ATP-dependent ubiquitin-proteasome pathway is a highly organised and complex cascade of enzymatic events in which, ubiquitin (ub) molecules are covalently linked to proteins marking them for degradation. The tagging of a protein with ub is a process that is coordinated by the activity of three enzymes E1, a ub- activating enzyme, E2, a ub- conjugating enzyme, and E3, a ub- ligase. The key enzyme is the E3 ligase, which provides the system with specificity. There are in the region of 1000 different E3 ligases, each recognising a different protein (Rajan and Mitch, 2008).

Before ub can be conjugated to the target protein it must first be activated by the binding of ubiquitin activating enzyme E1 via a high energy thioester bond in an ATP-dependent process. Once activated, ub is then transferred to an E2 carrier protein followed by an E3 ub ligase which then covalently joins the ub molecule by an isopeptide bond to an ε-amino group of a lysine residue on the target protein. This process is then repeated and the C-terminal glycine of another ub molecule is linked to the lysine residue on the first until a chain of four or more ub molecules is made producing a polyubiquiniated protein. This can then be recognised and degraded by the 26S proteasome (Lecker et al. 1999; Murton et al. 2008).

The 26S proteasome is a large protein complex of approximately 50 subunits and is located in the nucleus and cytoplasm of all cells. It comprises a 20S catalytic core and two 19s regulatory sub units. The 19s "cap" protein complex, recognises and binds polyubiquitinated molecules, unfolds the protein to its primary structure and guides it into the catalytic core following the disassembly of the ub chain enabling the ub molecules to be recycled (Hasselgren, et al. 2002). The barrel shaped 20S subunit is made up of four stacked rings of proteins each containing approximately seven subunits around a central cavity (Goldberg et al. 1995). The subunits of the two inner rings have proteolytic sites that cleave the protein into peptides that are six to twelve amino acids in length (Mitch and Goldberg, 1996). These peptides are then degraded into individual amino acids by cytosolic exopeptidases (Debigaré and Price, 2003), (Figure 1.7).
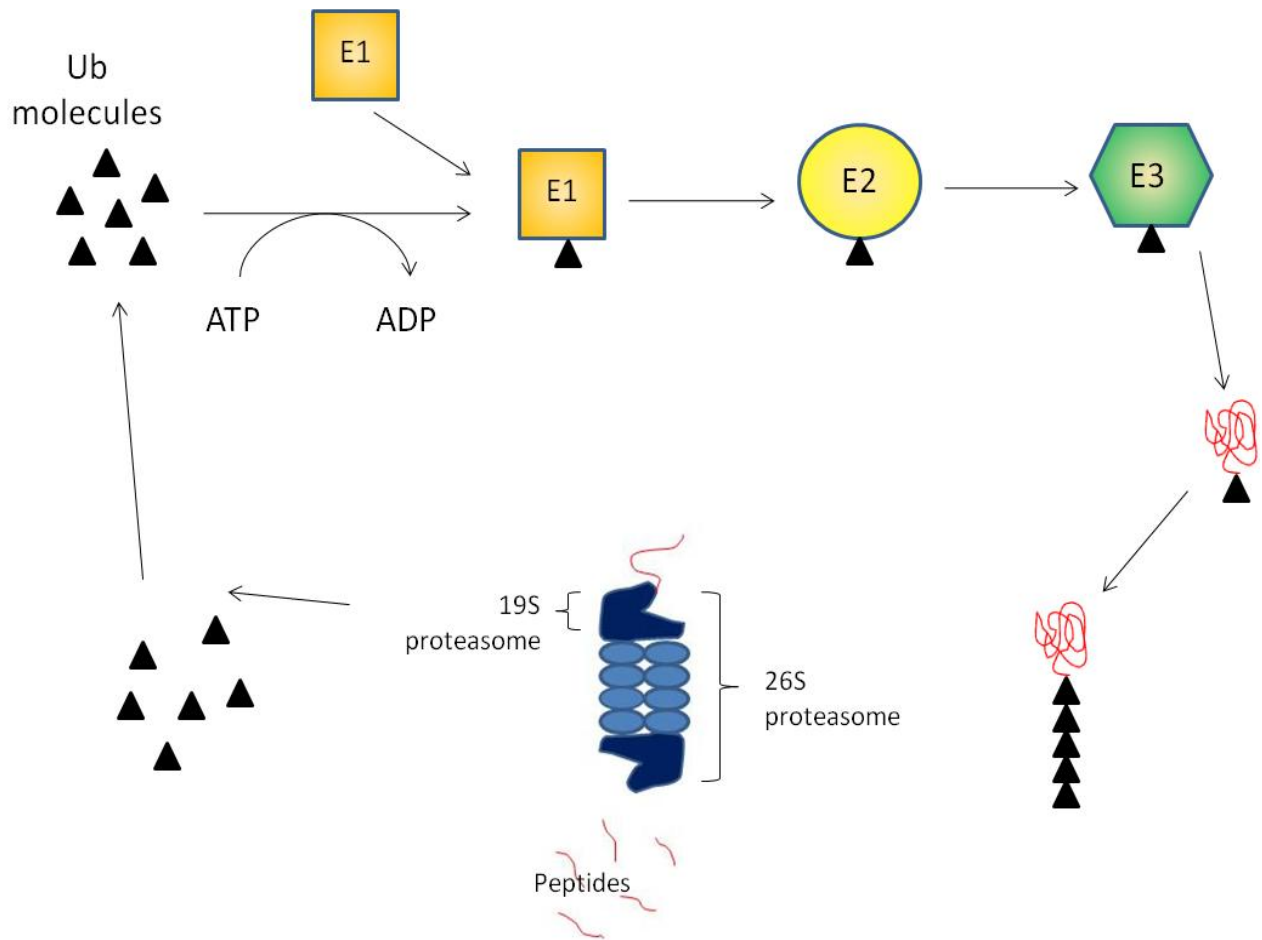
**Figure 1.7. Ubiquitin conjugation of a protein and its degradation by the ubiquitin proteasome system.**

This diagram depicts ubiquitination of a protein catalysed by E1, E2 and E3 ligases and subsequently degraded by the 26S proteasome into small peptides. This figure was adapted from the information in Debigaré and Price, (2003); Lecker et al. (1999); Murton et al. (2008).

## 1.11.2 Degradation of myofibrillar proteins

The majority of muscle protein (50-70%) exists as myofibrils and actomyosin complexes (Du et al. 2004), but the ubiquitin proteasome system is unable to degrade these large complexes (Solomon and Goldberg, 1996) so they must first be cleaved into smaller protein fragments by caspase-3, creating substrates that are rapidly degraded by the proteasome. This process leaves behind a characteristic "footprint", a 14-kDa C-terminal fragment of actin that is found in the insoluble fraction of muscle (Du et al. 2004). The presence of this fragment has been seen to

increase in catabolic states and the level of this is closely related to the rate of protein degradation (Workeneh et al. 2006). In conjunction with other methods, this fragment can be used to detect changes in muscle proteolysis (Workeneh et al. 2006; Workeneh and Mitch, 2010).


**1.11.3 Muscle Specific E3 Ligases and FOXO transcription factors**

Muscle contains two specific E3 ligases, muscle atrophy F box (MAFbx, also known as atrogin-1) and muscle-specific RING finger-1 (MuRF-1) (Sandri et al. 2004, Bodine et al. 2001b) collectively called atrogins. The expression of these E3 ligases is controlled by the phosphorylation status of the forkhead box gene group O (FOXO) family of transcription factors. FOXO is phosphorylated by PKB rendering it inactive and inhibiting its transcriptional activity by confining it to the cytoplasm. A reduction in phosphorylation of FOXO by PKB results in their translocation to the nucleus where they increase the transcription of the two E3 ligases and thereby increasing protein degradation through the ubiquitin proteasome system.

The expression of these ligases increases dramatically in many different catabolic states, including uraemia (Lecker et al. 2004). Bodine and colleagues (2001b) observed that in response to denervation, MAFbx or MuRF-1 knockout mice experienced a 56% and 36% slowing of muscle atrophy respectively in comparison to a wild type, identifying these genes as critical regulators of muscle atrophy. Subsequently, many research groups have used the mRNA expression of these E3 ligases as a marker of myofbrillar proteolysis.

However, caution is required when interpreting this expression data from humans when measures of proteolysis have not been made, as recent evidence suggests that in healthy individuals the relative expression of MuRF-1 and MAFbx do not always correlate with measured protein degradation rates (Greenhaff et al. 2008). However, this study was carried out using healthy individuals and there are currently no studies of its kind in humans in a chronic disease state. It is possible that in healthy individuals, the increase in MuRF-1 and MAFbx mRNA expression is a side effect of an increase in FOXO activity, which has an involvement in muscle fuel selection. In the case of an individual in a chronic disease state characterized by muscle wasting, the increase in the mRNA expression of these E3 ligases may have physiological significance and correlate with measured rates of protein degradation. This has certainatly been shown in animal models. Wang and colleagues (2006) studied the influence of

insulin resistance, a common feature of many catabolic diseases, on muscle atrophy using the *db/db* mouse model. They observed that these insulin resistance mice had reduced muscle mass and increased rates of muscle protein degradation as measured by tyrosine release into the media. Rosiglitazone, an insulin sensitizer, was administered to a group of the *db/db* mice, and resulted in an increase in muscle mass, and in PI3-K activity, and a reduction in muscle proteolysis rates. This was also seen with a significant reduction in the mRNA expression of MuRF-1 and MAFbx, consistent with the decline seen in muscle protein breakdown rates. A similar finding was reported by Crossland et al. (2008) who reported in a model of sepsis, a reduction in muscle protein:DNA ratio was seen with increases in the expression of MuRF-1 and MAFbx. However, these results are yet to be demonstrated in humans.

Recent studies by Crossland et al. (2008, 2010) and Constantin et al. (2007) have highlighted a potential role for FOXO transcription factors in the generation of muscle insulin resistance by identification of another target, pyruvate dehydrogenase kinase 4 (PDK4). Activation of FOXO (reduced phosphorylation) results in an increase in transcription of PDK4 which phosphorylates and inactivates pyruvate dehydrogenase complex, preventing the conversion of pyruvate to acetyl-CoA and impairing carbohydrate oxidation. This suggests there is a common pathway that activates the loss of muscle protein and impairs muscle carbohydrate oxidation involving activation of the FOXO family of transcription factors.

## 1.12 Insulin signalling pathway

In human skeletal muscle, insulin and IGF-1 exert a major anti-catabolic effect preventing initiation of protein degradation (Goldspink, 2006).

Insulin signalling propagates through three major pathways, Phosphatidylinositol 3 kinase (PI3-K), MAPK (mitogen-activated protein kinase) and Cbl/CAP (Cbl-associated pathways). As this thesis focuses on the initiation of protein synthesis and signalling to protein degradation, only the PI3-K pathway will be discussed.

**1.12.1 PI3-K pathway**

Insulin and IGF-1 signalling through the PI3-K pathway plays a key role in promoting muscle growth, repair and the maintenance of muscle mass (Goldspink, 2006). The insulin receptor has two α extracellular subunits and two transmembrane β subunits connected by disulphide bridges (Seino et al. 1989). Insulin binds to its receptor on the outside surface of the cell causing a conformational change that triggers tyrosine kinase activity on the β subunits and autophosphorylation of the β insulin receptor subunit at several tyrosine residues (Sykiotis and Papavassiliou, 2001). This activation of tyrosine kinase causes phosphorylation of insulin receptor substrates 1 and 2 (Sykiotis and Papavassiliou, 2001), which then act as a docking site for proteins containing Src-homology-2 (SH-2) domains including the p85 regulatory subunit of PI3-K. The binding of the SH-2 domain to the insulin receptor substrate – 1 (IRS-1) stimulates the activity of the P110 catalytic subunit of the PI3-K molecule resulting in the conversion of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to $PIP_3$ which is an important second messenger in the action of insulin. $PIP_3$ recruits PDK1 (3-Phosphoinositide-dependent kinase 1) and protein kinase B (PKB, also known as Akt) to the plasma membrane where PKB becomes activated (Cohen, 2006). Downstream of PKB, but upstream of the mammalian target of rapamycin (mTOR) are the Tuberous Sclerosis Complex proteins (TSC), TSC1 also known as hamartin and TSC2, also known as tuberin. When present as a hetrodimer, the TSC1-TSC2 complex inhibits mTOR indirectly by converting a small G protein, ras homolog enriched in brain (rheb) from the active GTP-bound state into the inactive GDP-bound state (Corradetti and Guan, 2006; Jastrzebski et al. 2007; Frost et al. 2009). PKB phosphorylates TSC2 at $Ser^{939}$ and $Thr^{1462}$ residues (Rosner et al. 2008) degrading the TSC1/TSC2 complex and therefore increasing the activity of rheb and ultimately mTOR. It has recently been shown that rheb up regulates the activity of mTOR via the association with FKBP38, a member of the FK506 binding protein family (FKBP) relieving FKB38's inhibition on mTOR (Rosner et al. 2008). Downstream targets of mTOR are $P^{70}S6K$ and 4E-BP1, which are both regulators of the translational machinery (Hay and Sonenberg, 2004) and allows initiation of protein synthesis (Figure 1.8).
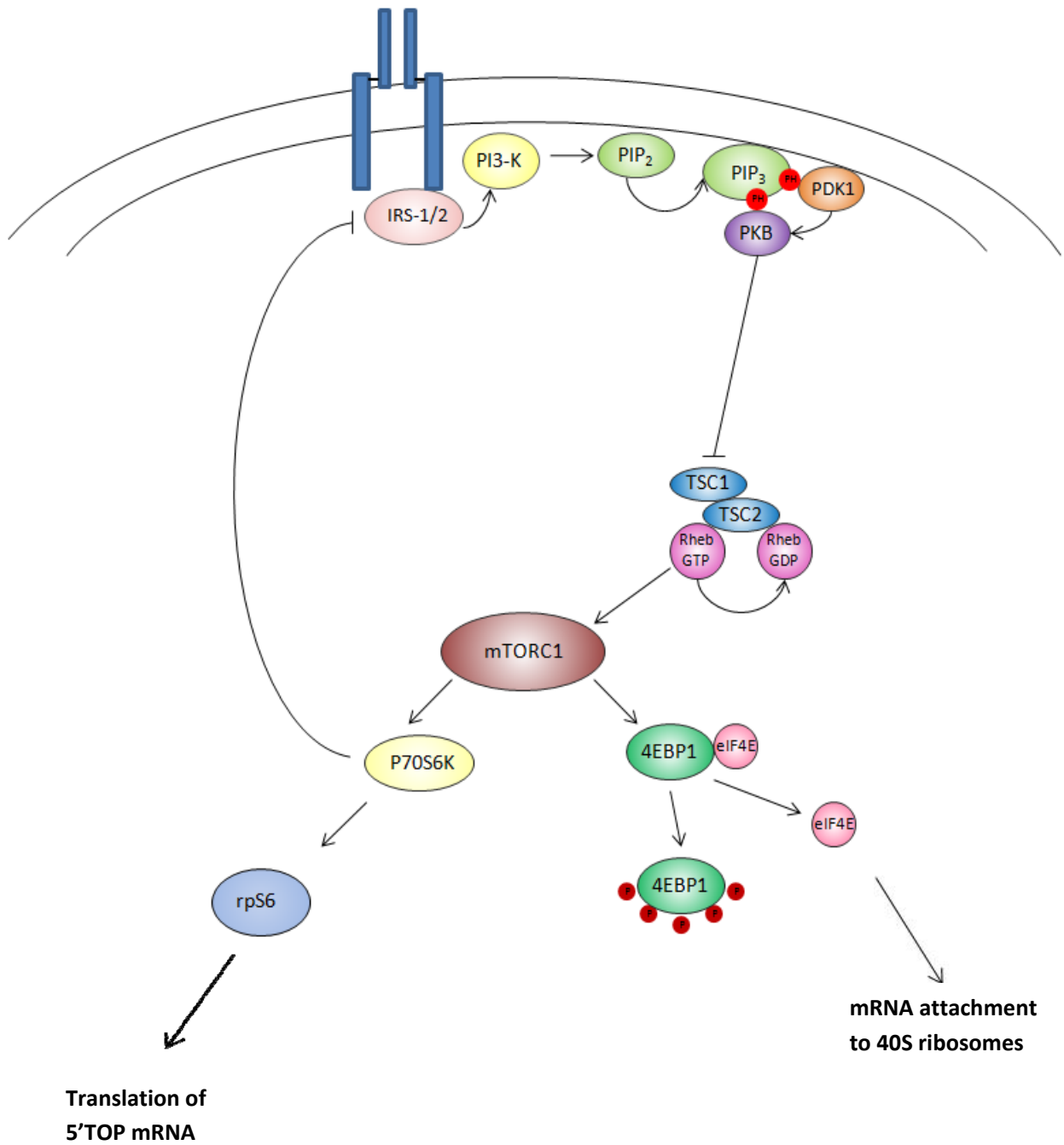
**Figure 1.8. Schematic representation of the PI3-K/PKB pathway.**

This figure was adapted from the information cited in Cohen (2006); Sykiotis and Papavassilou (2001); Hay and Sonenberg (2004); Lizcano and Alessi (2002).

**1.12.2 PI3-K**

PI3-K is a lipid kinase consisting of a p110 catalytic subunit and a p85 regulatory subunit that contains two SH2 domains that interact with the IRS proteins and is a key protein in the metabolic action of insulin. There are three classes of PI3-K, but the most likely form to be involved with insulin signalling is Class 1a (Shepherd et al. 1998) that is sensitive to activation by growth factors, but is not sensitive to amino acids. Upon IRS-1 phosphorylation, PI3-K is recruited to the membrane where it converts $PIP_2$ to $PIP_3$. Recent evidence shows PI3-K is an important regulator of proteolysis and suppressed activity of this kinase by acidosis has been implicated in the accelerated muscle protein degradation in CKD (Franch et al. 2004). The activation of mTOR complex 1 (mTORC1) by insulin appears to be mediated by P13-K as blockade using wortmannin inhibits the phosphorylation of 4E-BP1 and $P^{70}S6K$ which are downstream targets of mTORC1.

**1.12.3 Protein Kinase B (PKB)**

PKB is a serine/threonine phosphatidylinositol – regulated kinase and is a critical mediator of mTOR activity sitting at the crossroads between protein synthesis and degradation, and playing a central role in the regulation of glucose and protein metabolism, motility, and cell growth and survival (Fayard et al. 2005). There are three isoforms of PKB, α, β and γ. The most important isoform in muscle seems to be PKBβ which is also the dominant isoform in insulin responsive tissues (Kandel and Hay, 1999). In order to become fully activated, PKB must be phosphorylated on two sites, $Ser^{473}$ and $Thr^{308}$. $Ser^{473}$ is phosphorylated first by rictor that is bound to the mammalian target of rapamycin complex 2 (mTORC2) and is required for the localisation of - PKB to the membrane (Sarbassov et al. 2005). PKB contains a pleckstrin homology domain that binds to $PIP_3$ with high affinity, exposing the $Thr^{308}$ residue and allowing phosphorylation by PDK-1 (Cohen, 2006; Scheid et al. 2002). Once activated, PKB dissociates from the membrane and phosphorylates downstream targets such as TSC2 (Kimball, 2007) and AS160 (Sano et al. 2003). The phosphorylation of AS160 results in the inactivation of its GTPase activity leading to the accumulation of GTP-bound Rab (a small G-protein required for vesicle trafficking) and promotes the translocation of GLUT-4 to the plasma membrane (Cartee and Funai, 2009; Thong et al. 2005).

**1.12.4 Mammalian target of rapamycin (mTOR)**

The mammalian target of rapamycin is a 289kDa serine/theronine protein kinase that regulates many aspects of cell growth, notably the regulation of translation through the phosphorylation of $P^{70}S6K$ and 4E-BP1 (Corradetti and Guan, 2006) and the phosphorylation of these two proteins is often used as a marker of mTOR activity (Hay and Sonenberg, 2004). mTOR exists in two complexes, mTORC1 and mTORC2. mTORC1 consists of three proteins, mTOR, MLST8 and raptor (regulatory associated protein of mTOR) and is involved in the control of translation and of cell growth in response to nutrients and growth factors. mTORC2 consists of mTOR, MLST8 and rictor (rapamycin insensitive companion of mTOR) and is a rapamycin insensitive protein. It controls the actin cytoskeleton dynamics and the phosphorylation PKB on the $Ser^{473}$ residues (Cohen, 2006; Um et al. 2006). It is raptor that binds mTORC1 to $P^{70}S6K$ and 4E-BP1 and facilitates their phosphorylation (Corradetti and Guan, 2006). mTOR is activated by growth factors and is also a nutrient sensing protein that is capable of being activated by amino acids (Hay and Sonenburg, 2004). Insulin and IGF-1 activate mTOR through the PI3-K/PKB signalling pathway, whilst amino acids possibly signal to mTOR through a type III PI3-K (a nutrient sensing isoform) that is not activated by insulin and does not phosphorylate PKB (as in traditional PI3-K signalling), but phosphorylates mTORC1 directly (Kimball, 2007; Evans et al. 2008).

If signalling through mTOR is inhibited using rapamycin, cell growth and protein accretion is inhibited. Bodine and colleagues (2001a), demonstrated that 14 days of overload of the plantaris muscles by removal of the synergists resulted in an increase in the size of the overloaded leg by 45% compared to the control, however, this effect was abolished when rapamycin was administered demonstrating the powerful effect of mTOR signalling.

Studies have shown that mTOR activity can also be negatively regulated by DNA damage responses protein 1 (REDD-1) that acts to restore the TSC1-TSC2 complex and subsequently inhibits rheb (DeYoung et al. 2008; Frost et al. 2009). Expression of this protein has been found to be increased under conditions of hypoxia (Brugarolas et al. 2004).

**1.12.5 S6 kinases**

Mammalian cells contain two genes for S6 kinases, giving rise to S6K1 (also known as $P^{70}S6K$) and S6K2. $P^{70}S6K$ was discovered much earlier and as much more is known about the function of this protein it will be discussed here in more detail than S6K2. Downstream targets of S6Ks are ribosomal protein S6 (rpS6) and eIF4B (Yang et al. 2008; Holz et al. 2005).

The activity of $P^{70}S6k$ is regulated by mTORC1, specifically by Raptor (Corradetti and Guan, 2006) and is phosphorylated in a sequential manner on a number of different residues, $Ser^{411}$, $Ser^{418}$, $Thr^{421}$ and $Ser^{424}$ by mTOR which then allows phosphorylation of $Ser^{389}$ and $Ser^{371}$ also by mTOR and finally allowing PDK1 to phosphorylate $P^{70}S6K$ on its $Thr^{229}$ residue (Jastrzebski et al. 2007). In the inactive form, $P^{70}S6K$ along with 4E-BP1 are bound to eIF3, part of the pre-initiation complex and, following stimulation by growth factors and nutrients, both $P^{70}S6K$ and 4E-BP1 are phosphorylated by mTORC1 causing their dissociation from this complex (Corradetti and Guan, 2006).

The importance of S6K as a growth effecter became apparent when the gene for $P^{70}S6K$ was successfully knocked out (Shima et al. 1998). These knockout mice are 20% smaller at birth, glucose intolerant and have low insulin levels due to a reduction in β cell volume. It was observed that there was an increase in S6K2 expression, but this was unable to reverse the growth defect caused by this genotype. Interestingly, the transcription of the 5' terminal oligopyrimidine (5' TOP) mRNA's (see Section 1.12.6) was intact indicating that the low birth size was not due to a decrease in the translational machinery (Shima et al. 1998). Those mice null for the $P^{70}S6K$ gene also had a muscle atrophy phenotype despite no alteration in phosphorylation of PKB or 4E-BP1, indicating that $P^{70}S6K$ plays a key role in IGF-1 induced skeletal muscle hypertrophy (Um et al. 2006)

**1.12.6 Ribosomal protein S6 (RpS6)**

RpS6 is a component of the 40S subunit of the ribosome and is phosphorylated on five residues, $Ser^{235}$, $Ser^{236}$, $Ser^{240}$, $Ser^{244}$ and $Ser^{247}$ by ribosomal protein S6 kinase (rpS6K) (Ruvinsky et al. 2005). Evidence from knockout mice show both $P^{70}S6K$ and S6K2 are required for the activation of rpS6, but predominantly S6K2 is required (Pende et al. 2004). Despite its discovery many years ago, the precise biological function of rpS6 remains obscure (Proud, 2007). However, the

phosphorylation of rpS6 correlates with protein synthesis rates and phosphorylated rpS6 is found at the mRNA binding site of 40S ribosomes, so it probably has a significant translational function. It has been suggested that rpS6 is involved in the translational control of the 5'TOP mRNA's that encode proteins associated with the assembly of the translational machinery (Ruvinsky et al. 2005). However, as described above, $P^{70}$S6K-/- mice have no defects in the translation of these mRNA's indicating that rpS6 phosphorylation is not required for this (Shima et al. 1998) and in fact these mice did not display any translation related phenotype. In support of these findings by Shima and colleagues (1998), rpS6-/- mice have a reduction in cell size, but not body size and a defective glucose homeostasis, but normal rates of protein synthesis (Ruvinsky, 2005).

## 1.12.7 Eukaryotic Initiation Factor 4E- Binding Protein 1 (4E-BP1)

The efficiency of mRNA binding to the 40S ribosome depends upon the availability of eukaryotic initiation factor 4e (eIF-4E) (see Section 1.10.1 above). When signalling through the PI3-K pathway is low and therefore anabolic signalling to protein synthesis is low, eIF4E is bound to 4E-BP1 sequestering it away from other initiation factors. However, when demand for protein synthesis increases, 4E-BP1 is hyper-phosphorylated. This dissociates the eIF4E:4E-BP1 complex thereby releasing eIF4E. Once released, eIF4E can bind with eIF4G and eIF4A creating the initiation complex eIF4F and allowing cap-dependent mRNA translation initiation (Shah et al. 2000). 4E-BP1 is phosphorylated at many sites by mTORC1 stimulated by growth factors and nutrients. In humans these are $Thr^{37}$, $Thr^{46}$, $Ser^{65}$, $Thr^{70}$, $Ser^{83}$ (Bai and Jiang, 2010), with the first four being of particular importance, and upon phosphorylation of $Ser^{65}$, eIF4E is released from its complex with 4E-BP1 (Hay and Sonenberg, 2004).

## 1.12.8 Activation of PKB/mTOR in relation to protein synthesis in humans

Results from studies using cell culture and small mammals suggest that insulin activates mTOR and up-regulates protein synthesis rates (Kimball et al. 1994; Biolo et al. 1995). However, there is evidence to suggest that there is some dissociation between insulin signalling and protein synthesis rates in humans. Greenhaff et al. (2008) investigated the effect of insulin on muscle protein synthesis and breakdown rates and changes in phosphorylation of anabolic signalling proteins in the presence of a continuous supply of amino acids in healthy human subjects. Four

insulin concentrations were used, 5, 30, 70 and 180 mU/l that were maintained using the insulin clamp technique. They observed that with an increasing plasma insulin concentration there was a dose-response effect on the phosphorylation status of PKB and $P^{70}S6K$ and that the phosphorylation of mTOR and 4E-BP1 was increased when plasma insulin concentrations were increased from 5 to 30 uM/l, but no further changes were seen when insulin concentrations were increased to 70 and 180 uM/l. However, these changes in phosphorylation status was seen to occur with no corresponding change in muscle protein synthesis rates measured from $[1-^{13}C]$ Leucine incorporation into the quadriceps muscle. Glover et al. (2008) investigated the effect of 14 days unilateral knee immobilization on muscle protein synthesis rates in the quadriceps muscle. They found immobilization did result in decreased rates of protein synthesis compared to the non-immobilized leg and this was unable to be restored by amino acid infusion. The changes in phosphorylation status of PKB, mTOR and $P^{70}SK6$ were not easily interpreted and as Greenhaff et al. (2008) had observed previously, didn't follow the changes in protein synthesis rates. This discrepancy in muscle protein synthesis rates and phosphorylation status of proteins thought to be important in anabolic signalling has also been demonstrated by de Boar and colleagues (2007) who also reported that lower leg immobilization resulted in reduced rates of myofibrillar protein synthesis with no corresponding changes in the phosphorylation of any of the proteins in the PI3-K pathway. Finally, Wilkinson et al. (2008) compared the effects of 10 weeks of resistance or endurance training on muscle protein synthesis rates and activation of the signalling proteins in the PKB/mTOR pathway. They observed that resistance exercise training lead to a significantly greater increase in resting muscle protein synthesis rates compared to endurance exercise, which was associated with changes in mitochondrial protein synthesis rates. This increase in muscle protein synthesis was also seen to occur with no obvious change in the phosphorylation of the regulatory proteins involved in translation initiation. These studies suggest that this pathway may be more complex in humans than has previously been described in rodents and that using the phosphorylation status of these proteins alone may cause misleading conclusions on the rates of protein turnover in humans and so caution may be required when interpreting this data.

## 1.13 The coupling of insulin signalling, glucocorticoid and activation of the ubiquitin-proteasome system in metabolic acidosis

In early experiments in which rats with intact kidneys were subjected to metabolic acidosis, an increase in skeletal muscle protein degradation was observed (May et al. 1986). However, depletion of ATP from the muscle abolished this effect demonstrating that the acceleration of muscle wasting was via a pathway that consumed energy (Mitch et al. 1994), i.e. the ubiquitin proteasome system, that is now accepted as a major contributor to mscle wasting in acidosis and uraemia (Lecker et al. 2006). Evidence from uraemic patients has shown that there is an increase in the expression of mRNA encoding ubiquitin and some proteasome subunits in CKD (Mitch and Goldberg, 1996), and correction of acidosis by sodium bicarbonate results in a decrease in the expression of ubiquitin (Pickering et al. 2003). As discussed in Section 1.11.3 changes in expression of the E3 ligases MuRF-1 and MAFbx have been used to infer changes in proteolysis. To date, only one study has investigated the effect of CKD on MuRF-1 and MAFbx. Lecker et al. (2004) found by Northern blot that the expression of MuRF-1 and MAFbx was increased in uraemic rats, but as protein degradation was not measured directly, we are unable to conclude if this change in expression conferred an increase in protein degradation.

It was aslo discussed in Section 1.8.2, that under conditions of catabolic stress, glucocorticoids may exert a permissive effect for the catabolic action of acidosis *in vivo*. This possible catabolic action of glucocorticoids also appears to be primarily through activation of the ubiquitin proteasome pathway. Bodine et al. (2001b) observed that dexamethasone administered to normal rats resulted in a 10-fold increase in expression of both MuRF-1 and MAFbx. This was also seen *in vitro* by Sandri et al. (2004), an effect that was rapidly suppressed by insulin. However, the MAFbx promoter lacks a glucocorticoid response element (GRE) and so the action of glucocorticoid on this ligase must be indirect and is most likely to be through expression of the transcription factor FOXO that controls expression of both MuRF-1 and MAFbx (Sandri et al. 2004). MuRF-1 on the other hand, does have a GRE upstream of the promoter so it appears this E3 ligase is directly activated by glucocorticoids and FOXO-1 synergistically (Waddell et al. 2008). Expression of FOXO-1 and FOXO-3 have been seen to be raised in myotubes in response to glucocorticoids *in vitro* (Imae et al. 2003) and led to the conclusion that glucocorticoids activate a transcriptional programme thought to be responsible for activating muscle atrophy (Schakman et al. 2008).

Glucocorticoids have also been shown to reduce protein synthesis rates. Shah et al. (2000) demonstrated that L6 myotubes incubated with dexamethasone have a reduced phosphorylation of 4E-BP1 and $P^{70}S6K$, ultimately resulting in a reduction in protein synthesis. A study by Wang and colleagues (2006) found an enhanced REDD-1 expression following dexamethasone administration in vivo, an effect that has been blocked using RU38486 in vitro (Shah et al. 2002) resulting in an increase in phosphorylation of these downstream targets of mTOR, suggesting that glucocorticoids exert their effects upon protein synthesis via regulation of mTOR activity. However, as discussed earlier (see Section 1.8.2) these studies use relatively high doses of glucocorticoids, and in light of the study by Crossland et al. (2010) who found a low dose of dexamethasone was actually able to suppress muscle protein degradation induced by endotoxaemia, the applicability of this data to humans where glucocorticoid concentrations are much lower, is questionable. However, as endotoxaemia is not characterized by acidosis, it is possible that in when combination with acidosis, the effect of glucocorticoids is catabolic and not anabolic, but much more data is needed before firm conclusions can be drawn.

In CKD, metabolic acidosis blunts the action of insulin even in non-diabetic patients. This effect is not just the result of lower insulin or IGF-1 concentration, as it has been reported that uraemia and acidosis are typically associated with normal or high insulin levels (Defronzo et al. 1981).

This insulin resistance appears not to result from a defect of the insulin receptor (Cecchin et al. 1988) and has therefore been attributed to post-receptor defects, possibly through a decrease in signalling through the PI3-K/PKB pathway, an impairment of which has been observed during atrophy (Bodine et al. 2001a). In a study by Bailey et al. (2006), it was demonstrated that the muscles of acidotic CKD rats had abnormalities in the IRS-1-associated PI3-K activity that were accompanied by a reduction in the phosphorylation of PKB, leading to activation of the transcription factor FOXO. This PKB inhibition also activated caspase-3 which is known to lead to the initial cleavage of the myofibrils prior to degradation by the UPS (Du et al. 2004) and generates the characteristic 14kDa actin cleavage fragment (Section 1.11.2) which has been found to be higher in the muscle of maintenance haemodialysis patients than in healthy controls (Workeneh et al. 2006). There was also an increase in the activity of IRS-2 to compensate for the decrease in IRS-1-associated PI3-K activity, but this was unable to prevent the decrease in PKB phosphorylation (Bailey et al. 2006). In addition to this, as previously described in Section 1.11.3, activation of FOXO and therefore PDK4 may also have a role to play in the impairment

of muscle carbonhydrate oxidation and therefore in the generation of insulin resistance in CKD (Constantin et al. 2007; Crossland et al. 2010). However, to date, there is no data to support this speculation.

The defect in the IRS-1-associated PI3-K activity during metabolic acidosis may arise partly from the secondary increase in glucocorticoid secretion during acidosis (May et al. 1986, Pickering et al. 2003). In rats, a pharmacological dose of dexamethasone results in a reduced content of both IRS-1 and PI3-K in muscle and a reduced IRS-1 associated PI3-K activity (Saad et al. 1993). Hu et al. (2009) discovered that endogenous glucocorticoids bind to the glucocorticoid receptor which then competes with IRS-1 for PI3-K binding, further reducing IRS-1-associated PI3-K activity and PKB phosphorylation, thus promoting atrophy. This mechanism has yet to be confirmed in humans, and the direct effect upon protein degradation rates would need to be determined in light of the suggestion of a dissociation between anabolic signalling and protein turnover in humans (Greenhaff et al. 2008).

## 1.14 Depletion of intramuscular amino acids

It is well established that branched chain amino acids (BCAA), in particular Leucine, are anabolic in muscle, capable of stimulating muscle protein synthesis (Rennie et al. 2006). Under conditions of catabolic stress, depletion of these BCAA could potentially be a limiting factor for protein synthesis (Bergström et al. 1990). Therefore, the depletion of intramuscular amino aicds is thought to be an important early step in the muscle wasting process and abnormalities have been reported in both muscle and plasma amino acid concentrations and in the distribution of amino acids between cells and extracellular fluid in CKD patients compared to healthy individuals (Bergström et al. 1990). Early studies of patients with untreated chronic uraemia reported low intramuscular concentrations of threonine, lysine, arginine, valine and taurine with low plasma concentrations of leucine, lysine, phenylalanine and again, taurine (Bergström et al. 1990; Alvestrand et al. 1982; Bergström et al. 1989). It appears that some, but not all of these abnormalities can be corrected with nutrition (Alvestrand et al. 1978). However, it has also been observed that rats with chronic renal failure exhibit a similar pattern of amino acid depletion despite the same food intake as control rats (Haines et al. 1989) indicating that is not a result of malnutrition, but of factors relating to uraemia.

Once the patients began haemodialysis, depletion of intramuscular valine and serine was still observed and the muscle/plasma concentration gradients for glycine, citrulline and arginine were low (Bergström et al. 1990). A strong positive correlation was also observed between the intramuscular valine concentration and the pre-dialysis plasma bicarbonate concentration in these haemodialysis patients, suggesting that metabolic acidosis may have a role in intramuscular amino acid depletion (Bergström et al. 1990). This was confirmed in a later study by rigorous correction of metabolic acidosis by use of high bicarbonate haemodialysis fluid which significantly increased the intramuscular concentrations of alanine, isoleucine, leucine and valine; and increased the muscle/plasma amino acid concentration gradients for these amino acids as well as arginine, asparagine, glycine, serine and histidine (Lofberg et al. 1997). These changes were accompanied by a statistically significant fall in the plasma concentrations of glutamine, glycine and histidine. These effects on concentration gradients were observed in fasted patients, thus eliminating transient disturbances to amino acid concentrations caused by amino acid intake from the most recent meal. This implies that the active transport mechanisms responsible for pumping amino acids into the muscle cells, and for maintaining the intracellular concentrations higher than in extracellular fluid, are impaired in metabolic acidosis.


## 1.15 The SNAT/slc38 amino acid transporters

The System N and A Neutral Amino Acid Transporters (SNATs) are members of the slc38 gene family and are sub-divided into System A and System N transporters according to their substrate specificities (i.e. the solutes that they carry). System A has three isoforms, SNATs 1, 2 and 4, and System N has two isoforms, SNATs 3 and 5 (Mackenzie and Erickson, 2004).

System A transporters were originally described in the 1960s as sodium-dependent transporters that carry small zwitterionic (neutral) amino acids into the cell along with one $Na^+$ cation that binds to the transporter first (Mackenzie and Erickson, 2004). This is a secondary active process utilising the $Na^+$ gradient set up by the $Na^+,K^+$-ATPase pump (Palii et al. 2006). The property of System A transporters distinguishing them from the System N transporters is the ability to transport N-Methylamino acids, for example, α-Methylaminoisubyrate acid (MeAIB) (Yao et al. 2000), and the uptake of this synthetic amino acid is often used as a measure of System A transport activity (McGivan and Pastor-Anglada 1994). System A's naturally occurring substrates include alanine, asparagine, cysteine, glutamine, glycine, methionine and serine. In

contrast System N only carries glutamine, histidine and asparagine (Mackenzie and Erickson, 2004). System A is regulated by environmental conditions, for example, it is up-regulated by insulin and amino acid starvation (Yao et al. 2000), is activated by exercise (King et al. 1994) and, of particular relevance to amino acid abnormalities in metabolic acidosis, it is strongly inhibited by low pH (Mackenzie and Erickson, 2004).

The only SNAT transporter that is strongly expressed in skeletal muscle is SNAT2 (Mackenzie and Erickson, 2004) so, for the purposes of this thesis, System A activity in muscle will be regarded as a consequence of the SNAT2 transporter.

### 1.15.1 The SNAT2 amino acid transporter

SNAT2 is widely expressed in mammalian tissue and is found in skeletal muscle, brain, adrenal glands, liver, kidney, lungs and skin (Mackenzie and Erickson, 2004) and appears to be the most highly regulated isoform in response to endocrine and nutrient stimuli (Baird et al. 2006, McDowell et al. 1998). SNAT2 directly accumulates amino acids, for example L-Glutamine, inside the cell, maintaining an intracellular L-Glutamine concentration that is approximately 10 times greater than in the extracellular fluid (Mittendorfer et al. 2001). As with System A transporters in general (Section 1.15), the activity of this transporter is up-regulated in response to insulin (McDowell et al. 1998) and amino acid starvation (Mackenzie and Erickson, 2004) and is suppressed when the external pH falls, over the physiologically relevant range (pH 7.8 – 7.0) (Baird et al. 2006).

This suppressed activity by low pH was initially thought to be caused by $H^+$ directly competing with $Na^+$ for the cation binding site (Chaudhry et al. 2002). However, more recently, mutation of the c-terminal histidine residue of SNAT2 has been shown to blunt the pH sensitivity, suggesting that this terminal histidine has functional effects in the sensing of $H^+$, as it does in other membrane proteins (Zong et al. 2001; Uchiyama et al. 2003). Baird and colleagues (2006) found that reducing the extracellular pH resulted in a reduced affinity for $Na^+$ binding to SNAT2, and pre-treatment of the cells with the histidine specific reagent DEPC reduced the pH sensitivity, leading to the conclusion that extracellular $H^+$ affects SNAT2 transport activity by interacting with one or more conserved histidine residues which indirectly affect allosteric binding of $Na^+$.

As well as being readily detectable at the plasma membrane (Hyde et al. 2002), SNAT2 is also reported to be present in intracellular membranes and is translocated to the plasma membrane in response to insulin in a manner similar to GLUT4 translocation (Hyde et al. 2001, 2002; Hatanaka et al. 2006). However, there is no evidence that internalisation of SNAT2 has any role in the reduction of its activity in response to low pH.

**1.15.2 SNAT2, amino acid depletion and suppression of protein synthesis**

As discussed in Section 1.14, intramuscular amino acid depletion may be an early contributor to cachexia (Bergström et al. 1990), but the precise mechanism of this depletion *in vivo* is not well defined.

Previous work from our laboratory using cultured L6-G8C5 myotubes has suggested a role for SNAT2 in the regulation of intramuscular amino acid content and signalling through mTOR to protein synthesis (Evans et al. 2007). Inhibition or silencing of the SNAT2 transporter resulted in reduction of the intracellular concentration of a number of amino acids, notably L-Glutamine and the overall pattern of amino acid depletion was similar to the amino acid depletion observed in the muscles of acidotic CKD patients (Bergrstöm et al. 1990; Lofberg et al. 1997). A number of these depleted amino acids are not carried on SNAT2, for example, L-Leucine. This suggests that SNAT2 and the System L amino acid transporter (that is capable of transporting L-Leucine) are coupled, with System L exchanging the intracellular amino acids accumulated by SNAT2 for extracellular L-Leucine. The depletion of intracellular amino acids observed on inhibition or gene silencing of SNAT2 was accompanied by impaired signalling through mTOR, implying that SNAT2 has a role in the regulation of mTOR through the widely documented effect of free amino acids on this kinase (Kimball et al. 1999).

**1.15.3 SNAT2 and Proteolysis**

Further results from this laboratory have shown that silencing of SNAT2 using small interfering RNAs (SiRNA) stimulates proteolysis rates in L6-G8C5 myotubes, accompanied by an impairment in the activation of PI3-K and PKB in the presence of insulin (Evans et al. 2008).

The resemblance between these effects of SNAT2 silencing in myotubes *in vitro* and the effects of metabolic acidosis on skeletal muscle *in vivo* suggests that, owing to its sensitivity to pH, SNAT2 may have a functionally significant role in the control of protein synthesis and degradation in uraemic metabolic acidosis. The activity of System A transporters has been reported to be low in the skeletal muscle of rats with acute uraemia incubated *ex vivo* (Maroni et al. 1990); and the activity in skeletal muscle of CKD patients (assessed by measuring uptake of ^{11}C-MeAIB by positron emission tomography) is also reported to be low (Asola et al. 2001), but it is important to emphasise that there is no direct evidence yet for SNAT2 mediating the effects of acidosis *in vivo*.

## 1.16 Exercise in CKD patients

Recent published guidelines by the UK National Institute for Health and Clinical Excellence (NICE) gave the recommendation that CKD patients "should be encouraged to take exercise" (NHS, 2008), but there are currently no specific exercise guidelines for this population, unlike cardiac and pulmonary populations. However, exercise in CKD populations is an area that is gaining in popularity and momentum and the first sessions were dedicated to it at a recent congress (World Congress of Nephrology, Milan, 2009). There is a growing body of research in this area that will hopefully lead to the implementation of exercise programmes for these patients.

CKD patients have a number of biochemical abnormalities such as reduced insulin sensitivity and glucose tolerance (Goldberg et al. 1983, 1986), hypertension (Johansen, 2005) and dyslipidemia (Bronas, 2009). These, and an abnormal internal milieu, contribute to a vastly increased risk of cardiovascular events, which is the biggest cause of mortality in these patients and poses a significant challenge to clinicians (Bronas, 2009). In the healthy population, exercise helps to reduce risk factors for CKD and so exercise in this population may also help to reduce mortality and increase quality of life. As in the healthy population, sedentary behaviour carries with it an increased risk of all-cause mortality. O'Hare et al. (2003) observed 2,837 patients for a one year period and showed that the sedentary patients had a 62% greater risk of mortality than those patients who were more active. The majority of research into exercise implementation has focused on the dialysis population and has shown significant benefits (Kouidi et al. 1998; Kopple

et al. 2007; Koufaki, et al. 2002), but there has been very little research performed using the pre-dialysis population.

### 1.16.1 Exercise tolerance and capacity in CKD patients

Patients on dialysis are extremely inactive compared to their healthy counterparts (Johansen et al. 2000) and Painter et al. (2000) reported that 59% of HD patients participated in no physical exercise at all. This may be the result of a lack of encouragement to do so by their doctors or nurses (Johansen et al. 2003), an increase in co-morbidity as the disease progresses, a general feeling of being unwell, depression or cardiac dysfunction (Kouidi et al. 1998). This reduced exercise capacity is further compounded by anaemia (Painter, 2005), which it appears cannot be improved with exercise (Zabetakis et al. 1982; Painter et al. 1986).

Many early studies reported that CKD patients have a reduced $VO_{2peak}$ compared to the healthy population (Barnea et al, 1980) and this has been reported to be up to 60% less (Painter, 2005) ranging from 17.0 mL/kg/min to 28.6 mL/kg/min (Kouidi et al. 1998; Lundin et al. 1981). Many studies have shown that with aerobic exercise, CKD patients can improve their $VO_{2peak}$, with improvements ranging from 17% (Hagberg et al. 1983) up to 43% (Konstantinidou et al. 2002) and even improvements of 70% after one year of cycle ergometer exercise (Kouidi et al. 2004). A few studies have reported no improvement in $VO_{2peak}$ (Carney et al. 1983; Akiba et al. 1995) and this can probably be attributed to a low exercise intensity and a short duration of the exercise training period. Correction of anaemia with recombinant human erythropoietin (rHuEPO) results in a very small improvement in $VO_{2peak}$ (Painter and Moore, 1994) suggesting that this is not the main cause of a limited exercise capacity.

### 1.16.2 Muscular abnormalities in CKD patients

CKD patients exhibit severe muscular atrophy that appears to affect type II fibres more than type I (Kouidi et al. 1998). Many abnormalities are present at the ultra structural level, for example, swollen mitochondria, disappearance of the cristae and reduced matrix density (Kouidi et al. 1998). Electron microscopy has shown Z-band degeneration, myofilament loss and accumulation of intracellular glycogen (Diesel et al. 1993) and fibre splitting, internalized nuclei and moth-eaten fibres by light microscopy (Diesel et al. 1993). This atrophy has also been seen in non-locomotor muscles demonstrating that these changes are unlikely to be caused purely by disuse,

and are probably due to uraemia (Sakkas et al. 2003). As well as the abnormalities in mitochondrial structure described above, these patients also exhibit dysfunctional intramuscular energy metabolism (Johansen et al. 2005), but the cause of this is currently unknown. Following a bout of exercise, there is a slowing in the recovery of phosphocreatinine (PCr) (Kemp et al. 2004) and a reduced PCr level and pH and a higher Pi/ PCr ratio (Johansen et al. 2005), indicating their ability to recover from a bout of exercise is grossly impaired. In principle, exercise in CKD patients may worsen pre-existing metabolic acidosis through exercise-induced lactic acid generation and this may explain the abnormally prolonged and severe intramuscular acidification observed in these patients following exercise (Johansen et al. 2005).

Several studies have reported an improvement in these muscular abnormalities with exercise training in haemodialysis patients. Kouidi et al. (1998) saw an increase in the fibre cross sectional area for type I by on average 25.9%, and 23.7% for type II following a six month mixed exercise programme, and similar changes were seen by Sakkas et al. (2003). A regeneration of degraded fibres and an improved architecture was observed following exercise training (Kouidi et al. 1998; Sakkas et al. 2003). The mitochondria were of a normal shape and size with normal orientation of the cristae (Kouidi et al. 1998), and there was an increased capillary content of the muscle (Sakkas et al. 2003). This suggests that skeletal muscles of CKD patients are able to respond to exercise training in the same way as a healthy individual, but whether they are able to adapt to the same extent is unknown.

CKD patients generally have a reduced muscular strength compared to healthy controls (Johansen et al. 2003), but this can also be improved with resistance exercise training. Castaneda et al. (2001) saw a 32% increase in muscle strength in pre-dialysis patients following a 12 week programme compared to a 13% reduction in strength in the non-exercising CKD control group. Headley et al. (2002) conducted a 12 week resistance exercise programme in haemodialysis patients and saw an improvement in peak torque of the leg extensors and an improvement in several physical functioning tests, with no adverse event reported in either study. Muscle wasting and weakness are well established predictors of increased mortality (Kosmadakis et al. 2010) and so any improvement in these parameters may lead to a reduced mortality risk.

### 1.16.3 Cardiac Function

It is extremely important for renal patients to maintain good blood pressure control, as hypertension can accelerate progression of kidney disease. A study by Miller and colleagues (2002) was designed solely to examine the effect of an intradialytic exercise programme on blood pressure. They reported that after six months of cycling exercise there was no significant change in blood pressure compared to the control patients, but patients were taking on average 36% less antihypertensive medications, an annual saving that the authors calculated was US$885/patient. An improvement in blood pressure has also been reported by Boyce et al. (1997) following four months of exercise training; an improvement that was reversed by two months of detraining.

## 1.17 Signalling events in response to exercise and training adaptations

Resistance exercise training is mainly associated with muscle hypertrophy and an increase in strength and power. Endurance exercise training on the other hand is associated with adaptations that lead to an increase in oxidative capacity, resulting in a slower rate of muscle glycogen and blood glucose utilisation, an increased reliance upon fatty acid oxidation and a reduction in lactate production during submaximal exercise (Hollosky and Coyle, 1984). These adaptations include: increases in stroke volume and cardiac output resulting in a reduced heart rate, increased capillarisation and importantly, an increase in mitochondrial content (mitochondrial biogenesis) (Hood, 2009). Mitochondrial content can be doubled within six weeks of endurance training, but due to their short half life, repeated exercise is required to maintain these increased levels (Zierath and Hawley, 2004). The diverse adaptations to training that occur with endurance and resistance training result from different molecular events in the hours following the exercise bout.

A bout of resistance exercise results in an increase in protein synthesis rates that can be maintained for up to 48 hours (Phillips et al. 1997). This increase in protein synthesis is thought to be brought about through an increase in signalling through the PI3-K/PKB pathway (Baar, 2006; Nader, 2006). A single bout of resistance exercise can increase the activity of PI3-K (Hernandez et al. 2000) and increase PKB phosphorylation (Nader and Esser, 2001) and $P^{70}S6K$ phosphorylation (Baar and Esser, 1999; Koopman et al. 2006) resulting in rpS6 and 4E-BP1 activation, but only in the post-prandial state (Koopman et al. 2006; Witard et al. 2009). If

resistance exercise is performed in the fasted state, it appears that this increase in protein synthesis is suppressed due to AMPK activation, an important energy sensor that reduces the phosphorylation of 4E-BP1 (Dreyer et al. 2006).

Endurance exercise, on the other hand, is not usually associated with hypertrophy and large increases in protein synthesis rates, but primarily with an increase in mitochondrial biogenesis and therefore mitochondrial content (Holloszy and Coyle, 1984). This adaptation requires the activation of a different pattern of signalling events. The long exercise duration results in an increase in the AMP:ATP ratio that activates AMPK, inhibiting ATP-consuming pathways and increasing carbohydrate and fatty acid oxidation (Wackerhage, 2006; Hawley, 2009). Exercise also results in calcium release from the sarcoplasmic reticulum, increasing intramuscular calcium concentrations (Wackerhage, 2006) that activate calmodulin-activated kinase (CamK). CamK and AMPK allow myocyte-enhancing factor 2 (MEF2) to bind to the proliferator-activated receptor γ coactivator 1α (PGC-1α). PGC-1α then increases the expression of nuclear respiratory factors 1 and 2 (NRF-1,2) that bind to promoters and activate transcription of genes encoding mitochondrial respiratory chain proteins (Kelly and Scarpulla, 2004). NRF-1 also regulates the expression of mitochondrial transcription factor A (Tfam) that binds to mitochondrial DNA and induces mitochondrial biogenesis (Baar, 2006; Wackerhage, 2006; Hawley, 2009).

There is some evidence that moderate intensity (75% $VO_{2Max}$) endurance exercise can increase phosphorylation of mTOR that remains above basal for two hours after exercise (Mascher et al. 2007). An increase in phosphorylation of PKB was seen one and two hours after exercise which returned to baseline after three hours. This delay in PKB phosphorylation suggests that mTOR is activated by PKB, but also by an alternative mechanism. There was, however, no signalling downstream of mTOR to $P^{70}S6K$ (Coffey et al. 2006), suggesting another role for mTOR activation following endurance exercise. There is currently a lack of agreement on the effect of endurance exercise upon PKB phosphorylation. Some studies report no change (Widegren et al. 1998), but others do show an increase (Sakamoto et al. 2004). It is likely that this discrepancy arises from differences in the intensity and duration of exercise and in participant characteristics.

As well as an increase in mTOR phosphorylation, Mascher et al. (2007) also reported a dephosphorylation of eukaryotic elongation factor 2 (eEF2), that is activated by eEF2 kinase (eEF2k), following one hour of exercise at 75% $VO_{2Max}$ that occurred with a lack of $P^{70}S6K$

activation. It is thought that insulin acting through mTOR may inactivate eEF2 kinase thus dephosphorylating eEF2 that in its phosphorylated state serves to inhibit elongation, the major energy consuming step of protein synthesis (Proud et al. 2001).

However, recent evidence from two other studies that concomitantly measured protein synthesis rates and the phosphorylation status of some of the proteins in the PI3-K/PKB pathway suggest that this pathway may be a little more complicated in humans than previously thought. Wilkinson et al. (2008) showed that an acute bout of single-legged endurance exercise was associated with an increase in the phosphorylation of PKB, mTOR and $P^{70}S6K$, but this was seen without any changes in myofibrillar protein synthesis rates. Likewise, Greenhaff et al. (2008) saw that in healthy human subjects, a stepwise increase in plasma insulin concentrations resulted in stepwise increases in the phosphorylation of PKB and $P^{70}S6K$, but this was also seen with no changes in muscle protein synthesis rates. These results suggest that using the phosphorylation status of proteins such as these described above as markers of protein turnover in muscle, may be an oversimplification.

### 1.17.1 Exercise signalling in CKD

There is very little information regarding signalling events following exercise in CKD and the only studies that have been performed were in rodents. Wang and colleagues (2009) found that as previously reported (Bailey et al. 2006), the skeletal muscles from CKD mice (subjected to partial nephrectomy) had a reduced phosphorylation of PKB. However, they reported that two weeks of treadmill running or two weeks of muscle overload were able to correct this defect, returning phosphorylation levels back to those seen in the sham-operated control mice. This was accompanied by an increase in phosphorylation (and hence inactivation) of the FOXO transcription factors in both modes of exercise and ultimately a suppression of muscle protein breakdown, as evidenced by a reduced level of the 14kDa actin fragment in the muscle. Muscle overload was able to increase phosphorylation of mTOR and its downstream target $P^{70}S6K$ and reverse the CKD-induced suppression of protein synthesis. Treadmill running, however, was unable to increase the phosphorylation of these downstream targets despite a small increase in PKB phosphorylation, and only small increases in protein synthesis rates were seen, which is consistent with results from healthy humans undergoing endurance training (Coffey et al. 2006).

Chen and colleagues (2008) observed that rats (both with surgically induced CKD and sham-operated controls) were able to activate PKB and $P^{70}S6K$ to the same extent in muscle following muscle overload, which was also accompanied by a decrease in the accumulation of the 14kDa actin fragment in both groups and hence a decrease in myofibrillar degradation. This evidence suggests that overload can overcome the signalling defects that are present in uraemic muscle and can effectively activate the PI3-K/PKB pathway.

## 1.18 Cell stretch *in vitro*

Muscle protein synthesis is modulated by mechanical activity (Hornberger et al. 2006), but the mechanisms by which this occurs are only recently becoming understood. What is well known however, is that as discussed above in Section 1.12.1 myocyte growth is regulated by mTOR through activation of downstream substrates. Mechanically overloading a muscle results in hypertrophy and underloading leads to atrophy (Goldberg et al. 1975) suggesting that tension may be playing a role in muscle growth (Hornberger et al. 2006). Administration of rapamycin to rats with overloaded plantaris muscles prevents the occurrence of hypertrophy (Bodine et al. 2001a) suggesting a role for mTOR in mechanically induced hypertrophy.

In contrast, passive stretch may be exerting effects via a different mechanism. Vandenburg (1987) demonstrated that cyclic stretch applied to myotubes *in vitro* resulted in an increase in protein synthesis rates and an accumulation of cellular protein with a decrease in protein degradation. This has been confirmed *in vivo* by Hornberger and colleagues (2006) who observed that muscles from mice incubated and subjected to passive intermittent stretch *ex vivo* had increased the rates of protein synthesis, an effect that was completely blocked by rapamycin. They also saw an increase in the phosphorylation of $P^{70}S6K$, without any change in PKB phosphorylation, suggesting that the increase in protein synthesis that is seen following passive stretch occurs via a PI3-K independent mechanism.

However, more recent measurements *in vitro* suggest that the timing of the measurements of protein synthesis rate may be critical:  Atherton and colleagues (2009) found an *inhibition* of protein synthesis in cultured myotubes *during* cyclic stretch, in accordance with the inhibition of protein synthesis that is seen during (but not following) exercise *in vivo*. *Following* stretch *in*

*vitro* they found an increase in mTOR, $P^{70}S6K$ and 4E-BP1, but not PKB phosphorylation accompanied by an increase in protein synthesis rates (Atherton et al. 2009) supporting the earlier results of Hornberger et al. (2006).

## 1.19 Limitations of exercise performance

The factors limiting exercise performance in healthy individuals are a complicated and multi-factorial set of process that depends on the duration and intensity of the exercise bout. As this thesis is primarily concerned with muscle metabolism, only fatigue originating in the skeletal muscle will be discussed here.

During short duration high intensity exercise, ATP is replenished by the hydrolysis of intramuscular PCr stores, and through anaerobic glycolysis which results in the production of lactate. High intensity anaerobic exercise sees a rapid decline in the ATP and PCr concentration of the type II muscle fibres: following a 30 second maximal sprint, concentrations of ATP and PCr fall by approximately 40% and 70% respectively (Cheetham et al. 1986). This results in a reduced rate of glycolysis after approximately 20 seconds (Soderlund et al. 1992) and an accumulation of ADP resulting in a reduction in force production and ultimately, fatigue. Lactate production is also associated with the accumulation of $H^+$ (causing acidosis) which is a commonly cited cause of fatigue. However, recent studies have shown that reduced pH may have little effect on muscle contraction and therefore fatigue at physiological temperatures (Wiseman et al. 1996; Westerblad et al. 1997) and so fatigue must result from another product of anaerobic glycolysis. Inorganic phosphate produced during the breakdown of PCr has been shown to inhibit force generation in human muscle (Wilson et al. 1988) possibly by interfering with cross bridge formation or disruption of calcium handling and sensitivity (Westerblad et al. 2002; Allen et al. 2008), and is now a candidate for one of the main causes of muscular fatigue (Westerblad et al. 2002).

Fatigue during prolonged exercise in temperate conditions is mainly due to muscle glycogen depletion and a fall in muscle carbohydrate (CHO) oxidation rates (Sherman et al. 1983; Tsintzas et al. 1996). At fatigue, muscle glycogen concentrations have been found to be nearly depleted (Bergström et al. 1967) and the ingestion of CHO both before and during the exercise bout (Sherman et al. 1991; Coyle et al. 1986), improves performance. Muscle glycogen depletion

results in a reduced rate of ATP resynthesis, an increase in ADP and Pi concentrations, a decrease in the intermediates of the TCA cycle and therefore reduced oxidative ATP resynthesis (Maughan et al. 1997b).

Limitations to exercise in CKD patients appear to stem primarily from the muscular abnormalities described in Section 1.16.2 as CKD patients frequently attribute the early termination of exercise to muscle fatigue (McMahon et al. 1999; Lundin et al. 1987). CKD patients have defective mitochondrial energy metabolism, there is an accelerated rate of decline of PCr stores during exercise (Durozard et al. 1993) and a slower recovery following exercise (Reviewed in Kosmadakis et al. 2010), but the precise mechanism for this defect is currently unknown. Biopsy studies performed in haemodialysis patients have also shown fibre type grouping, which suggests denervation and subsequent re-innervation. However, central activation of motor units has been found to be normal (Johansen et al. 2003). It is possible that acidosis has a greater role to play in the development of fatigue in these patients. Nishida et al. (1991) observed using 31-P NMR that there was a greater decline in sarcosolic pH during aerobic exercise in CKD patients compared to healthy controls and a slower rate of its recovery, an observation that has been confirmed by other groups (Durozard et al. 1993; Kemp et al. 2004). Earlier studies have reported that an imposed mild metabolic acidosis by either $NH_4Cl$ administration or through diet manipulation was sufficient to reduce exercise capacity in healthy individuals during high intensity exercise (Jones et al. 1977; Greenhaff et al. 1987) and it was suggested that acidosis may have directly caused the early fatigue possibly by reducing the rate of glycolysis. However, the exact role acidosis played in the development of fatigue is unclear as it was later demonstrated that the acute correction of diet induced metabolic acidosis by bicarbonate supplementation failed to have an effect on time to exhaustion (Ball et al. 1996). It may be that under conditions of chronic metabolic acidosis, such as that in CKD, the role that acidosis plays in fatigue is significantly greater than when it is merely transient. CKD patients also appear to have a reduced ability to increase muscle blood flow during submaximal and maximal exercise compared to controls (Bradley et al. 1990) coupled with anaemia further reducing these patients ability to exercise.

## 1.20 Unanswered Questions and Aims

Muscle wasting is a clinically significant problem in CKD patients which appears to be partly caused by a combination of metabolic acidosis and glucocorticoids. The correction of acidosis by sodium bicarbonate therapy has been shown to improve nitrogen balance and morbidity in these patients. Exercise has also been shown to provide benefits to patients receiving haemodialysis therapy, but there is little information in the literature regarding potential benefits of exercise to skeletal muscle in the pre-dialysis population, nor is it known whether combining correction of acidosis with exercise would yield additional benefits. CKD is associated with impaired insulin signalling that in uraemic rat models has been shown to lead to the suppression of protein synthesis and the stimulation of protein degradation. It is not known if insulin signalling is suppressed in human CKD patients, or if this signalling has a tight relationship to protein synthesis and degradation, which has been shown in animal models, but seems to be more doubtful in healthy humans. If there is impairment in insulin signalling, it is not known if this can also be overcome using exercise and what effect this would have on protein turnover.

The acid load that CKD patients fail to excrete, leading to uraemic metabolic acidosis, arises largely from catabolism of the sulphur amino acid cysteine to sulphuric acid. The concentration of some of these sulphur metabolites is abnormal in uraemia, and there may be a defect in the activity of the enzyme CSAD resulting in a greater proportion of the flux from cysteine being metabolised to sulphuric acid rather than to taurine, thus adding to the burden of metabolic acidosis in these patients. However, few direct measurements of the flux from cysteine to sulphuric acid and taurine have been reported in CKD patients and the relative excretion rates of these metabolites of cysteine oxidation would shed more light on this.

Results from animal models have shown that glucocorticoids when in combination with metabolic acidosis, may have a role in the muscle wasting process that is commonly seen in uraemia via activation of the ubiquitin proteasome system. A functionally significant role for the pH-sensitive neutral amino acid transporter SNAT2 has also been identified in mediating the effects of acidosis on protein synthesis and degradation in a culture model of skeletal muscle *in vitro*. Whether a combined effect of acid and glucocorticoids upon SNAT2 might contribute to the synergism between acidosis and glucocorticoid in muscle wasting is currently unknown.

The specific hypothesises of this project were therefore:

In Chapter 3:

- It was hypothesised that six months of walking exercise would increase LBM (as measured by DEXA) compared to the control group. This would be associated with increased basal insulin signalling (increased phosphorylation of PKB, $P^{70}$S6K and 4E-BP1) suggesting and increase in muscle protein synthesis rates, and a reduction in MuRF-1 and MAFbx mRNA expression, reduced levels of 14kDa fragment, reduced 3-MH excretion rates and an increase in muscle protein:DNA ratio, indicating reduced rates of myofibrillar proteolysis.

- The effects of exercise and additional acidosis correction using sodium bicarbonate in these patients would be additive.

In Chapter 4:

- It was hypothesised that CKD patients would excrete more sulphuric acid and less taurine than healthy individuals and that the sulphate excretion (and hence sulphuric acid production) would vary widely between individuals, but correlate with myofibrillar protein degradation, thus possibly allowing identification of those patients who may be high acid producers and therefore may be at a greater risk of muscle wasting.

- Exercise and bicarbonate supplementation would reduce sulphuric acid production in CKD patients.

In Chapter 5

- It was hypothesised that glutamine would reduce sulphuric acid production *in vitro*.
- Glucocorticoids, like acidosis, would reduce the transport activity of SNAT2.
- Passive and cyclical stretch would increase mRNA expression and transport activity rates of SNAT2.

Chapter 2


General Methods

All the methods described in this chapter are used in more than one experimental chapter.

**Note:** For convenience, throughout this thesis all microcentrifuge speeds are quoted in rpm because all centrifuges used have approximately the same rotor radius of 7cm. All other centrifuge speeds have been quoted as the maximum g force generated at the outer edge of the rotor.

## 2.1 Blood sampling, handling and analysis

Venous blood samples, approximately 7ml, were collected for studies described in Chapters 3 and 4. Samples were taken from a superficial antecubital vein, drawn into a dry syringe and dispensed into a tube containing either the anticoagulant $K_2$ EDTA or lithium heparin, depending upon its intended analytical use. Arterial blood samples were also taken during the study described in Chapter 3 at each time point prior to the muscle biopsy, this data is also presented in Chapter 4. A 3ml sample was taken from the brachial artery following the administration of 1% lignocaine, drawn into a blood gas syringe containing heparin and taken to the Pathology Department at the Leicester General Hospital for analysis of arterial pH and bicarbonate.

## 2.2 Urine sampling and handling

Consenting patients and healthy volunteers underwent a diet free from animal protein for 4 days and collected a 24h urine sample on the final day of the diet. The urine volume was measured and aliquots were stored at $-20^{\circ}C$ prior to assay for sulphate, and amino acid derivatives by High Performance Liquid Chromatography (HPLC) as described below.

## 2.3 Measurement of amino acids and their derivatives by high performance liquid chromatography

Muscle and plasma samples were analysed for their full amino acid profiles. Urine samples were analysed for the derivatives of sulphur amino acid catabolism, cysteinesulphinic acid (CSA) and taurine and for the myofibrillar catabolite 3-Methyl Histidine (3-MH).

## 2.3.1 Preparation of muscle samples for amino acid analysis

Approximately 2mg (dw) muscle was homogenised (T8.01 Netzgerät, IKA, Germany) in 0.3M PCA (400μl) containing 1mM Norvaline as an internal standard and incubated on ice for 30 minutes. The samples were centrifuged in a microcentrifuge at 13,000rpm for 15 minutes at 4°C. The resulting supernatant was stored on ice and the pellet was retained for determination of total DNA concentration (see Chapter 3.2.7.2). An equal volume of freon-trioctylamine (78% v/v 1,1,2-trichlorotrifluoroethane, 22% v/v trioctylamine) was added to the supernatant and vortexed for 1 minute. The tubes were centrifuged briefly (for approximately 10 seconds at 13,000rpm) resulting in the formation of three phases. Approximately 80% of the top (aqueous) phase was taken off and filtered through a 0.45 microfilter into a glass HPLC vial.

## 2.3.2 Preparation of the plasma and urine samples for amino acid analysis

Plasma (500μl) was deproteinsed using 12M PCA (12.5μl) containing 40mM Norvaline as an internal standard. Urine (1ml) was deproteinsed using 12M PCA (25μl) containing 40mM Norvaline. The tubes were vortexed for 1 minute and incubated on ice for 30 minutes. From this point, the method of amino acid extraction from plasma and urine was the same as described above for muscle, with the exception that the pellets formed following centrifugation were discarded.

## 2.3.3 Amino acid separation by reverse-phase HPLC

Amino acid master standard was made fresh daily and comprised 25μl Agilent amino acid standard (5061-3330), 75μl ultra pure water and 1μl supplementary amino acid mixture. Amino acid concentrations were determined by reverse phase HPLC. All samples were separated on an Agilent (1100 Series) chromatograph with ultraviolet (UV) detection using a Zorbax eclipse AAA column (4.6 x 75mm, 3.5μm) with pre-column derivatisation using Orthophthalaldehyde, (OPA; Agilent, UK, 5061-3335) and Fluorenylmethyloxycarbonyl chloride (FMOC; Agilent, UK, 5061-3337). Samples were eluted with 40mM $Na_2HPO_4$, pH 7.8 supplemented with a linear gradient of 0-57% v/v Acetonitrile:Methanol:water (45:45:10) applied between t = 1 and 9.8 minutes in each run. At the end of each run, the column was purged with 100% aceonitrile:methanol:water for two minutes and re-equilibrated with 100% 40mM $Na_2HPO_4$, pH

7.8 for two minutes before injecting the next sample. Peaks were integrated using the Agilent Chemstation software and concentrations calculated in relation to the standard.

## 2.4 Protein Assays

Total protein concentration of acid precipitates from muscle and cell cultures was measured using the Folin Lowry protein assay. Protein in lysates intended for Western blotting was analysed using the BioRad Detergent Compatibile (DC) assay (BioRad, UK, 500-0116).

### 2.4.1 Folin Lowry protein determination

Acid-precipitated protein pellets were dissolved in 1ml 0.5M sodium hydroxide and heated at 70°C in a water bath for 30 minutes.  Just before use, Folin Reagent A was mixed with Reagent B (see Appendix A) at a ratio of 50:1 to give Reagent C and Folin Ciocalteu's Phenol reagent (Sigma, Dorset, UK, 47641) was diluted 1:2 using water. Samples were diluted to bring them within the range of the standard calibration (0-500μg/ml). Generally, muscle sample precipitates were diluted 1:10 using 0.5M sodium hydroxide and L6 precipitates were diluted 1:1. Standards and diluted samples (50μl) were then mixed with Folin C reagent (600μl) with immediate vortexing. After 10 minute incubation, diluted Folin Ciocalteu reagent (60μl) was added with immediate vortexing and tubes were incubated for a further 40 minutes at room temperature and subsequently read at 660nm on a Titertek Multiscan spectrophotometer.

### 2.4.2 Bio-Rad DC Assay

Reagent A (1ml) and Reagent S (20μl) were mixed immediately prior to use, producing reagent $A_S$. The muscle lysates to be assayed were diluted 1:10 with 1% v/v IGEPAL CA-360 detergent (Sigma, Dorset, UK, I3021) and the L6 lysates were diluted 1:1 in order to bring them into the range of the standard curve. Standards (0-20μg/ml) and diluted samples (5μl) were pipetted in triplicate into a clean 96 well plate. Reagent $A_S$ (25μl) was added to each well, followed by Reagent B (200μl). The plate was gently agitated to mix the reagents and left to incubate for 15 minutes and read at 750nm on a Titertek Mulitscan spectrophotometer.

## 2.5 Protein techniques

### 2.5.1 Cell Membrane Preparation

Following incubation with test media, cells were washed three times in 5ml ice cold 0.9% w/v NaCl. Cells were immediately scraped in 3ml per 9cm Petri UIC3 Protease Inhibitor Cocktail (see Appendix A) using a plastic cell scraper. Cells were centrifuged at 200g for 5 minutes at 4°C to pellet the intact cells. The supernatant was aspirated and cells were re-suspended in 12ml fresh UIC3 and incubated on ice for 30 minutes. Cells were then lysed by drawing them through a pre-chilled 33 gauge needle twice. Lysates were then microcentrifuged at 7500rpm for 10 minutes at 4°C. The pellets were discarded and supernatants centrifuged at 170,000g for 60 minutes at 2°C. The resulting membrane pellets were dissolved in UIC3 plus 1% IGEPAL CA-630 detergent (Sigma, Dorset, UK, I3021) and stored at -80°C. Protein concentration was determined using the BioRad DC assay as described in Section 2.4.2 and 30µg protein was mixed with an equal volume of SDS-PAGE reducing sample buffer and run on an SDS-PAGE gel as described below.

### 2.5.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE gels)

All samples were run on 12.5% gels using Mini PROTEAN 2 and 3 systems (Bio-Rad, Hertforshire, UK) and blotted using a semi-dry transfer cell (Bio-Rad, Hertfordshire, UK). All protein extracts were resolved under reducing conditions.

### 2.5.3 Casting the gels

The resolving gel was poured between two glass plates and over-laid with butanol. Once set, the butanol was removed and the stacking gel was poured over the top of the resolving gel and the well comb inserted in between the top of the glass plates creating loading wells. The gels were made up as follows,

**Table 2.1. SDS-PAGE resolving gel, all volumes are shown per gel**

| Final Acrylamide % | 12.5% |
|---|---|
| Water | 3.19ml |
| Acrylamide (30% w/v) | 4.17ml |
| 1.5M Tris-HCl pH 8.8 | 2.5 ml |
| SDS (10% w/v) | 0.1 ml |
| APS[1] (10% w/v) | 0.05ml |
| TEMED[2] | 5 µl |

**Table 2.2. SDS-PAGE stacking gel, all volumes are shown per gel**

| Final Acrylamide % | 4% |
|---|---|
| Water | 3.03 ml |
| Acrylamide (30% w/v) | 0.65 ml |
| 0.5M Tris-HCl pH 6.8 | 1.25 ml |
| SDS (10% w/v) | 0.05 ml |
| APS[1] (10% w/v) | 0.025ml |
| TEMED[2] | 5µl |

[1] *Ammonium Persulphate*

[2] *Tetramethylethylenediamine*

### 2.5.4 Preparation of muscle samples for protein SDS-PAGE

As well as skeletal muscle from CKD patients and healthy volunteers, skeletal muscle samples from rats were also taken as a positive control. Freeze-dried muscle samples to be analysed by Western blotting were homogenised in SDS-PAGE Muscle Lysis Buffer (see Appendix A) and run on a gel on the same day as the homogenisation in order to prevent any loss of signal by freeze-thawing. Samples were processed according to the method used by Karlsoon et al. (2004). Approximately 4mg dw muscle was homogenised (T8.01 Netzgerät, IKA, Germany) in 90µl/mg dw SDS-PAGE Muscle Lysis Buffer and rotated at 4°C for 90 minutes. The tubes were microcentrifuged at 13,000rpm for 15 minutes at 4°C and the resulting lysate supernatant was taken off and transferred to a new tube. The pellet was retained and stored at -80°C to be used later for determination of 14kDa actin fragment concentration. The lysate supernatant was assayed for protein concentration using a Bio-Rad DC assay (see Section 2.4.2) and a volume yielding 150µg protein was mixed with an equal volume of Laemelli Reducing Sample Buffer (see Appendix A), heated at 100°C and briefly centrifuged in a microcentrifuge (13,000rpm for 10 seconds at room temperature).

The protocol for the detection of the 14kDa fragment was based on the protocols developed by Du et al. (2004) and Workeneh et al. (2006) modified as follows. The pellets were resuspended in 150μl SDS-PAGE Muscle Lysis Buffer without Bromophenol Blue, vortexed and heated at 100°C for 5 minutes. Samples were then sonicated and heated again at 100°C for 5 minutes and this step was repeated until the pellet had gone back into solution. A volume yielding 50μg of protein was loaded onto an SDS-PAGE gel with a small amount of Bromophenol Blue. The protein concentration in this solution was assayed by mixing 50μl with an equal volume of 20% w/v Trichloroacetic Acid (TCA). This was vortexed and placed in the refrigerator at +4°C for 30 minutes. The samples were microcentrifuged at 13,000rpm for 10 minutes at 4°C, the supernatant was aspirated and the pellet was washed twice in 200μl 10% TCA. Following the final centrifugation, the supernatant was aspirated and the pellet dissolved in 50μl 0.5M NaOH. The tubes were placed in a water bath at 70°C to ensure that all the protein had dissolved and protein concentration was determined using the Folin assay (see Section 2.4.1).

## 2.5.5 Preparation of L6 skeletal muscle cell samples for SDS-PAGE

Following incubation with test media, medium was aspirated and the plates were scraped on ice in SDS-PAGE L6 Sample Buffer (see Appendix A) and the cell lysates were microcentrifuged at 13,000rpm at 4˚C for 10 minutes. The supernatant was assayed for protein using the Bio-Rad DC assay.

A volume yielding 30μg protein was mixed with an equal volume of Laemmli Reducing Sample Buffer. The tubes were heated at 100˚C on a heating block for 5 minutes and briefly centrifuged in a microcentrifuge as described above.

## 2.5.6 Running the gels

The gel rig was assembled and transferred to a tank containing Running Buffer (See Appendix A). The molecular weight markers (Full-Range Molecular Weight Markers, GE Health Care Life Sciences, Little Chalfont, UK, RPN800E) and prepared samples were loaded into the wells. The gel was run at 200V until the Bromophenol Blue dye front had almost reached the bottom of the glass plates (approximately 50 minutes). Gels run for 14kDa fragment and 4E-BP1 were stopped

slightly earlier (approximately 40 minutes) to ensure these small proteins did not run off the bottom of the gel.


### 2.5.7 Western Blotting

Once the gels had been run, they were soaked in Transfer Buffer (see Appendix A) along with blotting paper and the appropriate transfer membrane for 15 minutes. Western blots for P-S6 were transferred onto Polyvinylidine Fluoride (PVDF) membranes (Bio-Rad, Hertfordshire, UK). Western blots for 14KDa fragment, P-PKB and P-4E-BP1 were transferred onto nitrocellulose membranes (GE Health Care Life Sciences, Little Chalfont, UK) and the semi-dry transfer was performed at 15V for 30 minutes. The membranes were blocked using 5% w/v non-fat dry milk powder (NFDM) in TTBS (see Appendix A) for 1 hour at room temperature to prevent non-specific binding of the antibody to proteins. In some experiments the PVDF membranes were treated with an enhancer reagent (Qentix Western Blot Signal Enhancer, Thermo Scientific, Northumberland, UK) and rinsed in ultra pure water prior to blocking. After blocking, the membranes were washed 3 times in TTBS As the enhancer reagent had little obvious effect on signal intensity and prevented the membranes from being re-probed with other antibodies, its use was discontinued in later experiments.


### 2.5.8 Immunostaining

Membranes were then probed with primary antibodies made up in TTBS and milk powder or Bovine Serum Albumin (BSA) (Sigma, Dorset, UK) as indicated in Table 2.3. The membranes were incubated with the primary antibody overnight at 4˚C with continuous agitation. They were then washed 3 times with TTBS and incubated with horseradish peroxidise conjugated secondary antibody: goat anti rabbit (Dako, Denmark, P0448) or rabbit anti-mouse (Dako, Denmark, P0260) at a dilution of 1:1500. After 1 hour incubation, the membranes were again washed 3 times in TTBS. The bands were visualised using enhanced electrochemiluminescence (ECL) detection system according to the manufacturers' instructions (Thermo Scientific, Northumberland, UK).

**Table 2.3. Details of Antibodies**

| Antibody | Source | Ref No | Dilution | Blocking Agent | Secondary Antibody |
|---|---|---|---|---|---|
| $P^{Ser235/236}rpS6$ | Cell Signalling | 2211 | 1:1000 | 5% BSA | R |
| Total rpS6 | Cell Signalling | 9456 | 1:1000 | 5% BSA | R |
| $P^{Ser473}PKB$ | Cell Signalling | 9271 | 1:750 | 5% BSA | R |
| Total PKB | Cell Signalling | 9272 | 1:1000 | 5% BSA | R |
| $P^{Thr389}P70S6K$ | Cell Signalling | 9206 | 1:1000 | 5% BSA | M |
| Total P70S6K | Cell Signalling | 2708 | 1:1000 | 5% BSA | R |
| $P^{Ser65}4E-BP1$ | Cell Signalling | 9456 | 1:1000 | 5% Milk Powder | R |
| Total 4E-BP1 | Cell Signalling | 9452 | 1:1000 | 5% Milk Powder | R |
| Anti-Actin Clone AC40 (14kDa) | Sigma | A4700 | 1:500 | 5% Milk Powder | M |
| SNAT2 Ap | JE* | - | 1:4000 | 5% Milk Powder | R |
| Na, K, ATPase | Abcam | Ab7671 | 1:4000 | 5% Milk Powder | M |
| Annexin II | Santa Cruz | Sc-1924 | 1:4000 | 5% Milk Powder | G |

M denotes rabbit anti-mouse HRP secondary antibody was used (Dako P0260)

R denotes polyclonal goat anti-rabbit HRP secondary antibody was used (Dako P0448)

G denotes rabbit anti goat HRP secondary antibody was used (Sigma A5420)

* A kind gift from Professor J Erickson, Louisiana State University Health Sciences Centre.

## 2.6 RNA techniques

All RNA techniques were performed using RNAse free consumables and diethylpyrocarbonate-treated (DEPC) water. Any glassware that was used was baked at 200°C for 4 hours and equipment including the homogeniser was treated with RNase Zap (Sigma, Dorset, UK).

### 2.6.1 RNA extraction

RNA was extracted from muscle tissue using the guanidinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (1987) commonly known as TRIzol. Approximately 2mg dw muscle was homogenised in TRIzol (1.5ml) (Invitrogen, UK, 15596) and incubated at room temperature for 15 minutes. The tubes were microcentrifuged at 13,000rpm for 15 minutes at 4°C to remove any insoluble material and the TRIzol solution was transferred to a fresh tube and Chloroform (200µl/ml TRIzol) was added. The tubes were vortexed thoroughly and following a 10 minute incubation at room temperature, they were microcentrifuged at 13,000rpm for 15 minutes at 4˚C resulting in the formation of three phases. The aqueous phase, containing RNA was taken off and transferred to a new tube. Isopropanol (500µl/ml TRIzol) was added to precipitate the RNA and the tubes were vortexed and incubated at room temperature for 10-15 minutes, then microcentrifuged at 13,000rpm for 15 minutes at 4°C. The resulting pellet was washed in 75% ethanol and dissolved in DEPC treated water (20µl). The RNA concentration was quantified from the optical density using a Nanodrop (ND 1000) Spectrophotometer (Thermo Scientific, Northumberland, UK). RNA extracts were stored at -80°C.

RNA was extracted from L6-G8C5 cells also using the TRIzol technique. At the end of the incubation time, test media was aspirated and 1ml TRIzol was added. The cells were then frozen at -80˚ until a time when the RNA was extracted using the method described above.

### 2.6.2 Reverse transcription

RNA was reverse transcribed to cDNA using the Reverse Transcription System (Promega, Hampshire, UK A3500). RNA (1µg) was heated at 70˚C for 10 minutes to remove any secondary

structure, briefly centrifuged as previously described and incubated on ice.  The reaction buffer was made up as follows:

**Table 2.4. RT- Reaction**

| For a single 20µl reaction | Volume (µl) |
|---|---|
| MgCl$_2$ | 4 |
| 10X Reverse Transcription Buffer | 2 |
| 10mM dNTP Mix | 2 |
| Rnasin Ribonuclease Inhibitor | 0.5 |
| AMV Reverse Transcriptase | 15 Units |
| Primers[1] | 1µl |
| RNA | 1µg |
| Water | to 20µl |

[1]A mix of random and oligo dT primers was used in a 1:1 ratio. Preliminary experiments showed this gave the greatest RNA yield over and above that gained by using each primer individually.

The tubes were then placed in a controlled heat block at 42˚C for 1 hour, 95˚C for 5 minutes and 4˚C for 5 minutes. The resulting cDNA was then stored at -20˚C.

## 2.6.3 Validation of an appropriate reference gene

To control for the variation in the loading of cDNA into the PCR reaction it is important to have a reference gene whose level of expression remains stable over all the experimental conditions. Commonly used exercise reference genes are β-actin, β-microglobulin and GAPDH (Jemiolo and Trappe, 2004; Mahoney et al. 2004). qPCR primers for these genes were available in our laboratory, but preliminarily experiments showed that the expression of all of these genes in skeletal muscle from the exercising patients varied outside the acceptable limits (1 comparative threshold, cT value) to be used as a reference gene. Therefore, in order to identify the most appropriate reference gene, an endogenous control plate (Applied Biosystems, Warrington, UK, 4396840) was used to screen 32 different control genes using the cDNA derived from one patient before and after exercise training. The results showed ribosomal protein L30 (RPL30) and beta-glucuronidase (GUSB) were the most stable with exercise training (see Table 2.5) and the most highly expressed.

Primers were designed for RPL30, GUSB and SNAT2 using Primer-3 software version 0.4.0 ([www.primer3.Sourceforge.net](www.primer3.Sourceforge.net)) using gene sequences obtained from the NCBI nucleotide database. They were all designed to span an intron-exon boundary to allow any product amplified contaminating genomic DNA to be distinguished. Primers were checked for sequence homology to other genomic sequences using the NCBI Basic Local Alignment Search Tool (BLAST) and were commercially synthesized (Invitrogen, UK). All primers were designed and optimised to amplify with an annealing temperature of 56°C allowing all genes to be run on the same plate. Primer's PCR efficiency was calculated by performing a serial dilution reaction and drawing a standard curve (see Table 2.6 for efficiencies).

**Table 2.5. Results of endogenous control plate**

| Gene Name | Coefficient of Variation (%) | Gene Name | Coefficient of Variation (%) |
|---|---|---|---|
| 18S | 4.7 | MT-ATP6 | 0.7 |
| ABL1 | 0.6 | PES1 | 0.01 |
| ACTB | 5.8 | PGK1 | 1.6 |
| B2M | 2.3 | POLR2A | 3.2 |
| CASC3 | 0.4 | POP4 | 1.7 |
| CDKN1A | 2.1 | PPIA | 1.7 |
| CDKN1B | 0.2 | PSMC4 | 1.7 |
| EIF2B1 | 1.6 | PUM1 | 1.3 |
| ELF1 | 1.7 | RPL30 | 0.17 |
| GADD45A | 1.3 | RPL37A | 0.4 |
| GAPDH | 3.3 | RPLPO | 2.4 |
| GUSB | 0.6 | RPS17 | 3.3 |
| HUBS | 1.3 | TBP | 1.7 |
| HPRT1 | 1.4 | TFRC | 0.2 |
| IPO8 | 1.3 | UBC | 2.5 |
| MRPL19 | 2.1 | YWHAZ | Undetermined |

### 2.6.4 DNA sequencing

The amplified products generated from the SNAT2, RPL30 and GUSB primers were sequenced to confirm that the correct amplicon was produced.

### 2.6.5 Principle of the DNA sequencing technique

Primer amplification products were sequenced at the Protein Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester using Fluorescence-Based Sequencing and Dye Terminator Chemistry on an Applied Biosystems 3730 sequencer (Applied Biosystems, Warrington, UK). This technique requires the DNA template, a modified DNA polymerase, nucleotides (dNTPs), and dideoxynucleotides (DdNTPs) with each of the four bases labelled with a different colour fluorescent dye, and a buffer. This is then subjected to cycles of annealing, extension and denaturation in a thermo cycler. The modified DNA polymerase allows the incorporation of DdNTP into the product. As DdNTP lacks a 3-hydroxyl (3-OH) group on its deoxyribose sugar which is already lacking a 2-OH group, no further nucleotides can be added as a phosphodiester bond is unable to be formed and this terminates the fragment. The ratio of dNTP and DdNTP is balanced to ensure that a mixture of long and short extension products are produced. This has the result of producing many extension products of different sizes. At the end of the reaction, a high voltage charge is applied to the buffered reaction products forcing the negatively charged fragments into a capillary separating them by size based upon their total charge. Before reaching the positive electrode, the fragments pass through a laser beam causing the dyes to fluoresce emitting a wavelength specific to the base. This signal is then converted to a digital signal producing a series of peaks indicating the DNA sequence.

### 2.6.6 Sequencing protocol for fluorescence-based sequencing using dye terminator chemistry

The PCR product was run out on a 1% agarose gel in DNA sample buffer (see Appendix A) plus Ethidium Bromide with a DNA molecular weight ladder ($\Phi$X174 *Hae* III Digest, Sigma, Dorset, UK). An electric current was applied at 120V until the DNA sample buffer dye front had moved approximately three quarters of the way down the gel. The bands were visualized under UV light, cut out of the gel and placed in pre-weighed tubes. The DNA was extracted from the gel

using a Qiaex II gel extraction kit according to the manufacturer's instructions (GE Health Care Life Science, Little Chalfont, UK). The extracted DNA was quantified by running on a 0.8% agarose gel with the DNA markers ($\varphi\chi$174 DNA Hae III digest, Sigma, Dorset, UK). Two sequencing reactions were then performed, one using the forward primer and the second using the reverse primer. 20µg DNA template was required for each sequencing reaction plus the primer (4pmol) and 8µl BigDye terminator V1.1 (PNACL Laboratory, University of Leicester). The sequencing reaction was performed in a controlled heat block at 94˚C for 30 seconds then 30 cycles of the following, 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 4 minutes. Any unincorporated dyes were removed using the ethanol/sodium acetate precipitation method; 3M sodium acetate (2µl) and 95% ethanol (50µl) was added and incubated on ice for exactly 10 minutes. The tubes were then centrifuged at 13,000rpm for 30 minutes at room temperature. The supernatant was removed and pellets washed with 70% ethanol (150µl) by gently agitation and centrifuged at 13,000rpm for 1 minute at room temperature. The supernatant was taken off and pellets left to dry. They were then sent to the PNACL at the University of Leicester for the amplicon to be sequenced (3730 sequencer, Applied Biosystems, Warrington, UK).


## 2.6.7 Real time qPCR

All qPCR was carried out on an Applied Biosystems Light cycler real time PCR machine. Amplification reactions for SNAT2 were set up in 25µl volumes containing 12.5µl *Power* SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) with 10pmol per 25µl forward primer, 10pmol per 25µl reverse primer and 10.5µl sterile water. cDNA (or water if a negative control) (1µl) was added once 24µl of the mastermix detailed above had been added to the plate. All samples were run in triplicate with negative controls for each gene. All Primers were optimised to run at 56°C for the extension phase of the amplification. A dissociation step was added onto the end of the cycling to check that no non-specific product amplification had occurred. Primers and probes for MAFbx and MuRF-1 were supplied as Taqman gene expression assays from Applied Biosystems (Applied Biosystems, Warrington, UK, MAFbx Hs00369714_m1, MuRF-1 Hs00822397_m1, RPL30 Hs00265497_m1)

The stability of expression of RPL30 and GUSB in response to exercise training was then tested in five more patients. RPL30 proved to be the most stable (RPL30 = 1.7% CV and GUSB 1.9% CV)  and so was selected as the house keeping gene to be used in all qRT- PCR experiments

using RNA extracted from human muscle samples. For qRT-PCR experiments performed using L6 rat skeletal muscle cells, cyclophilin was used as the reference gene as it had been used previously in L6 cell cultures and in rat skeletal muscle in catabolic states (Pickering et al. 2003). qRT-PCR primers were designed and sequenced for rat SNAT2 and Cyclophilin using the same method as that described above.

**Table 2.6. Primer sequences and efficiencies**

| Gene | Forward Sequence | Reverse Sequence | PCR Efficiency (%) |
|------|------------------|------------------|--------------------|
| SNAT2 (Hu) | AGTGGAATCCTTGGGCTTTC | TCCTTCATTGGCAGTCTTCA | 96.1% |
| RPL30 (Hu) | GGGTACAAGCAGACTCTGAA | CCAGTTTTAGCCAACATAGC | 97.5% |
| GUSB (Hu) | CTTTTTCAACTACGCTGGAC | ACGACTTTGTTTTCTGCATC | 96.3% |
| SNAT2 (Rat) | GCTCATTCTCCCATTGTCAC | TTGCAAATCACCACAATCAG | 98.5% |
| Cyclophilin (Rat) | CACCGTGTTCTTCGACATC | TGCTGTCTTTGGAACTTTGTC | 96.0% |

PCR data were quantified and presented as the Pfaffl ratio according to the following equation cited in Pfaffl (2001).

$$\text{ratio} = \frac{(\text{E Target}) \, \Delta \text{CT Target (Control} * - \text{ Treatment)}}{(\text{E Reference}) \Delta \text{CT Reference (Control} * - \text{Treatment)}}$$

Where E = PCR efficiency and CT = comparative threshold value.

*In the exercise studies this control value was the baseline value at the start of the study

## **2.7 Quantification of bands from Western and Northern Blotting**

Band intensities were quantified using a Bio-Rad GS7000 densitometer and Molecular Analyst v1.4 software.

## 2.8 Statistical Analysis

Data were tested for normal distribution using the Kolmogorov–Smirno test. All normally distributed data are presented as mean ± SEM. All non-normally distributed data are presented as median and range values.  Western and Northern blotting data are expressed as a percentage of the positive control run on the same gel. For both the one month pilot study and the six month study, baseline vs post-treatment values were analysed using Paired t tests for normally distributed data where appropriate and non-parametric Wilcoxon Signed Ranks test for non-normally distributed data. Data from cell culture experiments were analysed for statistical significance by ANOVA and *post hoc* testing with Duncan multiple range test. Changes were regarded as significant when $P<0.05$. Correlation was expressed as the Spearman Rank Correlation Coefficient Rs. All statistical analysis was carried out using SPSS 16.0 software (SPSS, Chicago, IL).

Chapter 3

*In vivo* study of the effect of exercise and metabolic acidosis correction on amino acid and protein metabolism in skeletal muscle in CKD

## 3.1 Introduction

It is well documented that CKD patients have poor physical functioning (reduced ability to perform everyday activities) and low exercise capacity (Painter, 2005; Johansen et al. 2007). This is caused by a combination of inactivity (Tawney et al. 2003), muscle wasting and reduced muscle function and metabolism (Diesel et al. 1990), inflammation (Kaizu et al. 2003) and anaemia (Painter and Moore, 1994) It has been suggested that the abnormalities in muscle metabolism are the most important limiting factors to exercise capacity (Diesel et al. 1990).There is growing evidence that exercise can provide benefits to haemodialysis patients such as improved exercise capacity (Painter et al. 2002; Kouidi et al. 2004), a reduction in cardiac risk factors (Goldberg et al. 1986), improved insulin sensitivity and glucose disposal (Goldberg et al. 1983, 1986), increased quality of life and reduced depression (Painter et al. 2000). However, there has been little research performed in this area on the pre-dialysis population.

As previously described in Chapter 1.6, metabolic acidosis is a common problem in CKD patients (Kovacic et al. 2003) and is an accepted cause of muscle wasting (Mitch, 2006). Acidosis is known to disrupt insulin action resulting in insulin resistance, but it has been shown that this can be improved following exercise training in haemodialysis patients (Goldberg et al. 1980, 1986) and there is some evidence that muscle catabolism (indicated from accumulation of the 14kDa fragment) can be slowed by combined strength and endurance exercise training in dialysis patients (Workeneh et al. 2006). Furthermore, Storer and colleagues (2005) found eight weeks of intradialytic exercise (three times a week) significantly increased quadriceps strength, power and fatigability. These authors attributed this effect of endurance exercise on muscle strength to the significant deconditioned state of the patients prior to the exercise training. However, Kopple et al. (2007) were unable to detect any increases in lean body mass following an 18 week exercise programme of either aerobic or resistance training, alone or in combination. The reason for this failure of exercise to induce any detectable increase in muscle mass is unknown, but exercise in non-dialysed patients could potentially result in a transient worsening of acidosis through exercise-induced lactic acid generation, and could therefore off-set any potential benefits gained from the exercise. It might be possible to neutralise this acid through the administration of extra alkali, but there are no studies to date that have investigated the effect of exercise training in combination with sodium bicarbonate therapy in these patients. Favourable gains in body mass have been achieved however, in peritoneal dialysis patients,

using a high alkali dialysis fluid (Stein et al. 1997) and additional alkali in the form of sodium bicarbonate (Szeto et al. 2003).

As described above, acidosis correction alone (Kooman et al. 1997), and exercise alone (Workeneh et al. 2006) can both reduce accelerated muscle protein degradation, but the effect of the two therapies combined has not been investigated. Therefore, the aim of this study was to implement a six month aerobic training study in pre-dialysis patients with and without additional bicarbonate therapy, and to investigate the resulting effects upon muscle amino acid and protein metabolism. Owing to the results of Storer et al. (2005), this study used walking exercise to investigate these changes. Exercise had not previously been implemented in our renal unit and although there are no reports in the literature of any adverse effects of exercise in this population, it was believed walking exercise would be safer and easier to implement in a non-supervised exercise programme.

It was hypothesised that six months of walking exercise would increase LBM (as measured by DEXA) compared to the control group. This would be associated with increased myofibrillar protein synthesis rates and reduced protein degradation rates. Increased protein synthesis would be detected by increased basal insulin signalling (increased phosphorylation of PKB, $P^{70}S6K$ and 4E-BP1). Increased myofibrillar protein degradation would be detected by a reduction in MuRF-1 and MAFbx mRNA expression, reduced levels of 14kDa fragment, reduced 3-MH excretion rates and an increase in muscle protein:DNA ratio. It was also hypothesised that additional sodium bicarbonate therapy would further correct acidosis causing the effects of exercise and acidosis correction to be additive.

## 3.2 Methods

### 3.2.1 Ethical approval

The studies described in Chapters 3 and 4 which required patient involvement received ethical approval from the Medicines and Health Regulatory Authority (MHRA) and the Local Ethics Committee. The sampling of skeletal muscle from healthy volunteers in Chapter 3, received ethical approval from the Loughborough University Ethical Advisory Committee. Potential participants from the patient population were approached in person at a routine hospital appointment. They were contacted a few days later by telephone to confirm their interest to participate. Potential healthy volunteer participants were approached in person, or contacted by email or telephone.

All participants were given information regarding the study protocol, assessments and any associated risks. Following any questions, participants who wished to volunteer signed a written statement of consent and were made aware of their right to withdraw from the study at any point without the need to give a reason.

### 3.2.2 Participant information

Patient characteristics and basic clinical biochemical measurements for the participants are summarised in Table 3.1. Patient volunteers were recruited from the Leicester General Hospital. Participants were CKD patients classified as CKD stage 4-5. Exclusion criteria included a physical disability (sufficient to prevent involvement in an exercise programme), age under 18 years, pregnancy, regular intake of aspirin, (or any other substance that would affect blood clotting) and particularly for the main study, those patients judged to be in imminent need of renal replacement therapy. Healthy volunteers were recruited from the University or hospital staff, as well as spouses of those participants in the patient arm of the study. All healthy volunteers were required to be in good general health with no signs of renal impairment or other diseases associated with muscle wasting.

**Table 3.1.  Patient characteristics at baseline**. Values are displayed as median and range.

| Patient Involvement | Completed (n) | Males | Females | Age (years) | Weight (kg) | Height (m) | BMI | Serum Creatinine (µmol/l) | eGFR (ml/min) | Plasma Bicarbonate (mmol/l) | STD Bicarb (n) | XS Bicarb (n) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| One Month | 24 | 18 | 6 | 60 (30-76) | 78 (53-116) | 1.70 (1.45-1.92) | 26.6 (20.4-37.4) | 292 (132-960) | 19 (7-35) | 22 (19-29) | 13 | 11 |
| One Month + Biopsy | 16 | 12 | 4 | 60 (30-76) | 79 (53-116) | 1.70 (1.48-1.92) | 27.1 (20.4-37.4) | 256 (132-960) | 21 (9-35) | 22 (19-28) | 9 | 7 |
| Six Months | 18 | 11 | 7 | 62 (50-73) | 80 (53-116) | 1.72 (1.48-1.92) | 28 (20.4-33.5) | 205 (132-506) | 26 (11-36) | 25 (19-29) | 10 | 8 |
| Six Months + Biopsy | 15 | 8 | 7 | 62 (50-73) | 80 (53-116) | 1.70 (1.48-1.92) | 29.9 (20.4-33.5) | 190 (132-506) | 26 (11-36) | 25 (19-27) | 9 | 6 |
| Non-Exercising controls | 14 | 8 | 6 | 56 (31-83) | 85.5 (60-125) | 1.76 (1.55-1.87) | 29.2 (19-38) | 205 (139-427) | 28 (12-37) | 27 (19-30) | 8 | 6 |
| Non-Exercising controls + Biopsy | 11 | 5 | 6 | 50 (31-83) | 85.5 (66-115) | 1.73 (1.57-1.87) | 29.6 (19-38) | 214 (154-427) | 24 (12-33) | 26 (19-30) | 8 | 3 |

### 3.2.3 Explanation of study design

A flow diagram to illustrate patient's involvement and study design is shown in Figure 3.1. A one month pilot study preceded this six month exercise training study. The pilot study was designed to test the logistics of running a training study in this patient population and to address any problems that were identified prior to embarking on the main study. At baseline the patients underwent an exercise tolerance test, assessments of skinfold thickness and mid-arm circumference, blood sampling, if consent was given, a muscle biopsy, and were asked to keep a 3-day food diary. Patients also travelled to the Royal Derby Hospital for a full body Dual Energy X-ray Absorptiometry (DEXA) scan. These assessments were repeated after one month and six months of exercise training. At the end of the pilot study all patients were given the choice of continuing into the main study or finishing their involvement upon completion of one month of exercise. Those patients who consented to continue into the main study did so without a break to avoid contaminating the baseline results of the main study with the effects of a previous one month of exercise. Those who did not wish to participate any further in the study withdrew and were replaced with a new patient who entered the 6 month study with no assessments at the one month time point. The non-exercising control patients took part only in the 6 month study, with assessments made at baseline and after 6 months during which time physical activity was not encouraged. By volunteering, the patients self-selected themselves into the exercise group.

Participants were randomized to receive additional acidosis correction by sodium bicarbonate supplementation (abbreviated to XS throughout this thesis), or to remain on their standard sodium bicarbonate prescription (abbreviated to STD throughout this thesis). The target serum bicarbonate level in the XS group was 28mmol/l and 25mmol/l in the STD group, the level that clinicians at the Leicester Renal Unit routinely aim to achieve.

Muscle biopsies were also taken from a group of healthy volunteers. This was a single biopsy (with no intervention) and served only to provide reference material for comparison with the CKD patients for some of the analyses.
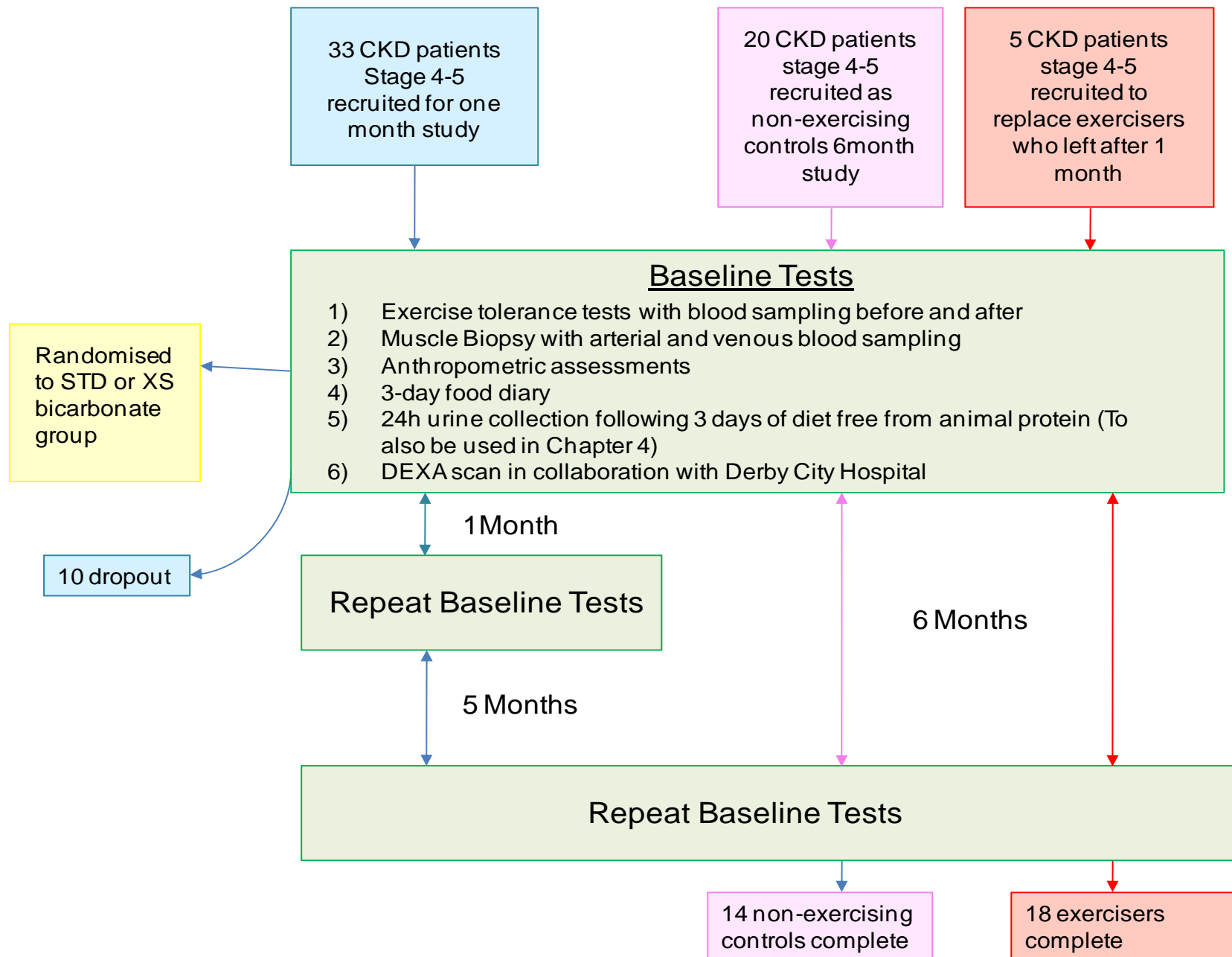
Figure 3.1. Flow diagram to illustrate patient involvement and study design.

**3.2.4 Description of the exercise tolerance test**

The exercise test was performed at the Leicester General Hospital on a treadmill (Life Fitness, UK) with a physician present. The test was a 30 minute submaximal exercise tolerance test, the intensity of which was measured throughout using Rating of Perceived Exertion (RPE) using the Borg scale (Borg, 1982) and heart rate (HR). Despite the reproducibility of RPE being questionable, it is frequently used in exercise tests and in exercise prescription in the CKD population (Sakkas et al. 2003; Koufaki et al. 2002; Segura-Orti et al. 2009; Fuhrmann and Krause, 2004) due to the inherent variability in HR in these patients and also because it provides a sensitive index of how the patients feel during the exercise bout (Koufaki et al. 2002). Patients were familiarized with the RPE scale and the treadmill and were fitted with a polar heart rate monitor (Polar Electro, Kempele, Finland). The treadmill was set at a 1% incline to mimic the energy cost of walking outside and the speed was increased to elicit a RPE of 12-14, somewhat hard. Every two minutes the patient's heart rate was recorded, they were asked their RPE and the treadmill speed was then altered or remained the same depending upon their response. In the event of the patient being unable to complete the 30 minute test, the time completed was noted along with their heart rate and RPE. After the one and/or six month period, the baseline exercise test was replicated exactly as at baseline: treadmill speeds were the same at each corresponding 2 minute time point over the 30 minute test, and heart rate and RPE were recorded as before. Blood samples were drawn from a superficial antecubital vein prior to, and immediately upon completion of the exercise test. Blood samples were handled as described in Chapter 2.2.


**3.2.5 Exercise prescription**

The results from the exercise test were used to prescribe individual exercise programmes that were reassessed on a monthly basis. Patients were asked to exercise for 30 minutes five times a week at a level of intensity that they deemed to be "somewhat hard". The exercise usually involved brisk walking, although a few of the patients were fit enough to begin light jogging. They were informed of their heart rate range at the desired exercise intensity during the test and were given a heart rate monitor that was to be used as an individual guide of exercise intensity when exercising at home. Patients were also asked to record all their exercise in a diary stating what they did, the duration and how hard they rated it overall on the Borg scale. Once a month the patients attended an exercise class held at the physiotherapy gymnasium at the Leicester General Hospital to meet with a doctor to discuss any problems that may have arisen, update

exercise programmes, check bicarbonate levels and dosage for those patients on XS bicarbonate treatment, and to help increase motivation.

### 3.2.6 Blood sampling, handling and analysis

Prior to the muscle biopsy, a 7ml venous blood sample was taken from a superficial antecubital vein, drawn into a dry syringe and dispensed into a tube containing the anticoagulant $K_2$ EDTA. This sample served as the baseline or pre-exercise sample and was also the sample that was used in the analysis of plasma amino acid concentrations. Immediately following the exercise test a second venous blood sample was taken from a superficial antecubital vein and treated as above. In additional to this, an arterial blood sample was also taken prior to the muscle biopsy. A 3ml sample was taken from the brachial artery following the administration of 1% lignocaine, drawn into a blood as syringe containing heparin and taken to the Pathology Department at the Leicester General Hospital for analysis of arterial pH and bicarbonate.  Unless otherwise stated, all assays described below were performed in duplicate.

Duplicate 100μl aliquots of whole blood in $K_2$ EDTA were immediately deproteinsed in 1ml ice cold 0.3M Perchloric Acid (PCA). These tubes were then centrifuged and the resulting supernatant was used in the spectrophotometric determination of blood glucose concentration measured on the day of the assessment using a commercially available kit (GodPAP; Randox, Co.Antrim, UK). The remaining acid supernatant was then frozen at -80°C for the fluorometric determination of blood lactate and β- hydroxybutyrate using the methods described by Maughan (1982).

 The EDTA-treated whole blood was used in the spectrophotometric determination of haemoglobin (Hb) using the cyanmethaemoglobin method and Packed Cell Volumes (PCV) measured in triplicate on a microhaematocrit centrifuge. Both were measured on the day of assessment. The remaining EDTA-treated blood was centrifuged at 1500g for 15 minutes at 4°C. The plasma was taken off and 1ml aliquots were stored at -80°C for the analysis of plasma amino acid concentration.  The blood collected from healthy volunteers was dispensed into a plain tube and allowed to clot at room temperature for approximately 1 hour. The clot was removed and the sample centrifuged at 1500g for 10 minutes at 4˚C. The serum was removed and placed into a fresh tube and analysed for creatinine concentration using a commercially available colorimetric

assay kit (BioAssay Systems, CA, USA, DICT-500) according to the manufacturer's instructions.

### 3.2.7 Muscle sampling and handling

Muscle biopsies were taken from patients using a Bergström needle by a trained physician at the Leicester General Hospital. Patients reported to the procedure room on the nephrology ward after an overnight fast. Fasting conditions were used to allow for the investigation of amino acid metabolism under the same conditions as those used by Bergström et al. (1990).

All biopsies were taken using a Bergström needle from the vastus lateralis muscle of the right leg with the patient in the supine position, and any subsequent biopsies were taken 1cm above or below the initial site. The area was first cleaned using an antiseptic and 1% lignocaine was administered superficially under the skin and fascia. A 1cm incision was made and the needle was inserted to a depth of approximately 3cm. Suction was used to draw the muscle tissue into the needle. To minimise trauma, small samples (rather than one large sample) were drawn as rapidly as possible until approximately 100mg wet weight had been obtained. The sample was immediately extracted from the needle and following the removal of any visible fat or connective tissue it was placed into a pre-weighed Eppendorf tube and quickly frozen in liquid nitrogen. Pressure was applied to the biopsy site for a few minutes and steri-strips placed over the incision. In the laboratory, the Eppendorf tube was removed from liquid nitrogen and immediately re-weighed to the nearest 0.1mg to obtain the wet weight of the sample. This allowed for the calculation of the water content of the biopsy to investigate if XS bicarbonate had caused any water loading in these patients. A correction of 0.0027g was subtracted from all values to allow for frost formation on the outside of the tube. This was based on the result of the difference in the weight of Eppendorf tubes at room temperature and immediately following freezing in liquid nitrogen repeated ten times. The results ranged from 0.0026g to 0.0032g with a mean of 0.0027g. The Eppendorf tube containing the muscle tissue was placed into a glass flask and freeze-dried (Edwards, Modulyo, UK with RV8 vacuum pump). The sample was re-weighed at regular intervals and was considered to be dried when there were no further changes in weight. Approximately 20mg dry weight in total was obtained using the Bergström needle. The muscle sample was stored at -80°C.

All healthy volunteer muscle biopsies were taken in the department of Sport, Exercise and Health Sciences at Loughborough University by the same physician who performed the patient sampling, using the same method.

Samples from all time points and patient groups were processed and analysed at the same time and were subjected to three types of analysis; amino acid concentration determined by HPLC, gene expression using real time polymerase chain reaction (PCR) and protein detection using Western Blotting.

### 3.2.7.1 Determination of the protein content of muscle biopsies

The total soluble protein content of muscle biopsies was measured using the Folin Lowry assay (see Chapter 2.4.1). Determination of protein concentration in muscle lysates to be run on SDS-PAGE was done using the Bio-Rad DC assay (see Chapter 2.4.2).

### 3.2.7.2 Determination of the concentrations of intramuscular and plasma amino acids

Muscle and plasma were analysed for their full amino acid profiles by High performance liquid chromatography (HPLC) (see Chapter 2.3) Results are shown for the 18 amino acids that were readily detectable in the muscle biopsies.

### 3.2.7.3 Determination of DNA content of muscle biopsies using Burton DNA Assay

Total DNA concentration in muscle extracts was measured using the Burton DNA assay. The pellet obtained following acid precipitation and centrifugation in Section 2.3.1 above, was resuspended in 10% PCA (400μl). The tubes were placed in a water bath at 70°C for 20 minutes to hydrolyse the DNA and vortexed at regular intervals throughout. They were then incubated at 4 °C for 30 minutes and microcentrifuged at 13,000rpm for 15 minutes at 4°C. The resulting supernatant was used in the determination of DNA concentration using the Burton assay.

Standards (0 - 200μg/ml of calf thymus DNA) and samples (100μl) were added to Eppendorf tubes followed by the addition of 4% w/v diphenylamine (DPA) (100μl) and 0.2% v/v acetaldehyde (20μl), (both of which were made up in glacial acetic acid) and vortexed. The tubes were incubated overnight at room temperature in the dark. The following morning, 190μl from

each tube was pipetted into a 96 well plate and read at 595nm and immediately again at 710nm with subtraction of the 710nm value as a blank from all samples.

**3.2.7.4 Determination of the chloride concentration of muscle samples**

Chloride was determined by turbidimetry by a modification of the method of Mesquita et al. (2002). This assay was performed on the neutralised muscle and plasma samples obtained in Sections 2.3.1 and 2.3.2. Prior to the assay, the plasma samples were diluted 1:100 using ultra pure water. A 50μl aliquot of Cl⁻ standard (0 – 1mM) or sample was pipetted into a 1.5ml Eppendorf tube with 50μl Precipitating Reagent (5mM Silver Perchlorate, 4% dextran in 2M PCA) and vortexed. From this, 95μl was transferred into a 96 well plate and incubated for 2h before reading at 405nM on a Titertek Mulitscan spectrophotometer.

**3.2.7.5 Determination of phosphorylated signals and 14kDa accumulation by Western blotting**

The phosphorylation status of proteins in the PI3-K/PKB signalling pathway were analysed by Western Blotting before and after exercise training. Proteins analysed were PKB, rpS6, $P^{70}S6K$ and 4E-BP1. The accumulation levels of the 14kDa actin fragment were also analysed using this technique. Western Blotting method used is detailed in Section 2.5.2.

**3.2.7.6 Determination of protein mRNA expression by real-time PCR**

The mRNA expression levels of SNAT2, MuRF-1 and MAFbx in the muscle biopsies were analysed before and after exercise training. Primers for MuRF-1 and MAFbx were supplied as Taqman gene expression assays from Applied Biosystems (Applied Biosystems, Warrington, UK, MAFbx Hs00369714_m1, MuRF-1 Hs00822397_m1). SNAT2 gene expression was quantified using SYBR green real-time PCR. Methods for RNA extraction and PCR experiments are detailed in Section 2.6.

### 3.2.8 Urine sampling and handling

Those patients who consented, underwent a diet free from animal protein for four days prior to the exercise training, with a 24h urine collection on the final day. This was repeated after one and/or six months of exercise training. Urine samples were analysed for volume and aliquots stored at -20°C for later analysis of 3-MH by HPLC as described in Section 2.3.2.

### 3.2.9 Analysis of food diaries

Patients were asked to keep a 3-day food diary prior to the commencement of exercise training, following one month, and at the end of the six month training period. Food diaries were analysed for energy content and macronutrients using the CompEat Pro version 5.8.0 nutritional analysis software (Grantham, UK).

## 3.3 Results

### 3.3.1 Bicarbonate therapy and completion rates

No ill effects were reported as a result of taking extra bicarbonate; however, technical difficulties were experienced in obtaining muscle biopsies at the one month point from patients receiving XS bicarbonate: specifically the biopsies were found to contain considerable amounts of fibrotic tissue and will be discussed in more detail in Section 3.4.6. For this reason, data from this group will not be presented.

Throughout these results, standard bicarbonate therapy is referred to as STD and additional bicarbonate as XS.

**Table 3.2. Effect of STD and XS bicarbonate therapy on blood bicarbonate levels and pH for those patients in the one month pilot study.** Data are presented as median and range. * denotes significant difference from corresponding baseline value ($P<0.05$). [a] denotes significant difference from STD group at corresponding time point($P<0.05$).

|  |  | Baseline | One Month |
|---|---|---|---|
| **Arterial pH** | 1 Mth Ex STD Bicarbonate | 7.38 (7.31 – 7.42) | 7.4 (7.27 – 7.49) |
|  | 1 Mth Ex XS Bicarbonate | 7.36 (7.33 -7.40) | 7.4 (7.36 – 7.42)* |
| **PCO$_2$ (kPa)** | 1 Mth Ex STD Bicarbonate | 3.5 (1.8 – 4.2) | 3.4 (1.3 – 4.3) |
|  | 1 Mth Ex XS Bicarbonate | 3.5 (1.8 – 4.6) | 3.6 (1.4 – 5.6) |
| **Venous Bicarbonate (mmol/l)** | 1 Mth Ex STD Bicarbonate | 21 (19 -27) | 22 (16 – 28) |
|  | 1 Mth Ex XS Bicarbonate | 21 (19 – 29) | 27 (21 -30)*[a] |

**Table 3.3. Effect of STD and XS bicarbonate therapy on blood bicarbonate levels and pH for those patients in the six month study.** Data are presented as median and range. * denotes significant difference from corresponding baseline value (P<0.05). [a] denotes significant difference from STD group at corresponding time point (P<0.05).

| | Condition | Baseline | Six Months |
|---|---|---|---|
| **Arterial pH** | 6 Mth Ex STD Bicarbonate | 7.41 (7.31 – 7.43) | 7.39 (7.34 – 7.48) |
| | 6 Mth Ex XS Bicarbonate | 7.36 (7.28 – 7.40) | 7.41 (7.35 – 7.44) |
| | Non-exercising Control STD Bicarbonate | 7.39 (7.30 – 7.45) | 7.42 (7.35 – 7.44) |
| | Non-exercising Control XS Bicarbonate | 7.39 (7.31 – 7.48) | 7.36 (7.32 – 7.42) |
| **PCO$_2$ (kPa)** | 6 Mth Ex STD Bicarbonate | 3.7 (1.8 – 4.5) | 3.7 (2.4 – 4.5) |
| | 6 Mth Ex XS Bicarbonate | 3.6 (1.1 – 4.6) | 3.8 (3.3 – 4.6) |
| | Non-exercising Control STD Bicarbonate | 4.1 (2.8 – 4.7) | 3.6 (2.6 – 4.7) |
| | Non-exercising Control XS Bicarbonate | 2.9 (1.9 – 4.8) | 3.7 (3.2 – 4.9) |
| **Venous Bicarbonate (mmol/l)** | 6 Mth Ex STD Bicarbonate | 25 (21 – 26) | 25 (20 – 28) |
| | 6 Mth Ex XS Bicarbonate | 22 (19 – 29) | 27 (26 – 30)*[a] |
| | Non-exercising Control STD Bicarbonate | 25 (19 – 30) | 26 (23 – 27) |
| | Non-exercising Control XS Bicarbonate | 27 (24 – 32) | 30 (26 – 31)[a] |

The effect of bicarbonate therapy on arterial pH and partial pressure of $CO_2$ are displayed in Tables 3.2 and 3.3. The XS bicarbonate therapy successfully raised venous bicarbonate concentrations above those in the STD group after both one and six months, and in the one month study, XS therapy was also able to significantly increase the arterial pH.

The patient completion rates are shown in Table 3.4. The lowest dropout rate of 10% was seen in the six month exercising patients, while the non-exercising control group and one month pilot

study saw a 30% dropout rate each. A higher dropout was seen in those taking XS compared to STD bicarbonate therapy but this did not reach statistical significance P>0.05 $\chi^2$ test. Reasons for withdrawal from the study were progression onto haemodialysis, clinical deterioration and family or work commitments. There were no fatalities.

**Table 3.4. Patient completion rates.**

| Group | Patients Consented (n) | Patients Completed (n) |
|---|---|---|
| All patients One Month Ex | 30 | 24 |
| All Patients Six Months Ex | 20 | 18 |
| All  Non-exercising Controls | 20 | 14 |
| One Month Ex STD | 16 | 13 |
| One Month Ex XS | 14 | 11 |
| Six Months Ex STD | 10 | 10 |
| Six Months Ex XS | 10 | 8 |
| Non-exercising Controls STD | 10 | 8 |
| Non-exercising Controls XS | 10 | 6 |

**3.3.2 Exercise tolerance tests**

Mean response in the exercise tolerance test is shown in Figures 3.2 – 3.4 and average walking speeds during the test are presented in Table 3.5. Exercise tolerance (individuals' RPE averaged over the whole 30 minute test) significantly improved after one (P<0.001) and six months (P<0.001) of exercise which is demonstrated by a lower RPE to the same absolute exercise intensity as that recorded prior to the training period (Figures 3.2 and 3.3). The decline in RPE following one month of training was more apparent in the XS bicarbonate group (Figure 3.2B) than in the STD bicarbonate group (Figure 3.2A). However, this apparent effect of XS bicarbonate was not sustained at six months; indeed the decline in RPE seemed less marked at

this time in the XS group (Figure 3.3B) compared to the STD group (Figure 3.3A). The non-exercising control group showed only small time-dependent changes in RPE over the six month period (Figure 3.4).

**Table 3.5. Patient walking speeds during the exercise tolerance test.**

| Time (minutes) | Walking Speed (km/h) | | |
| --- | --- | --- | --- |
| | One Month Study | Exercising Patients: Six Month Study | Non-Exercising Control Patients: Six Month Study |
| 2 | 2.8 ± 0.2 | 2.7 ± 0.2 | 2.6 ± 0.2 |
| 4 | 3.5 ± 0.3 | 3.5 ± 0.3 | 3.3 ± 0.3 |
| 6 | 4.0 ± 0.3 | 3.8 ± 0.3 | 3..8 ± 0.4 |
| 8 | 4.2 ± 0.3 | 4.0 ± 0.3 | 4. 0 ± 0.4 |
| 10 | 4.4 ± 0.3 | 4.1 ± 0.3 | 4.2 ± 0.4 |
| 12 | 4.5 ± 0.3 | 4.2 ± 0.3 | 4.2 ± 0.4 |
| 14 | 4.6 ± 0.3 | 4.4 ± 0.4 | 4.3 ± 0.4 |
| 16 | 4.6 ± 0.3 | 4.6 ± 0.4 | 4.3 ± 0.4 |
| 18 | 4.7 ± 0.3 | 4.6 ± 0.3 | 4.3 ± 0.4 |
| 20 | 4.7 ± 0.3 | 4.7 ± 0.3 | 4.4 ± 0.4 |
| 22 | 4.8 ± 0.3 | 4.7 ± 0.3 | 4.2 ± 0.3 |
| 24 | 4.8 ± 0.3 | 4.8 ± 0.3 | 4.2 ± 0.3 |
| 26 | 4.8 ± 0.4 | 4.7 ± 0.4 | 4.2 ± 0.3 |
| 28 | 4.7 ± 0.4 | 4.7 ± 0.4 | 4.2 ± 0.3 |
| 30 | 4.8 ± 0.4 | 4.8 ± 0.3 | 4.2 ± 0.3 |
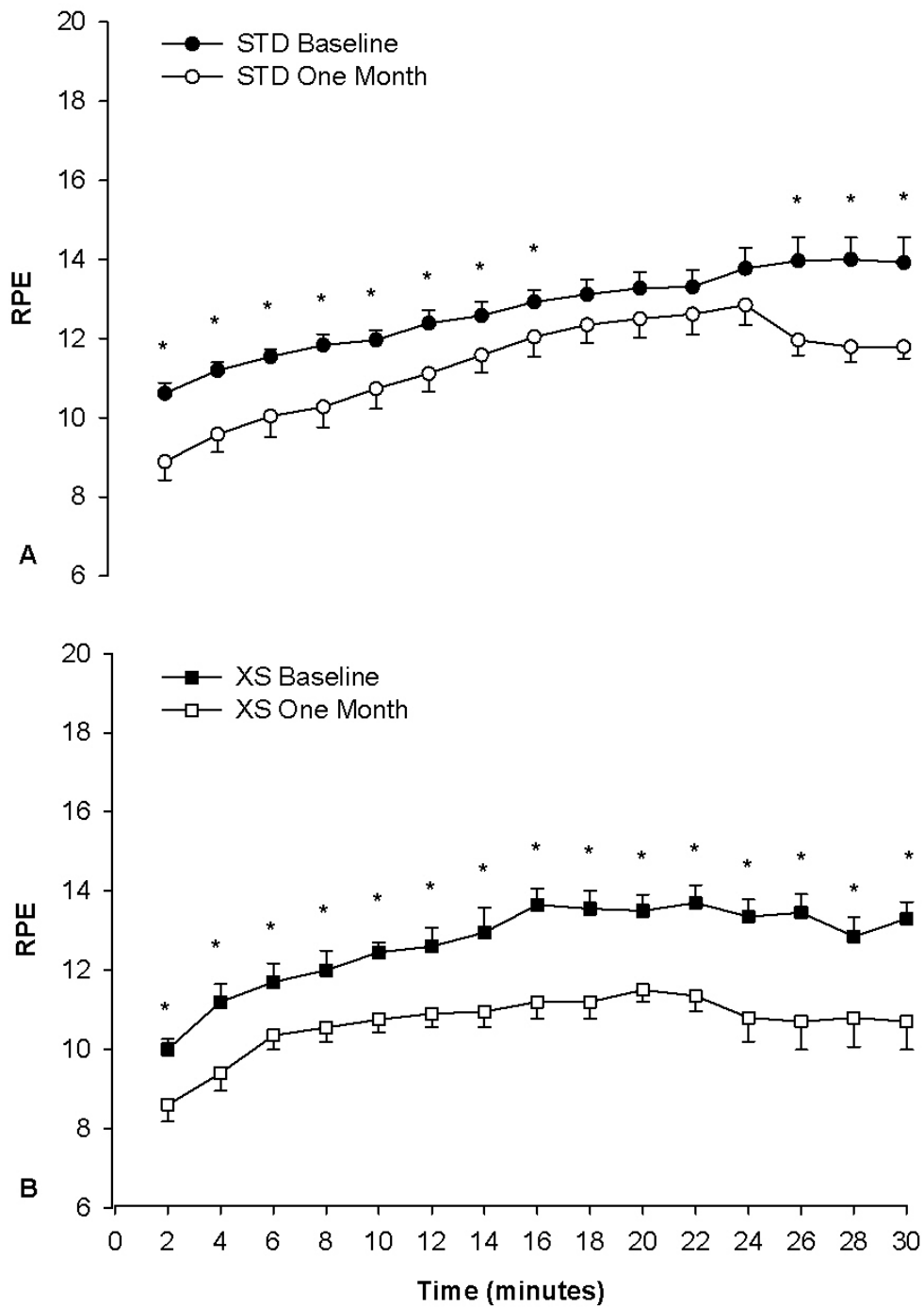
**Figure 3.2. Rating of perceived exertion (RPE) during an exercise tolerance test before (baseline) and after one month of exercise with STD (A) or XS (B) bicarbonate therapy. \*** Denotes a significant difference from corresponding time point during the baseline trial (P<0.05).
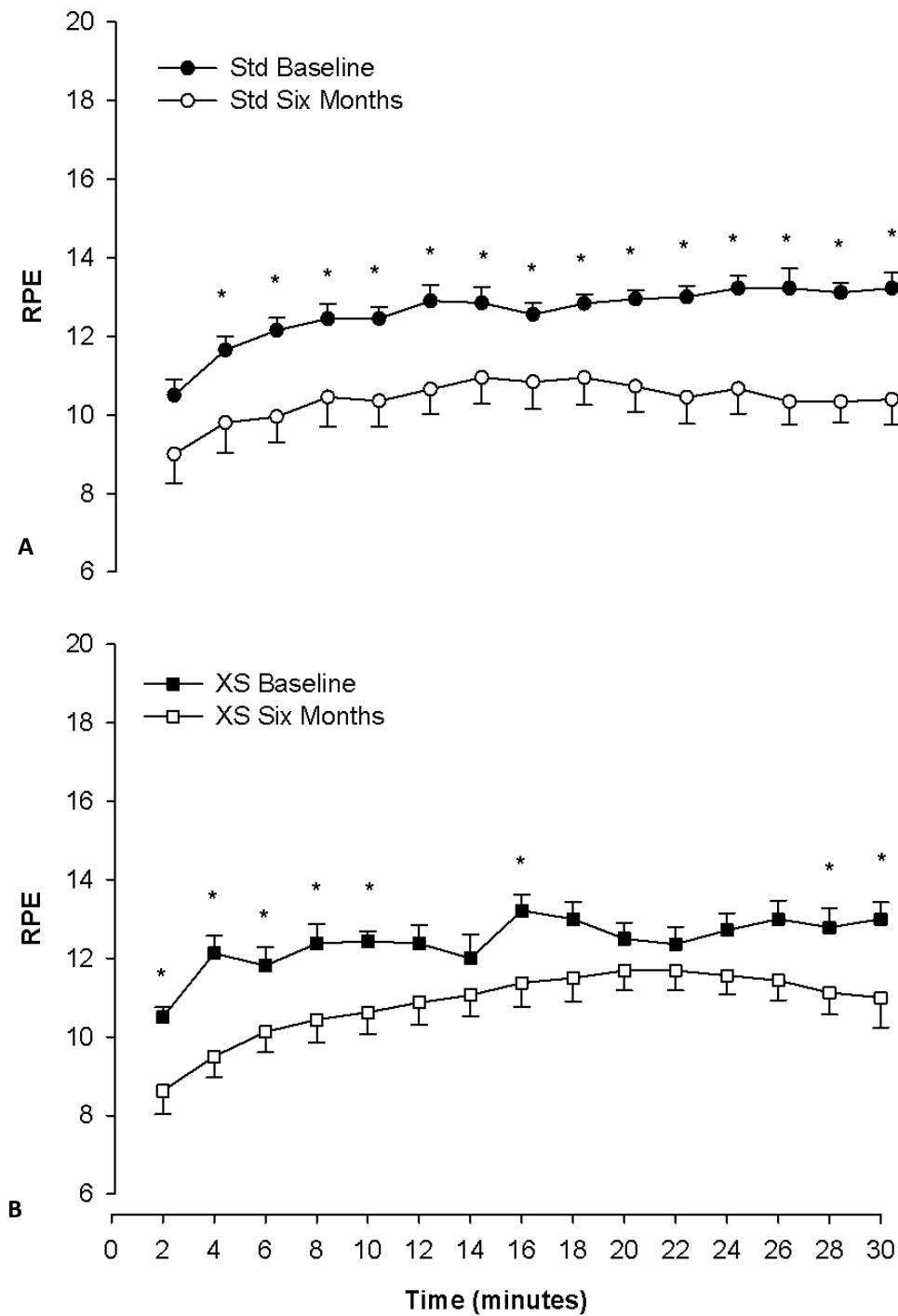
**Figure 3.3. Rating of perceived exertion (RPE) during an exercise tolerance test before (baseline) and after six months of exercise with STD (A) or XS (B) bicarbonate therapy.** * Denotes a significant difference from corresponding time point during the baseline trial (P<0.05).
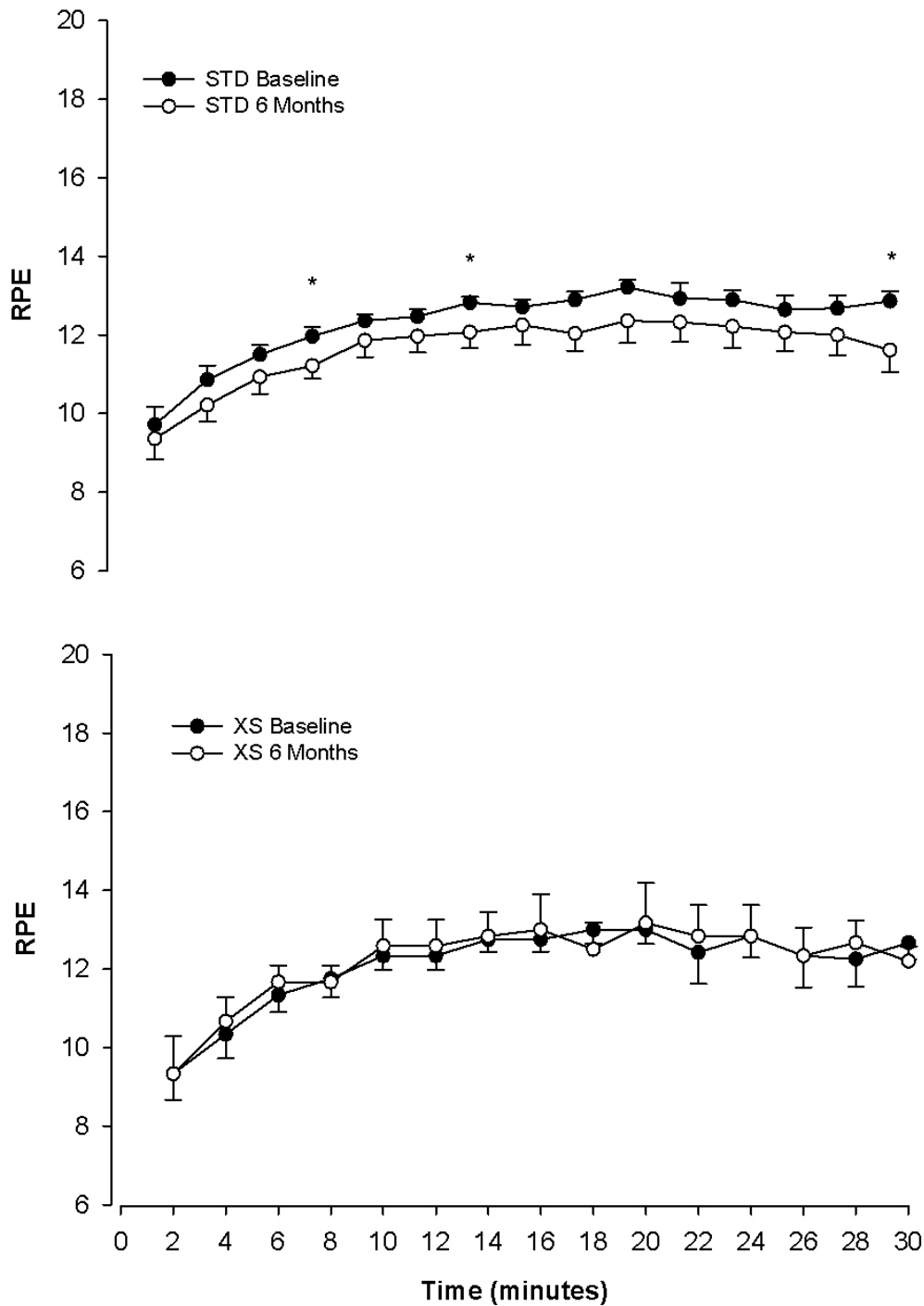
**Figure 3.4. Rating of perceived exertion during an exercise tolerance test before (baseline) and after six months without exercise with STD (A) or XS (B) bicarbonate therapy.** * Denotes a significant difference from corresponding time point during the baseline trial (P<0.05).

### 3.3.3 Blood lactate and glucose response to acute exercise

At baseline, the acute 30 minute treadmill exercise test led to a significant increase in the blood lactate concentration (Tables 3.6 and 3.7). One or six months of exercise with STD bicarbonate therapy had no effect on this lactate response to acute exercise ($P = 0.582$ and $P = 0.674$ respectively) (Figures 3.5A and 3.6A), and the magnitude of this rise with exercise was similar at both time points. However, after one month of exercise with XS bicarbonate therapy the lactate response to exercise was markedly suppressed, declining to less than a quarter of the baseline value (Table 3.6; Figure 3.5B) ($P = 0.005$). This trend was still present after six months, but was less marked and only reached marginal statistical significance (Figure 3.6B) ($P = 0.057$). There was no significant time-dependent change in the lactate response to exercise in the non-exercising control group receiving STD bicarbonate therapy ($P = 0.197$), nor in the non-exercisers receiving XS bicarbonate (Figure 3.6C and 3.6D) ($P = 0.072$).

No acute effect of exercise was observed on the blood glucose concentration in any of the groups ($P>0.05$, Tables 3.6 and 3.7). There were no abnormalities in the concentration of β-hydroxybutyrate in any group, all basal values falling within the normal range of 20-270μmol/l (data not shown) suggesting that keto acidosis was not a contributor to the metabolic acidosis in these patients.

**Table 3.6. Effect of one month of exercise training on blood lactate (mmol/l) and glucose (mmol/l) concentration at rest and following the acute 30 minute treadmill exercise test. \*** Denotes a significant difference between one month and the corresponding value at baseline ($P < 0.05$). $^a$ Denotes a significant difference from the corresponding pre-exercise value in each trial ($P < 0.05$).

| | Condition | Baseline | | | One Month | | |
|---|---|---|---|---|---|---|---|
| | | Pre-Exercise | Post Ex | Post-Pre | Pre-Exercise | Post Ex | Post-Pre |
| Lactate (mmol/l) | 1 Mth Ex STD Bicarbonate | 1.1 ± 0.1 | 2.1 ± 0.2$^a$ | 1.0 ± 0.2 | 1.0 ± 0.1 | 1.8 ± 0.2$^a$ | 0.8 ± 0.2 |
| | 1 Mth Ex XS Bicarbonate | 1.0 ± 0.1 | 2.5 ± 0.4$^a$ | 1.4 ± 0.4 | 1.2 ± 0.2 | 1.5 ± 0.2 | 0.3 ± 0.2* |
| Glucose (mmol/l) | 1 Mth Ex STD Bicarbonate | 5.4 ± 0.5 | 5.6 ± 0.6 | 0.2 ± 0.3 | 5.6 ± 3.6 | 5.6 ± 2.7 | 0.1 ± 0.3 |
| | 1 Mth Ex XS Bicarbonate | 6.1 ± 0.9 | 5.9 ± 0.8 | 0.9 ± 0.6 | 6.1 ± 1.7 | 6.0 ± 1.5 | 0.2 ± 0.4 |

**Table 3.7. Effect of six months of training or without training on blood lactate (mmol/l) and glucose (mmol/l) concentration at rest and following the acute 30 minute treadmill exercise test.** * Denotes a significant difference between six months and the corresponding value at baseline ($P < 0.05$). [a] Denotes a significant difference from the corresponding pre-exercise value in each trial ($P < 0.05$). $\Delta$ Denotes a near significant difference from the corresponding value at baseline ($P = 0.057$)

| | | Baseline | | | Six Months | | |
|---|---|---|---|---|---|---|---|
| | Condition | Pre-Exercise | Post Ex | Post-Pre | Pre-Exercise | Post Ex | Post-Pre |
| Lactate (mmol/l) | 6 Mth Ex STD Bicarbonate | 1.3 ± 0.1 | 2.4 ± 0.4[a] | 1.2 ± 0.2 | 1.4 ± 0.3 | 2.4 ± 0.5[a] | 1.0 ± 0.3 |
| | 6 Mth Ex XS Bicarbonate | 0.9 ± 0.1 | 2.8 ± 0.4[a] | 1.8 ± 0.5 | 1.2 ± 0.1 | 2.0 ± 0.3[a] | 0.8 ± 0.3$\Delta$ |
| | Non-exercising Control STD Bicarbonate | 1.2 ± 0.2 | 2.1 ± 0.4[a] | 0.9 ± 0.3 | 0.8 ± 0.1* | 1.2 ± 0.1[a] | 0.4 ± 0.1 |
| | Non-exercising Control XS Bicarbonate | 1.0 ± 0.2 | 1.7 ± 0.2[a] | 0.8 ± 0.2 | 0.9 ± 0.1 | 2.2 ± 0.4[a] | 1.3 ± 0.4 |
| Glucose (mmol/l) | 6 Mth Ex STD Bicarbonate | 5.4 ± 0.6 | 5.8 ± 0.6 | 0.4 ± 0.3 | 5.2 ± 0.9 | 5.0 ± 0.7 | 0.2 ± 0.2 |
| | 6 Mth Ex XS Bicarbonate | 5.2 ± 0.8 | 6.0 ± 0.7 | 0.7 ± 0.2 | 3.0 ± 0.1 | 3.8 ± 0.4 | 0.8 ± 0.1 |
| | Non-exercising Control STD Bicarbonate | 3.6 ± 0.3 | 4.5 ± 0.4 | 0.9 ± 0.3 | 6.2 ± 0.2 | 5.9 ± 0.4 | 0.3 ± 0.4 |
| | Non-exercising Control XS Bicarbonate | 4.4 ± 1.0 | 4.6 ± 1.0 | 0.2 ± 0.1 | 8.3 ± 2.1 | 8.7 ± 2.1 | 0.5 ± 0.2 |
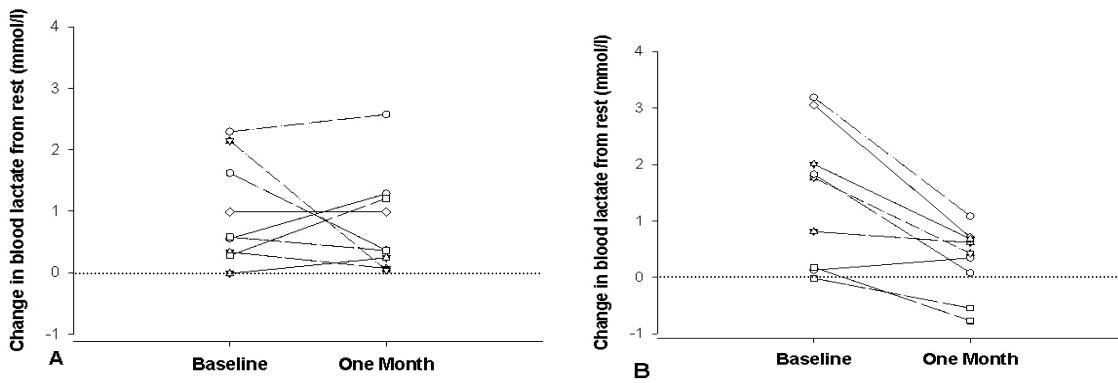
**Figure 3.5. Effect of one month of exercise training on the lactate response to exercise with STD (A) or XS (B) bicarbonate.** Due to sampling difficulties, n = 9 in both graphs A and B.
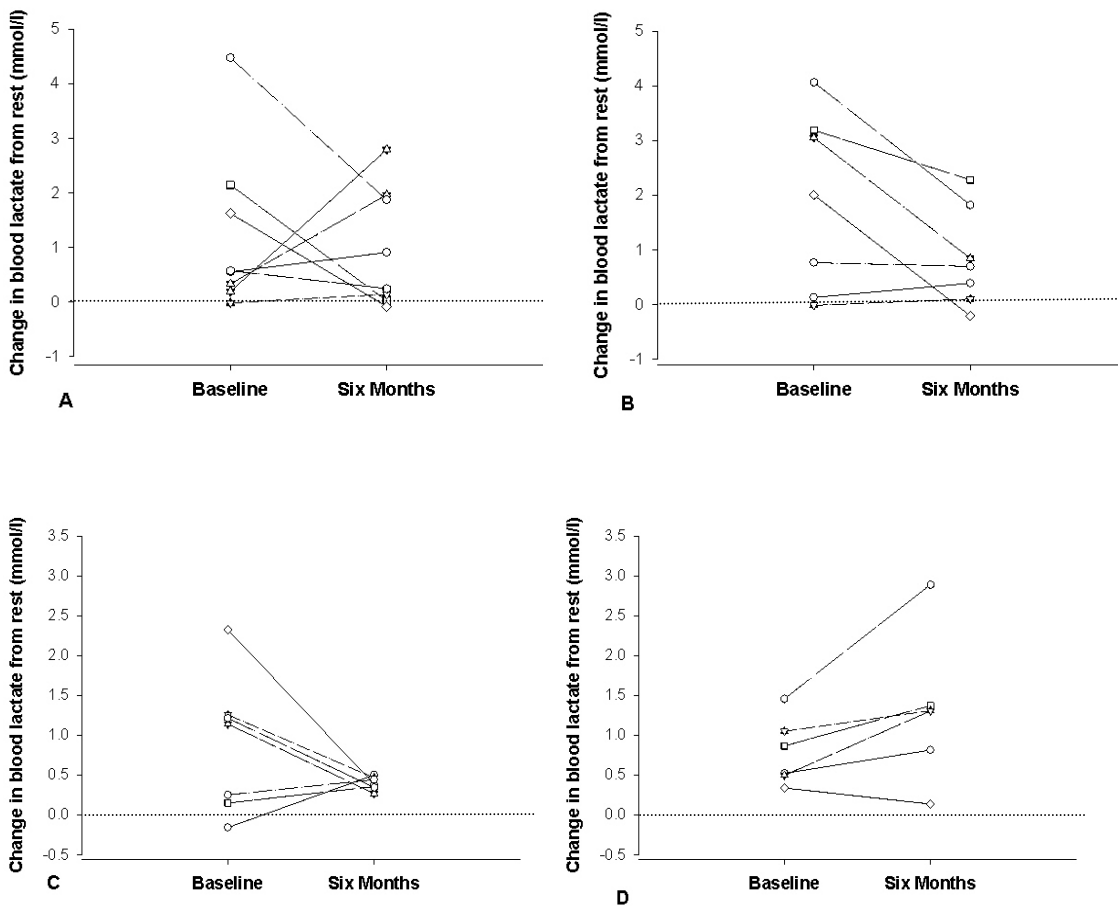


**Figure 3.6. Effect of six months with (A,B) or without (C,D) exercise training on blood lactate response to exercise with STD (A,C) or XS (B,D) bicarbonate.** Due to sampling difficulties, n = 8, n = 7, n = 7 and n = 6 in graphs A, B, C and D respectively.

### 3.3.4 Haemoglobin and Haematocrit Response to Acute Exercise

Basal haemoglobin (Hb) concentration and haematocrit (Hct) were not affected by one or six months of exercise training with STD or XS bicarbonate therapy (Tables 3.8 and 3.9). There was also no acute effect of the 30 minute treadmill exercise test on Hb concentration or Hct in any of the groups (data not shown). It is possible, however, that the erythropoietin treatment received by a number of the patients during the study obscured the effects of exercise.

**Table 3.8. Basal blood haemoglobin concentration (Hb; g/dl) and haematocrit (Hct; %) at baseline and after one month of exercise with STD or XS bicarbonate therapy.**

|  |  | Baseline | One Month |
|---|---|---|---|
| Hb (g/dl) | 1 Mth Ex STD Bicarbonate | 13.9 ± 0.6 | 13.2 ± 0.4 |
|  | 1 Mth Ex XS Bicarbonate | 13.0 ± 0.5 | 13.4 ± 0.6 |
| Hct (%) | 1 Mth Ex STD Bicarbonate | 38.0 ± 2.5 | 36.0 ± 1.0 |
|  | 1 Mth Ex XS Bicarbonate | 36.0 ± 1.5 | 36.5 ± 1.5 |

**Table 3.9. Basal blood haemoglobin concentration (Hb; g/dl) and haematocrit (Hct;%) at baseline and after six months of exercise or no exercise with STD or XS bicarbonate therapy.**

|  |  | Baseline | Six Months |
|---|---|---|---|
| Hb (g/dl) | 6 Mth Ex STD Bicarbonate | 13.9 ± 0.6 | 13.2 ± 0.3 |
|  | 6 Mth Ex XS Bicarbonate | 13.2 ± 0.6 | 13.6 ± 0.7 |
|  | Non-exercising Control STD Bicarbonate | 13.2 ± 0.4 | 13.0 ± 0.6 |
|  | Non-exercising Control XS Bicarbonate | 13.6 ± 0.6 | 12.2 ± 1.2 |
| Hct (%) | 6 Mth Ex STD Bicarbonate | 38.0 ± 1.5 | 38.0 ± 1.0 |
|  | 6 Mth Ex XS Bicarbonate | 36.0 ± 1.5 | 36.5 ± 1.5 |
|  | Non-exercising Control STD Bicarbonate | 36.0 ± 1.0 | 36.0 ± 1.5 |
|  | Non-exercising Control XS Bicarbonate | 35.5 ± 2.0 | 33.0 ± 2.5 |

### 3.3.5 Effect of exercise training on body composition data

Body composition data is tabulated in Table 3.10. There was no change in body weight following one or six months of exercise ($P>0.05$). There was a tendency for a decrease in body weight to occur in those patients who had exercised for six months with XS bicarbonate, but this failed to reach statistical significance ($P = 0.063$). A significant decline in the body weight of the non-exercising controls on STD bicarbonate was observed after six months ($P = 0.025$), but not in the XS group. There were, however, significant decreases in the body mass index (BMI) of those patients who exercised for one month regardless of bicarbonate treatment ($P = 0.018$) and for six months ($P = 0.006$), but the apparent decrease seen in the control group was not significant ($P = 0.103$). Furthermore, this decrease in BMI at one and six months was seen only in those patients who received XS bicarbonate therapy (Table 3.7) ($P = 0.051$ and $P = 0.009$ respectively) and not in those on STD bicarbonate therapy ($P = 0.209$ and $P = 0.223$ respectively). There was no change in BMI in either of the non-exercising control groups

suggesting there was no effect of bicarbonate supplementation in these patients or an effect of seasonal variation.

When bicarbonate groups were combined, full body DEXA scans revealed that there was a significant increase in LBM after one month of exercise (P = 0.013), that was not seen after six months of exercise (P = 0.188). No effect of bicarbonate was observed on LBM following one and six months of exercise or after a six month control period without exercise (P>0.05). However, expressing LBM data as % of total body mass reveals a significant increase following one month of exercise in both STD and XS bicarbonate groups (from 59.1 ± 2.6% to 59.8 ± 2.5%; P = 0.038 and from 65.4 ± 3.6% to 66.3 ± 3.8; P = 0.027 respectively) (Figure 3.7). Following six months of exercise this increase persisted only in the XS bicarbonate group, with 6 of the 7 patients showing an apparent increase (P = 0.028) (Figure 3.8B).

There were also demonstrable effects of upon fat mass measured by DEXA. One month of exercise with STD bicarbonate reduced fat mass by 0.6 ± 0.2kg (P = 0.018). A similar reduction of 0.6 ± 0.3kg was also seen in the XS bicarbonate group, but this fell short of significance (P = 0.063). After six months, the decline in fat mass on STD bicarbonate therapy had disappeared (P = 0.593). However, following six months of exercise with XS bicarbonate there was a reduction in fat mass of 1.5 ± 0.6kg achieving marginal significance (P = 0.054). These changes were reflected in an increase in the lean / fat mass ratio in the six month exercising patients receiving XS bicarbonate from 2.3 ± 0.5 to 2.9 ± 0.7 (P = 0.043). No detectable effect of exercise or bicarbonate on bone density was seen in any group (data not shown).

Two anthropometric measurements were also made, mid-arm circumference (an indicator of muscle mass) and skin fold thickness (a measure of fat mass). The only significant effect observed was a decrease in mid-arm circumference from 33.6 ± 1.1cm to 32.2 ± 1.2cm in the non-exercising control group on STD bicarbonate therapy (P = 0.028). No other changes were observed in any of the groups with either measure.

**Table 3.10. The effect of one and six months of exercise training or a no-exercise control period on indices of body composition.**

\* Denotes a significant difference from the corresponding baseline value (P<0.05). $^{\Delta}$ Denotes a near significant difference from baseline (P = 0.054).

| | Body Mass (kg) | | BMI | | Lean Body Mass (kg) | | Fat Mass (kg) | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Post Training | Baseline | Post Training | Baseline | Post-Training | Baseline | Post-Training |
| **All patients One Month Ex** | 78.7 ± 3.4 | 79.0 ± 3.5 | 27.3 ± 0.9 | 26.9 ± 0.9* | 49.4 ± 2.2 | 50.1 ± 2.3* | 28.1 ± 2.4 | 27.5 ± 2.4* |
| **All patients Six Months Ex** | 78.1 ± 3.4 | 78.0 ± 3.7 | 27.5 ± 1.0 | 26.9 ± 1.1* | 46.4 ± 2.3 | 46.8 ± 2.4 | 29.0 ± 2.4 | 28.5 ± 2.5 |
| **All non-exercising controls** | 84.2 ± 5.1 | 83.8 ± 4.8 | 29.2 ± 1.5 | 28.6 ± 1.3 | 49.1 ± 2.9 | 48.8 ± 2.7 | 32.3 ± 3.3 | 32.3 ± 2.7 |
| **One Month Ex STD** | 81.8 ± 5.0 | 81.9 ± 5.1 | 28.7 ± 1.4 | 28.5 ± 1.4 | 47.4 ± 3.4 | 48.1 ± 3.6 | 31.2 ± 3.2 | 30.7 ± 3.0* |
| **One Month Ex XS** | 78.1 ± 4.3 | 78.2 ± 4.5 | 25.8 ± 1.0 | 25.4 ± 0.9* | 53.0 ± 1.7 | 54.0 ± 1.7 | 24.1 ± 3.3 | 23.5 ± 3.4 |
| **Six Months Ex STD** | 81.2 ± 5.0 | 81.9 ± 5.3 | 29.1 ± 1.3 | 28.8 ± 1.3 | 46.0 ± 3.4 | 46.4 ± 3.6 | 32.4 ± 2.9 | 32.7 ± 2.7 |
| **Six Months Ex XS** | 73.8 ± 4.0 | 72.6 ± 4.4 | 25.5 ± 1.4 | 24.6 ± 1.5* | 47.0 ± 3.0 | 47.3 ± 3.2 | 24.1 ± 3.5 | 22.5 ± 4.1$^{\Delta}$ |
| **Non-exercising controls STD** | 84.4 ± 6.3 | 83.0 ± 6.3* | 30.3 ± 1.8 | 29.4 ± 1.7 | 45.6 ± 3.3 | 44.7 ± 3.1 | 36.2 ± 3.7 | 35.7 ± 3.8 |
| **Non-exercising controls XS** | 83.9 ± 9.1 | 84.9 ± 8.1 | 27.8 ± 2.5 | 27.6 ± 2.4 | 54.0 ± 4.6 | 54.2 ± 4.1 | 27.2 ± 5.6 | 27.9 ± 4.9 |
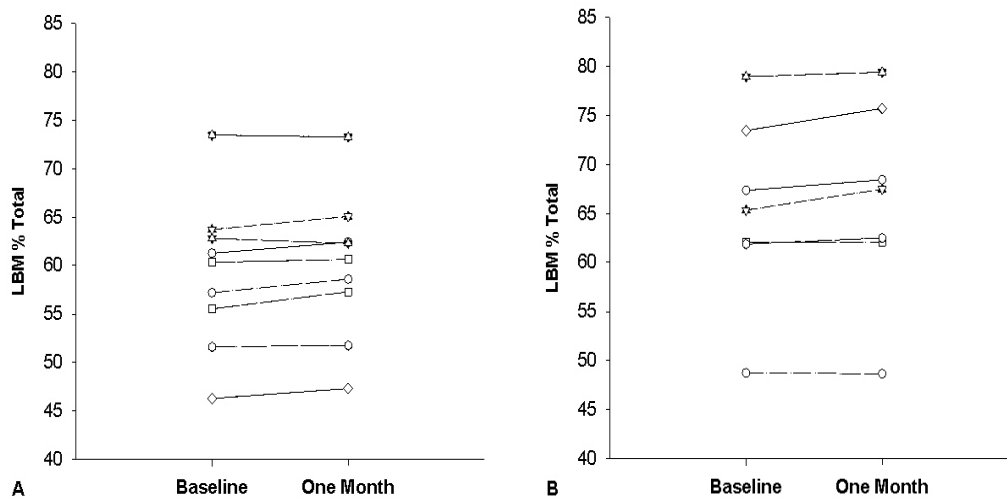
**Figure 3.7. The effect of one month of exercise on LBM expressed as % of total body mass with STD (A) or XS (B) bicarbonate therapy.** Due to logistical problems, n = 9 and n = 7 in graphs A and B respectively.
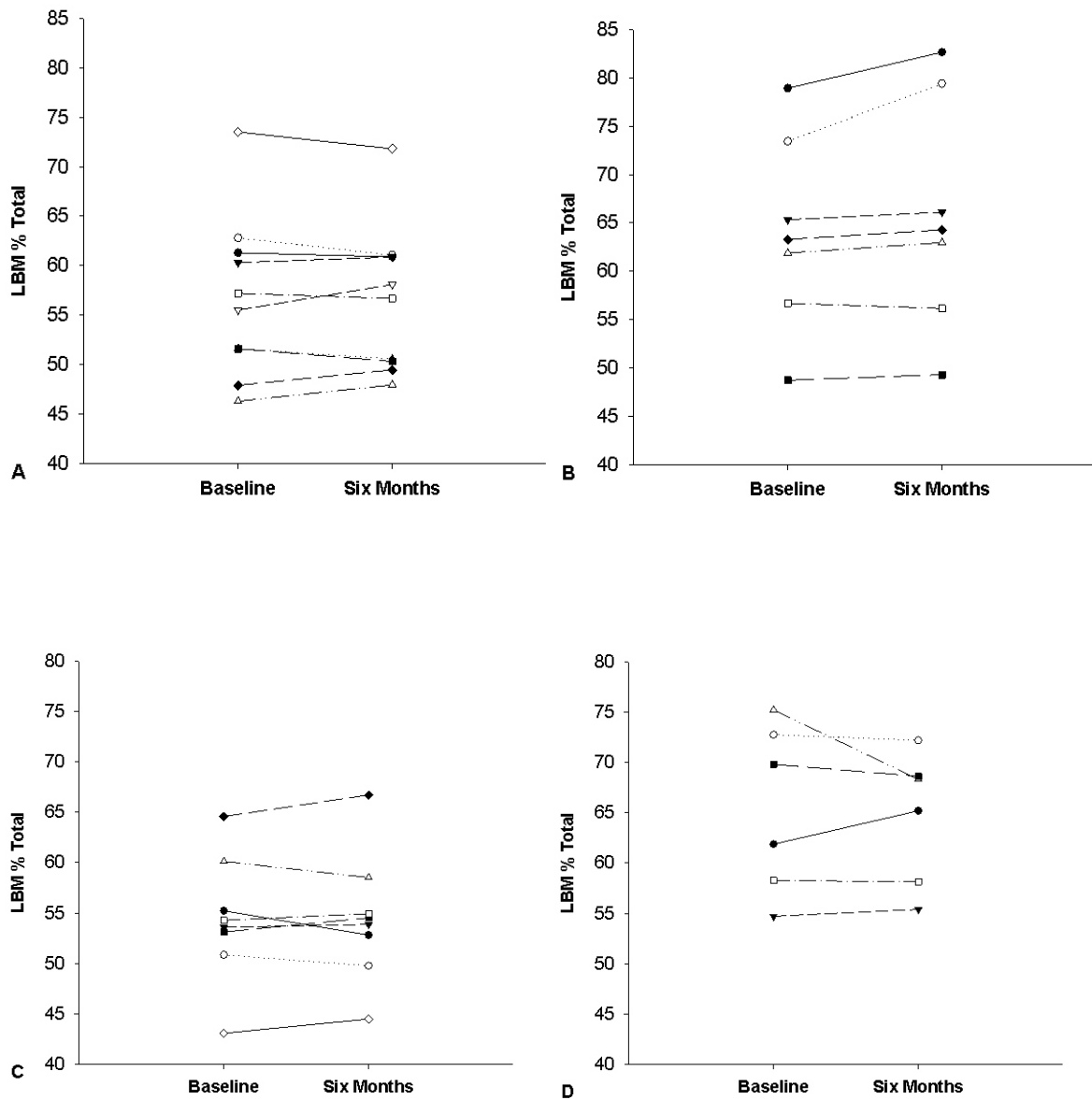
**Figure 3.8. The effect of six months of exercise training (A,B) or a 6 month control period (C,D) on LBM expressed as % of total body mass with STD (A,C) or XS (B,D) bicarbonate therapy.** Due to logistical problems, n = 10, n = 7, n = 8 and n = 6 in graphs A, B, C and D respectively.

### 3.3.6 Muscle biopsy composition

The gross composition of the muscle biopsies obtained from the patients is shown in Table 3.11. Muscle DNA content did not change under any condition. The RNA content of the muscle biopsies taken from those patients who had exercised for six months with STD bicarbonate therapy increased from $1.2 \pm 0.2$ µg/mg dw to $1.5 \pm 0.2$ µg/mg dw (P = 0.017), an effect that was not seen under any other condition. The water content was determined in the biopsies from the subtraction of dry weight from wet weight. Muscle biopsies were dried to constant weight, but the contribution to the weight from tightly locked water in the biopsy was unknown. No change was seen in the water content of the muscle biopsies from any group (P>0.05) indicating that the additional sodium load given to the XS bicarbonate group had not caused any detectable water loading in these patients.

There was an apparent decline in the muscle protein content in the biopsies taken from those patients who exercised for six months with XS bicarbonate therapy (P = 0.052) which did not occur in any of the other groups. A similar effect was observed on muscle Protein:DNA ratio (P = 0.008) and as no corresponding effect on lean body mass was detected by DEXA in this group of patients, it is unlikely that this decline in biopsy protein content reflects muscle protein wasting. Significant amounts of fibrotic tissue were observed in the biopsies obtained from the CKD patients. While macroscopic connective tissue was dissected away during processing of the sample (see Methods Section 3.2.7), this does not exclude microscopic infiltration of the muscle by scar tissue that may have been particularly likely to occur near the biopsy site (see Discussion Section 3.4.6). The soluble protein content of the biopsies was determined using the Folin Lowry assay (see Section 2.4.1). This assay works by oxidising the tyrosine and tryptophan residues in the protein producing a blue colour, and as collagen contains little or none of either of these amino acids, it would not be detected by the assay and therefore would not have contributed to the total protein content. No other effects on muscle protein:DNA ratio were seen in any other group.

In view of this uncertainty, all of the biochemical data in the remainder of this thesis are expressed in ways aimed at minimising artefacts arising from variation in the myocyte content of the biopsies. Data have therefore been presented as metabolite concentrations expressed in

relation to intracellular water (e.g. amino acid concentrations), relative abundance of proteins determined by Western blotting after loading of fixed quantities of total soluble protein onto the gels, and abundance of molecules (e.g. mRNA's and 14kDa actin fragment) expressed in relation to a reference molecule in the same sample. Intracellular water content was determined from intramuscular chloride concentrations assuming a normal membrane potential as described by Bergström et al. (1974). It was shown that changes in membrane potential result in only very small errors in intracellular water content and subsequently this method has frequently been to normalise amino acid concentration data.

**Table 3.11. The effect of six months of exercise or no exercise with STD or XS bicarbonate therapy on muscle biopsy composition.** * Denotes a significant difference between six months and the corresponding value at baseline ($P < 0.05$).

|  | Condition | Baseline | Six Months |
|---|---|---|---|
| Muscle Protein Concentration (mg/mg dw) | 6 Mth Ex STD Bicarbonate | 0.6 ± 0.1 | 0.6 ± 0.1 |
|  | 6 Mth Ex XS Bicarbonate | 0.5 ± 0.1 | 0.3 ± 0.1 |
|  | Non-exercising Controls STD | 0.5 ± 0.1 | 0.5 ± 0.1 |
|  | Non-exercising Controls XS | 0.5 ± 0.1 | 0.6 ± 0.2 |
| Muscle DNA Concentration (µg/mg dw) | 6 Mth Ex STD Bicarbonate | 2.6 ± 0.3 | 2.6 ± 0.2 |
|  | 6 Mth Ex XS Bicarbonate | 2.7 ± 0.2 | 2.7 ± 0.4 |
|  | Non-exercising Controls STD | 2.7 ± 0.2 | 2.8 ± 0.5 |
|  | Non-exercising Controls XS | 2.8 ± 0.3 | 2.9 ± 0.4 |
| Muscle RNA Concentration (µg/mg dw) | 6 Mth Ex STD Bicarbonate | 1.2 ± 0.2 | 1.5 ± 0.2* |
|  | 6 Mth Ex XS Bicarbonate | 1.6 ± 0.2 | 1.5 ± 0.3 |
|  | Non-exercising Controls STD | 1.6 ± 0.2 | 1.5 ± 0.2 |
|  | Non-exercising Controls XS | 1.2 ± 0.3 | 1.4 ± 0.3 |
| Muscle Protein:DNA ratio (mg/mg dw) | 6 Mth Ex STD Bicarbonate | 232.4 ± 124.1 | 263.6 ± 193.9 |
|  | 6 Mth Ex XS Bicarbonate | 202.5 ± 6.6 | 131.2 ± 35.1* |
|  | Non-exercising Controls STD | 200.3 ± 62.1 | 193.0 ± 86.1 |
|  | Non-exercising Controls XS | 202.9 ± 65.7 | 235.7 ± 66.5 |

**3.3.7 Muscle and plasma amino acid concentrations**

Tables 3.12 – 3.14 show the changes that occurred in fasted plasma amino acid concentrations over the course of the study. No dramatic changes were observed, but statistically significant increases in L-Asn, L-Gln, L-His, and L-Ala were observed after one or six months of exercise in the STD bicarbonate groups. These changes observed at six months were not paralleled by changes in the exercising patients on XS bicarbonate (Table 3.13) who showed statistically significant increases only in Gly and L-Met and the non-exercising patients on STD bicarbonate also failed to show these changes. All the changes that were observed were increases above baseline concentrations ($P<0.05$), except for the concentration of L-Arg that was seen to decline significantly in the six months exercise group with STD bicarbonate ($P = 0.008$). In contrast, this amino acid increased in the non-exercising patients on STD bicarbonate (Table 3.14) ($P = 0.008$).

**Table 3.12. Changes in fasted plasma amino acid concentrations (µmol/l) following one month exercise on STD bicarbonate.** * Denotes significant change from baseline (P<0.05).

| Amino Acid | STD Baseline | STD One Month | Δ |
|---|---|---|---|
| Glu | 61.9 ± 18.1 | 56.4 ± 14.5 | -5.5 ± 5.6 |
| Asn | 49.8 ± 5.7 | 63.1 ± 2.8 | 13.4 ± 5.3 |
| Ser | 68.5 ± 10.1 | 86.6 ± 16.2 | 18.1 ± 12.2 |
| Gln | 600.7 ± 59.4 | 732.3 ± 37.9* | 131.7 ± 51.1 |
| His | 51.3 ± 6.4 | 66.8 ± 6.6* | 15.6 ± 7.1 |
| Gly | 273.9 ± 81.6 | 366.5 ± 62.9 | 92.6 ± 66.7 |
| Thr | 83.1 ± 9.7 | 119.0 ± 13.5 | 35.9 ± 18.8 |
| Arg | 60.8 ± 13.0 | 80.8 ± 11.9 | 20.0 ± 11.3 |
| Ala | 329.2 ± 42.4 | 414.0 ± 33.1 | 84.4 ± 52.8 |
| Tyr | 50.7 ± 7.1 | 62.0 ± 5.6 | 11.3 ± 5. 0 |
| Cys | 68.3 ± 6.7 | 75.8 ± 6.9 | 7.4 ± 7.1 |
| Val | 145.9 ± 18.6 | 159.6 ± 18.3 | 13.7 ± 17.1 |
| Met | 14.8 ± 2.9 | 15.2 ± 1.5 | 0.5 ± 3.5 |
| Tryp | 38.7 ± 2.8 | 41.3 ± 2.8 | 2.6 ± 5.1 |
| Phe | 51.9 ± 7.1 | 60.7 ± 5.2 | 8.8 ± 4.6 |
| Ile | 45.8 ± 4.8 | 49.5 ± 4.3 | 3.7 ± 5.7 |
| Leu | 88.4 ± 8.3 | 93.3 ± 4.1 | 4.9 ± 7.1 |
| Lys | 154.0 ± 22.9 | 170.0 ± 8.5 | 15.9 ± 17.5 |

**3.13**. **Changes in fasted plasma amino acid concentration (μmol/l) following six months of exercise on STD or XS bicarbonate therapy.** * Denotes significant change from baseline in each group (P<0.05).

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 80.5 ± 19.0 | 94.3 ± 13.5 | 20.3 ± 17.0 | 74.2 ± 21.3 | 89.9 ± 14.8 | 3.1 ± 11. 0 |
| Asn | 49.1 ± 5.0 | 64.8 ± 5.4* | 15.7 ± 5.0 | 71.5 ± 5.2 | 68.4 ± 6.5 | -3.2 ± 7.2 |
| Ser | 81.1 ± 10.5 | 100.0 ± 5.8 | 18.9 ± 9.5 | 88.5 ± 5.8 | 99.0 ± 8.0 | 10.4 ± 7.2 |
| Gln | 599.4 ± 51.4 | 676.1 ± 27.8 | 76.6 ± 46.2 | 640.7 ± 41.4 | 712.1 ± 34.9 | 71.4 ± 5.3 |
| His | 49.8 ± 5.3 | 67.7 ± 7.0* | 19.9 ± 6.8 | 73.3 ± 7.8 | 74.2 ± 8.3 | 0.9 ± 8.5 |
| Gly | 293.5 ± 72.4 | 342.7 ± 51.3 | 49.2 ± 33.5 | 233.7 ± 23.0 | 290.0±29.5* | 56.3 ± 18.6 |
| Thr | 93.8 ± 10.4 | 127.6 ± 9.8 | 33.9 ± 14.4 | 134.9 ± 12.7 | 145.1 ± 12.7 | 10.2 ± 11.7 |
| Arg | 47.8 ± 11.7 | 29.3 ± 6.8* | -18.5 ± 8.2 | 57.2 ± 11.0 | 43.4 ± 8.4 | -13.8 ± 14.7 |
| Ala | 337.4 ± 38.9 | 448.5 ± 38.3* | 111.1 ± 43.6 | 497.8 ± 60.2 | 495.1 ± 60.0 | -2.6 ± 65.5 |
| Tyr | 56.0 ± 8.1 | 63.2 ± 5.1 | 7.2 ± 5.2 | 53.4 ± 5.1 | 50.7 ± 4.3 | -2.8 ± 4.5 |
| Cys | 66.5 ± 5.6 | 58.5 ± 3.1 | -8.0 ± 5.6 | 58.9 ± 8.2 | 58.1 ± 3.5 | -0.8 ± 5.1 |
| Val | 157.6 ± 18.5 | 170.0 ± 26.4 | 12.4 ± 25.1 | 205.4 ± 28.8 | 196.6 ± 19.7 | -8.8 ± 24.4 |
| Met | 15.7 ± 2.6 | 17.4 ± 1.8 | 1.7 ± 3.3 | 18.0 ± 2.9 | 23.5 ± 3.7* | 5.5 ± 1.6 |
| Tryp | 41.2 ± 5.8 | 40.1 ± 3.9 | -1.1 ± 5.8 | 41.8 ± 4.8 | 41.3 ± 4.2 | -0.5 ± 4.3 |
| Phe | 50.5 ± 5.5 | 59.9 ± 11.9 | 9.4 ± 7.1 | 54.6 ± 9.2 | 56.1 ± 7.3 | 1.5 ± 3.7 |
| Ile | 46.1 ± 4.2 | 48.3 ± 4.8 | 2.3 ± 5.9 | 62.8 ± 9.6 | 62.0 ± 11.3 | -0.8 ± 8.9 |
| Leu | 93.5 ± 8.2 | 108.7 ± 11.8 | 15.2 ± 13.2 | 113.9 ± 11.4 | 120.5 ± 15.6 | 6.6 ± 10.0 |
| Lys | 164.3 ± 21.1 | 179.1 ± 11.6 | 14.7 ± 21.6 | 189.1 ± 17.7 | 225.4 ± 37.5 | 36.3 ± 28.8 |

**Table 3.14. Changes in fasted plasma amino acid concentrations (µmol/l) following six months without exercise**. * Denotes significant change from baseline in each group (P<0.05).

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 51.7 ± 4.2 | 56.2 ± 5.8 | 4.4 ± 5.9 | 60.2 ± 8.2 | 49.2 ± 4.8 | -11. 0 ± 4. 0 |
| Asn | 51.1 ± 1.9 | 55.0 ± 3.4 | 3.9 ± 3.8 | 60.2 ± 5.3 | 56.6 ± 5.7 | -3.6 ± 7.1 |
| Ser | 89.3 ± 9.8 | 105.5 ± 16.3 | 16.2 ± 9.5 | 96.7 ± 21.0 | 87.6 ± 15.7 | -9.1 ± 16.0 |
| Gln | 598.3 ± 14.3 | 660.6 ± 32.0 | 62.3 ± 34.0 | 675.0 ± 55.2 | 684.0 ± 26.0 | 9.0 ± 55. 0 |
| His | 56.4 ± 3.2 | 64.9 ± 5.8 | 8.5 ± 5.5 | 82.2 ± 12.8 | 77.3 ± 15.3 | -4.8 ± 21.3 |
| Gly | 263.7 ± 31.8 | 306.0 ± 53.8 | 42.2 ± 27.1 | 345.0 ± 28.7 | 405.2 ± 14.4 | 60.2 ± 14.3 |
| Thr | 106.0 ± 11.2 | 117.7 ± 14.0 | 11.7 ± 9.5 | 141.8 ± 12.7 | 150.2 ± 11.3 | 8.4 ± 13.8 |
| Arg | 34.7 ± 6.4 | 58.0 ± 7.1* | 23.3 ± 6. 0 | 75.6 ± 15.1 | 83.2 ± 13.9 | 7.6 ± 3.8 |
| Ala | 322.6 ± 33.5 | 392.1 ± 32.4 | 59.5 ± 54.7 | 479.1 ± 45.8 | 455.4 ± 84.7 | -23.7 ± 87.2 |
| Tyr | 45.6 ± 2.2 | 51.8 ± 5.8 | 6.1 ± 5.2 | 80.5 ± 19.6 | 74.0 ± 13.4 | -6.5 ± 6.2 |
| Cys | 53.6 ± 5.6 | 69.5 ± 11.1 | 15.8 ± 8.1 | 63.6 ± 10.5 | 59.9 ± 15.4 | -3.7 ± 8.1 |
| Val | 156.7 ± 13.8 | 168.3 ± 13.6 | 11.5 ± 8.2 | 234.8 ± 20.4 | 203.4 ± 13.9 | -31.3 ± 14.2 |
| Met | 16.5 ± 0.8 | 21.0 ± 2.2 | 4.5 ± 8.2 | 29.5 ± 2.4 | 28.5 ± 3.7 | -1.0 ± 3.5 |
| Tryp | 32.0 ± 2.2 | 38.3 ± 4.2 | 6.3 ± 3.2 | 40.3 ± 3.9 | 38.2 ± 5.8 | -2.1 ± 2.4 |
| Phe | 52.8 ± 3.1 | 62.6 ± 7.3 | 9.8 ± 7.6 | 70.2 ± 0.4 | 65.9 ± 2.0 | -4.3 ± 1.7 |
| Ile | 44.0 ± 3.1 | 51.8 ± 4.8* | 7.8 ± 3.2 | 68.1 ± 2.9 | 61.7 ± 3.4 | -6.4 ± 2.6 |
| Leu | 87.5 ± 5.3 | 95.9 ± 7.8 | 8.4 ± 5.9 | 139.7 ± 12.3 | 116.2 ± 7.7 | -23.5 ± 5.4 |
| Lys | 127.9 ± 7.1 | 159.6 ± 22.7 | 31.7 ± 18.1 | 239.7 ± 67.5 | 188.9 ± 24.8 | -50.7 ± 50.2 |

Tables 3.15-3.17 show the changes in muscle amino acid concentrations in the fasted state. The median baseline concentrations were comparable with those previously reported by Bergström et al. (1990) (Figure 3.9). However, some of the patients had high baseline concentrations of some of the amino acids causing the data to be skewed, especially in the STD bicarbonate group. No artifactual explanation could be found for this and so all the data were included in the analysis (however see Chapter 4, Section 4.4.5(2)). Methionine concentrations were virtually undetectable in these muscle biopsies and so have been omitted from the tables. After six months of exercise with STD bicarbonate therapy, nine amino acids showed a striking statistically significant depletion relative to the baseline value. Of these Threonine, Valine, Phenylalanine, Leucine and Lysine are essential amino acids. Interestingly no significant effects were seen in the intramuscular concentration of histidine, which is kown to be highly involved in the muscle buffering process. This amino acid depletion is illustrated in Figure 3.10B. Such depletion was detectable after only one month on STD bicarbonate treatment, but was not yet statistically significant (Table 3.15; Figure 3.10A) This effect was not observed following six months of exercise with XS bicarbonate, nor was it seen in either the STD or XS non-exercising control groups (Figures 3.11 and 3.12). This depletion was also accompanied by a collapse in many of the muscle / plasma amino acid concentration gradients (Table 3.19). Again, this effect was apparently present at one month, but failed to reach statistical significance, except for L-Ser (Table 3.18). This collapse was not seen in any of the other groups (Tables 3.19. and 3.20).

**Table 3.15. Changes in muscle amino acid concentration (mmol/l cell H$_2$O) in the fasted state following one month of exercise with STD bicarbonate therapy.** Values are presented as median and range.

| Amino Acid | STD Baseline | STD One Month | Δ |
|---|---|---|---|
| Glu | 3.6 (0.3 – 16.7) | 1.6 (0.9 – 64.2) | -1.9 (-12.5 – 61.0) |
| Asn | 0.3 (0.3 – 1.9 ) | 0.4 (0.1 – 3.3) | -0.01 (-1.33 – 3.12) |
| Ser | 0.4 (0.08 – 7.78) | 0.3 (0 – 8.5) | -0.06 (-7.60 – 8.12) |
| Gln | 12.3 (3.0 – 92.1) | 8.4 (3.3 – 196.1) | -3.9 (-65.7 – 7.4) |
| His | 0.3 (0 – 2.9) | 0.2 (0.04 – 2.27) | -0.1 (-2.4 – 2.0) |
| Gly | 1.6 (0.6 – 9.9) | 1.5 (0.4 – 17.4) | -0.3 (-9.4 – 15.9) |
| Thr | 0.9 (0.3 – 5.7) | 0.8 (0.7 – 13.7) | 0.3 (-4.1 – 13.3) |
| Arg | 0.6 (0.09 – 4.2) | 0.4 (0.1 – 8.0) | -0.2 (-3.4 – 7.5) |
| Ala | 2.7 (0.7 – 29.6) | 2.5 (1.1 – 51.1) | -0.1 (-20.3 – 49.2) |
| Tyr | 0.78 (0.08 – 3.56) | 0.21 (0.05 – 1.19) | -0.03 (-3.5 – 1.1) |
| Cys | 0.09 (0.02 – 1.37) | 0.05 (0.01 – 0.55) | -0.01 (-1.50 – 0.18) |
| Val | 0.5 (0.1 – 6.9) | 0.2 (0 – 2.9) | 0.1 (-6.9 – 2.7) |
| Met | -- | -- | -- |
| Tryp | 0.27 (0 – 1.7) | 0.10 (0 – 2.1) | -0.03 (-0.45 – 0.16) |
| Phe | 0.5 (0.06 – 0.59) | 0.2 (0.06 – 1.68) | -0.05 (-1.01 – 1 .08) |
| Ile | 0.21 (0.06 – 2.38) | 0.15(0.05 – 0.87) | -0.02 (-2.33 – 0.18) |
| Leu | 0.35 (0.09 – 4.63) | 0.2 (0.09 – 1.57) | -0.08 (-4.53 – 1.36) |
| Lys | 0.5 (0.01 – 6.49) | 0.2 (0.1 – 10.5) | -0.2 (-6.3 – 10.0) |

**Table 3.16. Changes in muscle amino acid concentration (mmol/l cell H$_2$O) in the fasted state following six months of exercise with STD or XS bicarbonate therapy.** * Denotes significant change from baseline in each group (P<0.05). Values are presented as median and range.

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 3.6  (0.3 – 16.7) | 1.7 (0.3 – 3.1)* | -1.8 (-15.4 – 1.1) | 2.1 (0.5 – 2.6) | 3.3 (1.1 – 8.9) | 1.4 (-1.0 – 6.6) |
| Asn | 0.39  (0.11 – 1.87) | 0.18 (0.08 – 0.48)* | -0.25 (-1.66 – 0.06) | 0.14 (0.08 – 0.43) | 0.43 (0.11 – 0.61) | 0.3 (-0.3 – 0.4) |
| Ser | 0.4 (0.08 – 7.78) | 0.2 (0 – 0.50) | -0.09 (-7.40 – 0.08) | 0.3 (0.04 – 0.86) | 0.3 (0.05 – 2.15) | 0.2 (-0.2 – 1.3) |
| Gln | 11.9 (3.0 – 92.2) | 7.1 (2.2 – 10.6) | -5.7 (-83.1 – 7.6) | 7.6  (4.7 – 14.1) | 8.9 (1.5 – 19.8) | 4.3 (-6.4 – 9.2) |
| His | 0.3 (0 – 2.9) | 0.20 (0.06 – 0.76) | -0.10 (-2.8 – 0.6) | 0.2 (0.05 – 0.48) | 0.2 (0 – 2.4) | 0.05 (-0.5 – 2.3) |
| Gly | 1.7 (0.6 – 9.9) | 1.1 (0.7 – 10.6) | -0.4 (-8.3 – 8.9) | 1.0 (0.6 – 1.1) | 2.6 (0.4 – 16.7) | 1.6 (-0.7 – 11.7) |
| Thr | 0.7 (0.3 – 5.7) | 0.3 (0.1 – 0.6)* | -0.5 (-5.3 – 0.3) | 0.3 (0.1- 0.5) | 0.6 (0.3 – 1.9) | 0.4 (-0.2 – 1.6) |
| Arg | 0.7 (0.09 – 4.19 ) | 0.3 (0.1 – 0.5)* | -0.39 (-3.86 – 0.01) | 0.3 (0.09 – 0.48) | 0.3 (0.2 – 1.1) | 0.1 (-0.2 – 0.5) |
| Ala | 3.1 (0.7 – 29.6) | 1.4 (0.03 – 1.87)* | -2.0 (-26.8 – 0.1) | 1.8 (1.6 – 12.3) | 5.0 (0.8 – 7.3) | 0.02 (-4.94 – 3.37) |
| Tyr | 0.42 (0.08 – 3.56) | 0.09 (0.03 – 0.64) | -0.08 (-3.37 – 0.54) | 0.08 (0.03 – 0.37) | 0.13 (0.03 – 1.32) | 0.09 (-0.30 – 1.25) |
| Cys | 0.09 (0.02 – 1.37) | 0.08 (0.01 – 0.21) | -0.01 (-1.36 – 0.36) | 0.04 (0.02 – 0.39) | 0.02 (0 – 0.1) | -0.02 (-0.04 – 0.10) |
| Val | 0.62 (0.1 – 6.9) | 0.17 (0 – 0.3)* | -0.28 (-6.66 – 0.01) | 0.26 (0.08 – 0.3) | 0.52 (0.07 – 1.96) | 0.2 (-0.2 – 1.7) |
| Met | -- | -- | -- | -- | -- | -- |
| Tryp | 0.09  (0 – 1.7) | 0.06 (0 – 0.3) | -0.03 (-0.25 – 0.16) | 0.08 (0 – 0.6) | 0.2 (0.04 – 0.26) | 0.07 (-0.41 – 0.21) |
| Phe | 0.32 (0.09 – 2.58) | 0.11 (0.04 – 0.17)* | 0.26 (-2.24 – 0.05) | 0.11 (0.07 – 0.13) | 0.09 (0.04 – 0.49) | 0.09 (-0.2 – 0.4) |
| Ile | 0.22 (0.06 – 2.38) | 0.08 (0 – 0.6) | -0.07 (-2.15 – 0.48) | 0.08 (0.03 – 0.1) | 0.09 (0 – 0.1) | 0.05 (-0.06 – 0.21) |
| Leu | 0.5 (0.09 – 4.63) | 0.2 (0.09 – 0.48)* | -0.19 (-4.14 – 0.01) | 0.15 (0.12 – 0.17) | 0.3 (0.07 – 0.75) | 0.12 (-0.07 – 0.59) |
| Lys | 0.9 (0.01 – 6.49) | 0.2 (0.08 – 0.73)* | -0.4 (-4.7 – 0.2) | 0.3 (0.2 – 0.5) | 0.4 (0.2 – 1.0) | 0.06 (-0.09 – 0.55) |

**Table 3.17. Changes in muscle amino acid concentration (mmol/l cell H$_2$O) in the fasted state following six months without exercise with STD or XS bicarbonate therapy**. Data are shown as median and range.

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 1.4 (0.7-3.2) | 2.1 (0.9 – 4.0) | 0.4 (-1.6 – 2.1) | 1.6 (1.1 – 2.30) | 2.5 (0.3 – 2.8) | 0.5 (-0.8 – 1.0) |
| Asn | 0.2 (0.1 – 0.4) | 0.20 (0.05 – 0.56) | -0.03 (-0.09 – 0.35) | 0.14 (0.04 – 0.31) | 0.22 (0.23 – 0.24) | 0.08 (-0.06 – 0.16) |
| Ser | 0.3 (0.1 – 0.4) | 0.3 (0.1 – 1.0) | -0.09 (-0.1 – 0.7) | 0.3 (0.2 – 0.5) | 0.23 (0.08 – 0.45) | -0.06 (-0.1 - -0.05) |
| Gln | 8.3 (1.6 – 13.7) | 8.3 (0.6 – 20.9) | 0.2 (-8.1 – 19.7) | 8.7 (3.5 – 11.9) | 12.2 (0.3 – 15.1) | 0.3 (-3.2 – 6.4) |
| His | 0.14  (0.06 – 1.67) | 0.17  (0.09 – 2.71) | 0.05 (-0.36 – 1.36) | 0.2 (0.1 – 0.4) | 0.3 (0.1 – 0.4) | -0.01 (-0.09 – 0.27) |
| Gly | 0.9 (0.4 – 3.4) | 0.9 (0.5 – 2.7) | -0.2 (-0.8 – 1.2) | 0.9 (0.4 – 1.4) | 1.1 (0.5 – 1.5) | 1.1 (0.2 – 1.5) |
| Thr | 0.4 (0.1 – 0.9) | 0.20 (0.09 – 0.60) | -0.2 (-0.5 – 0.4) | 0.1 (0.1 – 0.3) | 0.29 (0.05 – 0.35) | 0.03 (-0.06 – 0.16) |
| Arg | 0.3 (0.1 – 0.6) | 0.4 (0.1 – 0.4) | -0.01 (-0.23 – 1.11) | 0.3 (0.2 – 0.5) | 0.43 (0.05 – 0.54) | -0.04 (-0.12 – 0.17) |
| Ala | 2.6 (0.7 – 3.7) | 2.1(0.2 – 0.4) | -0.3 (-0.8 – 6.9) | 2.6 (0.7 – 3.0) | 2.6 (0.7 – 3.0) | -0.06 (-0.52 – 2.28) |
| Tyr | 0.08 (0.03 – 1.10) | 0.20 (0.08 – 1.10) | 0.09 (-0.8 – 0.7) | 0.05 (0.05 – 0.40) | 0.06 (0.02 – 0.12) | -0.03 (-0.33 – 0.07) |
| Cys | 0.06 (0.02 – 0.53) | 0.10 (0 – 0.7) | 0.01 (-0.39 – 0.60) | 0.05 (0.03 – 0.07) | 0.2 (0.1 – 0.3) | 0.27 (0.03 – 0.30) |
| Val | 0.3 (0.2 – 1.6) | 0.6 (0.2 – 1.2) | 0.12 (-0.9 – 0.5) | 0.3 (0.1 – 0.3) | 0.3 (0.1 – 0.4) | -0.03 (-0.10 – 0.10) |
| Met | -- | -- | -- | -- | -- | -- |
| Tryp | 0.3 (0.1 – 0.6) | 0.5  (0 – 1.2) | 0.05 (-0.64 – 1.0) | 0.2 (0.1 – 0.2) | 0.16 (0.08 – 0.24) | -0.02 (-0.02 – 0.04) |
| Phe | 0.09 (0.03 – 0.3) | 0.17 (0.08 – 1.63) | 0.09 (-0.18 – 1.51) | 0.10 (0.06 – 0.16) | 0.11 (0.08 – 0.12) | 0.01(-0.03 – 0.01) |
| Ile | 0.18 (0.07 – 0.34) | 0.26 (0.07 – 1.25) | 0.05 (-0.01 – 0.96) | 0.12 (0.05 – 0.14) | 0.10 (0.06 – 0.13) | -0.01 (-0.02 – 0.01) |
| Leu | 0.19 (0.09 – 0.55) | 0.21 (0.08 – 0.57) | -0.01 (-0.26 – 0.23) | 0.15 (0.07 – 0.43) | 0.12 (0.11 – 0.21) | 0.05 (-0.31 – 0.06) |
| Lys | 0.3 (0.1 -1.0) | 0.7 (0.1 – 1.2) | 0.3 (-0.5 – 1.0) | 0.3 (0.1 – 0.3) | 0.28 (0.03 – 0.86) | -0.01 (-0.08 – 0.48) |
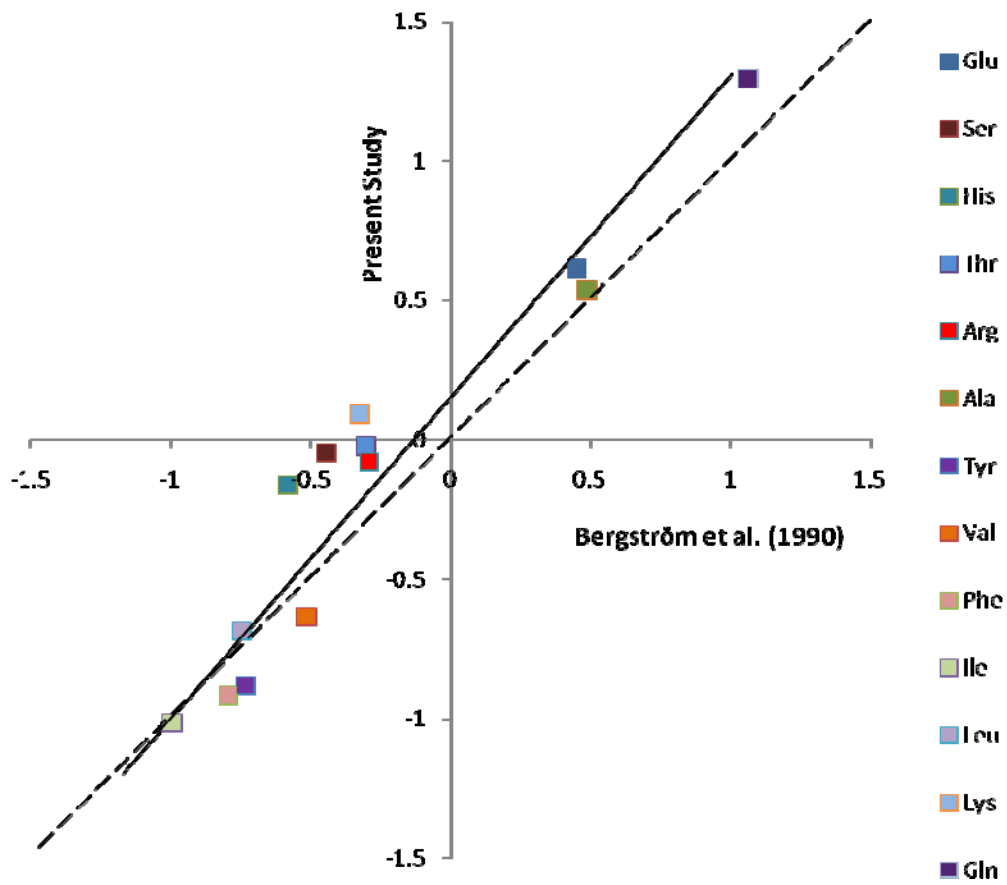
**Figure 3.9. Comparison of muscle amino acid concentrations recorded in the present study with those previously reported by Bergström et al. (1990).** Data are expressed as $\log_{10}$.mmol/l Solid black line denotes $r^2 = 0.957$, dashed black line symbolises $r^2 = 1.000$. Median values from the six month exercise group on STD bicarbonate at baseline were used in the comparison.

**Table 3.18. Changes in muscle / plasma amino acid gradients (mmol/l) following one month of exercise with STD bicarbonate therapy.** * denotes significant change from baseline (P<0.05). Values are presented as median and range.

| Amino Acid | STD Baseline | STD One Month | Δ |
|---|---|---|---|
| Glu | 13.9 (0.4 – 30.9) | 4.6 (0.8 – 301.2) | -1.9 (-30.0 – 278.2) |
| Asn | 7.2 (1.8 – 28.9) | 5.9 (2.0 – 61.7) | -1.2 (-38.0 – 52.2) |
| Ser | 5.9 (1.3 – 125.5) | 0.27(0.07 – 8.39)* | -2.7 (-125.4 – 0.1) |
| Gln | 25.9 (3.7 – 126.4) | 10.9 (3.7 – 337.7) | -14.2 (-91.9 – 309.2) |
| His | 4.7 (0 – 42.3) | 3.3 (0.7 – 33.4) | -2.11 (-37.4 – 29.0) |
| Gly | 9.6 (1.8 – 54.6) | 3.2 (1.8 – 56.6) | -2.9 (-52.7 – 43.9) |
| Thr | 11.4 (4.0 – 49.7) | 7.9 (2.2 – 114.0) | -1.6 (-47.5 – 106.1) |
| Arg | 15.6 (1.2 – 14.1) | 5.9 (1.8 – 157.1) | -8.0 (-51.7 – 143.0) |
| Ala | 10.6 (1.8 – 75.3) | 6.9 (3.9 – 114.1) | -2.1 (-54.0 – 102.1) |
| Tyr | 14.0 (2.5 – 41.0) | 4.1 (0.6 – 22.1) | -3.3 (-40.3 – 26.9) |
| Cys | 1.7 (0.9 – 23.6) | 0.8 (0.3 – 8.4) | -0.4 (-23.2 – 2.0) |
| Val | 4.1 (0.9 – 107.5) | 1.3 (0 – 18.6) | -1.4 (-106.2 – 1.2) |
| Met | -- | -- | -- |
| Tryp | 7.6 (0 – 33.0) | 2.8 (0 – 61.4) | -4.9 (-31.7 – 61.4) |
| Phe | 8.0 (1.5 – 74.7) | 3.4 (0.9 – 30.6) | -0.6 (-73.6 – 4.0) |
| Ile | 6.0 (1.3 – 50.1) | 3.5 (0.9 – 19.4) | -0.6 (-49.2 – 12.3) |
| Leu | 5.6 (0.9 – 45.2) | 2.3 (0.9 – 18.4) | -0.9 (-44.3 – 12.9) |
| Lys | 5.0 (0.06 – 31.1) | 1.3 (0.6 – 62.7) | -2.2 (-30.1 – 56.8) |

**Table 3.19. Changes in muscle / plasma amino acid gradients (mmol/l) following six months of exercise with STD or XS bicarbonate therapy.** * denotes significant change from baseline in each group (P<0.05). Values are presented as median and range.

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 11.1 (0.4 – 30.9) | 2.2 (0.6 – 8.3)* | -9.3 (-69.6 – 5.1) | 4.8 (2.1 – 12.5) | 4.9 (4.0 – 9.1) | 0.3 (-8.4 – 4.3) |
| Asn | 8.4 (1.8 – 28.9) | 2.5 (1.8 – 8.2)* | -6.0 (-32.9 – 0.1) | 2.4 (1.0 – 5.5) | 6.9 (1.15 – 11.5) | 4.5 (-4.0 – 8.7) |
| Ser | 3.23 (1.4 – 125.5) | 0.11 (0 – 0.39)* | -3.1 (-125.2 – 1.3) | 3.23 (0.4 – 12.3) | 0.24 (0 – 2.05) | -2.3 (-10.3 - -0.46) |
| Gln | 25.6 (3.7 – 126.4) | 10.7 (3.5 – 14.5)* | -13.8 (-113.5 – 9.2) | 12.5 (6.0 – 12.5) | 12.5 (2.4 – 31.0) | 2.2 (-10.5 – 12.5) |
| His | 4.6 (0 – 42.3) | 5.0 (0.6 – 10.4) | -1.8 (-39.8 – 6.7) | 2.0 (0.7 – 6.0) | 2.4 (0 – 45.6) | 0.4 (-5.5 – 43.8) |
| Gly | 12.6 (1.8 -54.6) | 3.4 (1.2 – 50.6) | 1.5 (-47.3 – 35.3) | 4.9 (1.9 – 28.0) | 8.7 (1.6 – 70.4) | 3.8 (-3.2 – 42.3) |
| Thr | 7.9 (1.0 – 49.7) | 2.4 (0.8 – 4.5)* | -4.3 (-41.0 – 3. 0) | 2.1 (1.5 – 5.1) | 4.5 (2.0 – 13.7) | 2.9 (-3.0 – 11.6) |
| Arg | 23.8 (1.2 – 34.0) | 14.3 (1.4 – 30.9)* | -8.8 (-43.8 – 0.5) | 5.9 (1.5 – 5.9) | 7.7 (4.2 – 42.5) | 6.2 (-17.1 – 36.0) |
| Ala | 11.5 (1.8 – 75.3) | 2.9 (0.07 – 7.08)* | -7.7 (-70.3 – 0.5) | 4.2 (2.9 – 41.8) | 12.1 (2.1 – 18.9) | -0.5 (-22.9 – 8.5) |
| Tyr | 8.2 (2.5 – 41.0) | 1.6 (0.4 – 11.5) | -2.4 (-38.9 – 7.6) | 2.3 (0.5 – 7.6) | 1.9 (0.6 – 30.2) | 1.4 (-6.0 – 29.1) |
| Cys | 1.5 (0.9 – 23.6) | 1.1 (0 -3.6) | -0.5 (-20.6 – 0.3) | 0.7 (0.3 – 7.4) | 0.5 (0 – 6.4) | 0.2 (-7.4 – 5.7) |
| Val | 5.0 (0.9 – 107.5) | 1.2 (0 – 5.0)* | -2.3 (-102.5 - -0.2) | 1.2 (0 – 5.0) | 2.2 (0.4 – 12.0) | 1. 0 (-1.6 – 10.3) |
| Met | -- | -- | -- | -- | -- | -- |
| Tryp | 2.6 (0 – 33.0) | 1.3 (0 – 5.7) | -1.4 (-27.6 – 5.0) | 2.6 (0 – 9.6) | 5.4 (1.2 – 9.6) | 0.3 (-6.9 – 7.4) |
| Phe | 5.2 (1.5 – 74.7) | 1.8 (0.8 – 5.5)* | -2.7 (-69.1 – 0.3) | 2.1 (1.3 – 6.8) | 3.2 (1.1 – 9.7) | 1.1 (-5.7 – 7.9) |
| Ile | 4.9 (1.3 – 50.1) | 1.8 (0 – 8. 0) | -3.9 (-45.5 – 9.6) | 1.6 (0.4 – 2.1) | 1.1 (0 – 7.4) | 0.5 (-1.6 – 5.8) |
| Leu | 5.8 (0.9 – 45.2) | 1.5 (0.5 – 4.2)* | -3.7 (-41.0 – 0.1) | 1.4 (0.8 – 1.7) | 1.7 (0.7 – 8.3) | 0.8 (-0.9 – 6.8) |
| Lys | 5.9 (0.06 – 31.3) | 1.4 (0.5 – 3.6)* | -3.6 (-27.9 – 1.3) | 2.1 (0.5 – 3.3) | 1.5 (1.2 – 2.4) | 0.2 (-1.0 – 2. 0) |

**Table 3.20 Changes in muscle / plasma amino acid gradients (mmol/l) following six months without exercise with STD or XS bicarbonate therapy.** * Denotes significant change from baseline in each group (P<0.05). Values are presented as median and range.

| Amino Acid | STD Baseline | STD Six Months | STD Δ | XS Baseline | XS Six Months | XS Δ |
|---|---|---|---|---|---|---|
| Glu | 24.9 (16.5 – 61.6) | 36.6 (13.6 – 91.6) | 4.6 (-23.3 – 66.2) | 26.8 (24.3 – 31.0) | 48.6 (7.4 – 56.8) | 17.7 (-16.8 – 29.9) |
| Asn | 3.7 (2.6 – 8.7) | 4.6 (0.8 – 8.0) | -0.3 (-2.6 – 4.4) | 2.4 (1.4 – 4.5) | 3.9 (3.8 – 4.9) | 2.4 (-0.5 – 2.5) |
| Ser | 4.0 (1.9 – 6.2) | 3.9 (1.8 – 7.6) | -0.01 (-3.3 – 4.9) | 2.7 (1.6 – 4.0) | 3.4 (0.6 – 3.8) | -0.2 (-1.0 – 0.7) |
| Gln | 13.1 (2.8 – 16.3) | 12.0 (0.9 – 32.8) | 1.1 (-16.3 – 30.7) | 11.1 (5.7 – 19.0) | 16.9 (0.4 – 21.8) | -2. 0 (-5.3 – 10.7) |
| His | 2.3 (1.1 – 33.3) | 3.8 (1.2 – 38.0) | 0.8 (-5.7 – 23.8) | 1.7 (1.2 – 6.4) | 5.8 (1.1 – 5.9) | -0.5 (-0.6 – 4.6) |
| Gly | 3.8 (3.1 – 9.3) | 4.4 (0.9 – 10.6) | -0.8 (-3.0 – 6.9) | 2.5 (1.3 – 4.7) | 2.9 (0.6 – 3.7) | -0.7 (-1.7 – 1.1) |
| Thr | 3.2 (1.1 – 7.8) | 2.2 (0.9 – 7.3) | -1.1 (-5.2 – 4.8) | 1.2 (0.7 – 2.3) | 2.0 (0.4 – 2.1) | -0.1(-0.3 – 0.8) |
| Arg | 12.3 (1.8 – 22.6) | 6.8 (1.8 – 33.8) | -2.4 (-16.1 – 25.4) | 3.4 (3.0 – 7.6) | 4.9 (0.9 – 5.5) | -2. 0 (-2.1 – 1.4) |
| Ala | 0.14 (0.05 – 0.56) | 0.18 (0.05 – 2.43) | -0.01 (-0.4 – 2.3) | 0.3 (0.1 – 0.8) | 0.18 (0.05 – 0.29) | 0.01 (-0.05 – 2.2) |
| Tyr | 1.6 (0.7 – 23.0) | 3.9 (1.1 – 12.8) | 2. 0 (-19.5 – 7.4) | 0.5 (0.5 – 9.3) | 1.3 (0.3 – 1.5) | -0.3 (-7.9 – 0.9) |
| Cys | 1.3 (0 – 11.4) | 1.7 (0 – 21.0) | -0.06 (-8.62 – 19.54) | 0.7 (0.7 – 1.4) | 3.2 (2.7 – 5.3) | 2. 0 (1.8 – 4.6) |
| Val | 2.1 (1.6 – 6.8) | 2.8 (1.9 – 8.1) | 0.06 (-3.70 – 3.85) | 1.2 (0.7 – 1.8) | 1.3 (0.7 – 1.8) | 0.01 (-0.05 – 0.06) |
| Met | -- | -- | -- | -- | -- | -- |
| Tryp | 10.3 (4.3 – 38.8) | 12.6 (0 – 36.5) | 0.9 (-8.5 – 31.9) | 4.1 (2.6 – 5.4) | 4.7 (2.4 – 5.4) | -0.1 (-0.6 – 0.8) |
| Phe | 1.6 (1.0 – 7.8) | 3.5 (1.4 – 28.8) | 1.9 (-4.5 – 26.7) | 1.6 (0.9 – 2.3) | 1.7 (1.1 – 1.9) | -0.2 (-0.3 – 0.3) |
| Ile | 4.1 (1.4 – 10.7) | 6.5 (1.6 – 13.3) | 0.3 (-2.6 – 11.5) | 1.7 (0.9 – 2.0) | 1.7 (1.1 – 1.9) | -0.01 (-0.07 – 0.2) |
| Leu | 2.2 (1.4 – 5.6) | 2.1 (1.1 – 6.5) | -0.4 (-2.7 – 3.4) | 0.9 (0.5 – 3.2) | 1.2 (1.0 – 1.7) | 0.6 (-2.2 – 0.7) |
| Lys | 2.6 (1.0 – 6.1) | 3.4 (0.8 – 13.5) | 1.4 (-3.7 – 5.7) | 1.2 (0.4 – 2.9) | 1.9 (0.1 – 4.8) | -0.2 (-0.9 – 3.5) |
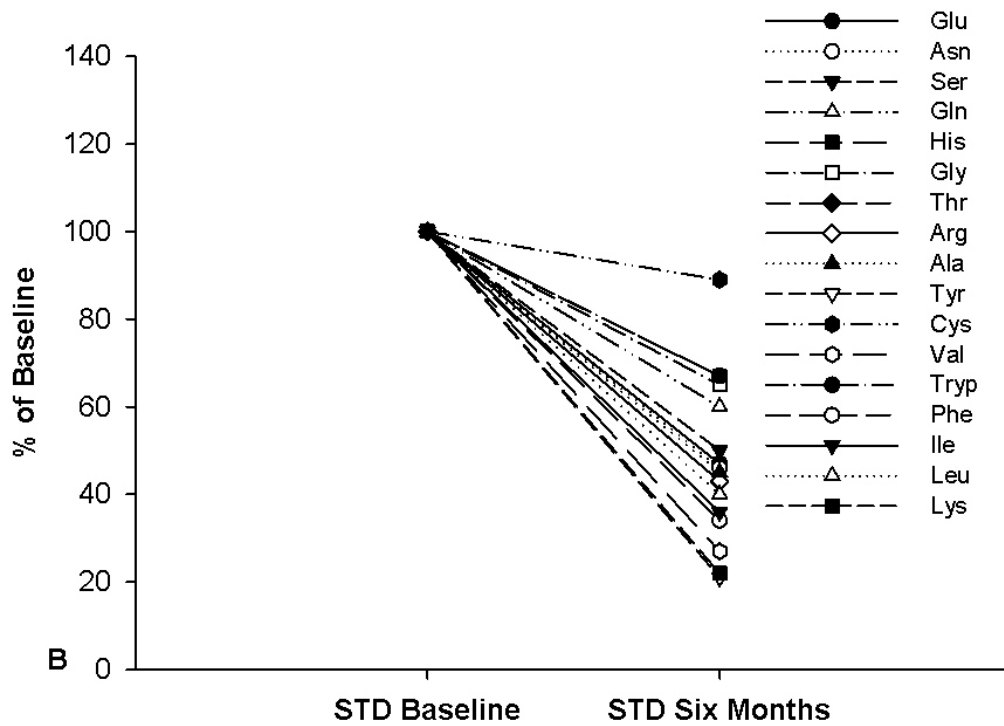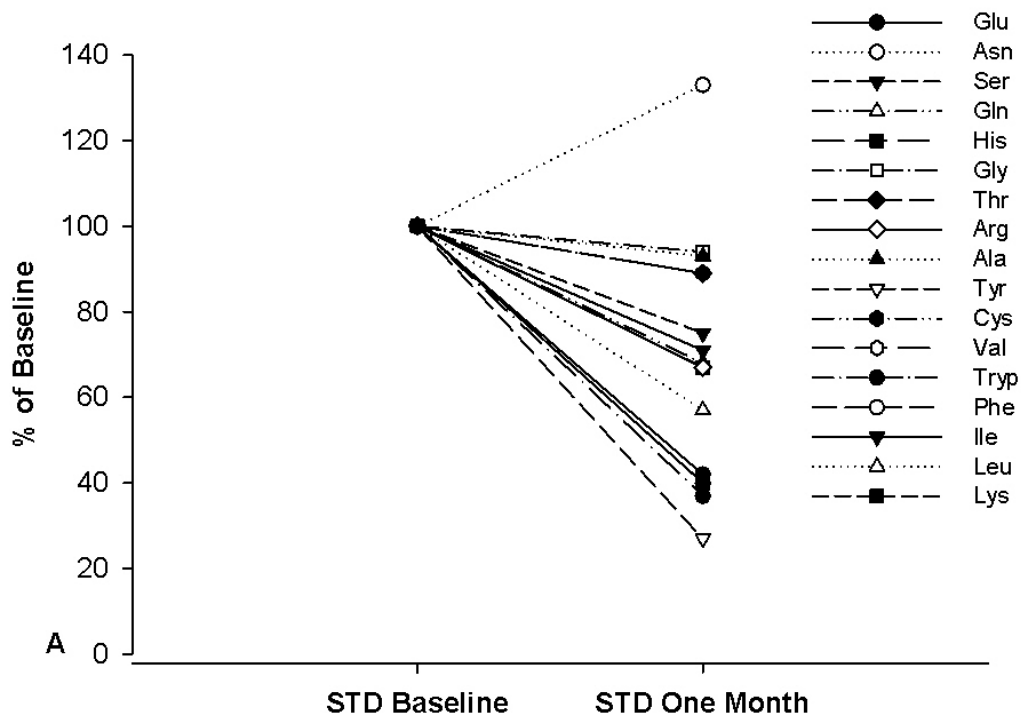
**Figure 3.10. Summary of changes in muscle amino acid concentration from baseline following one month (A) or six months (B) of exercise with STD bicarbonate therapy.** Values are calculated from median concentrations.
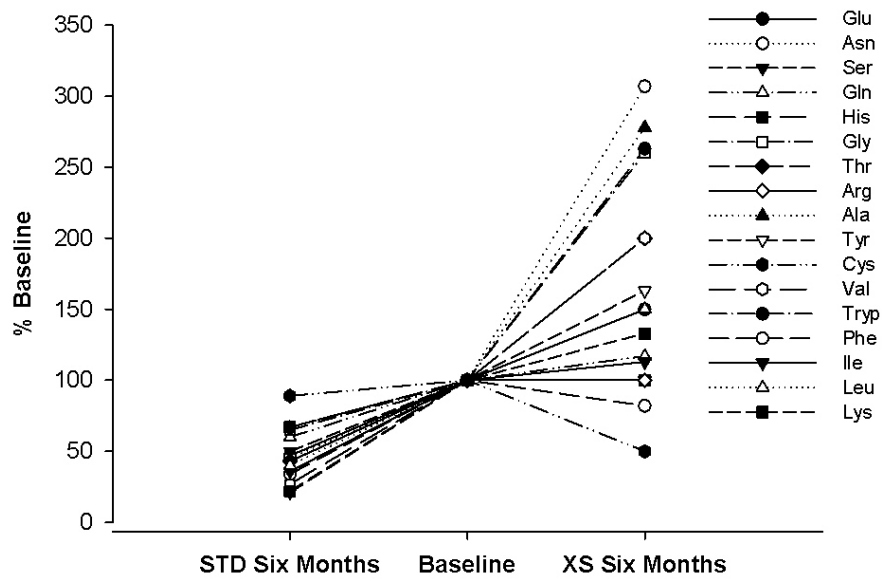
**Figure 3.11. Summary of changes in muscle amino acid concentrations from baseline following six months of exercise with STD or XS bicarbonate therapy.** Values are calculated from median concentrations
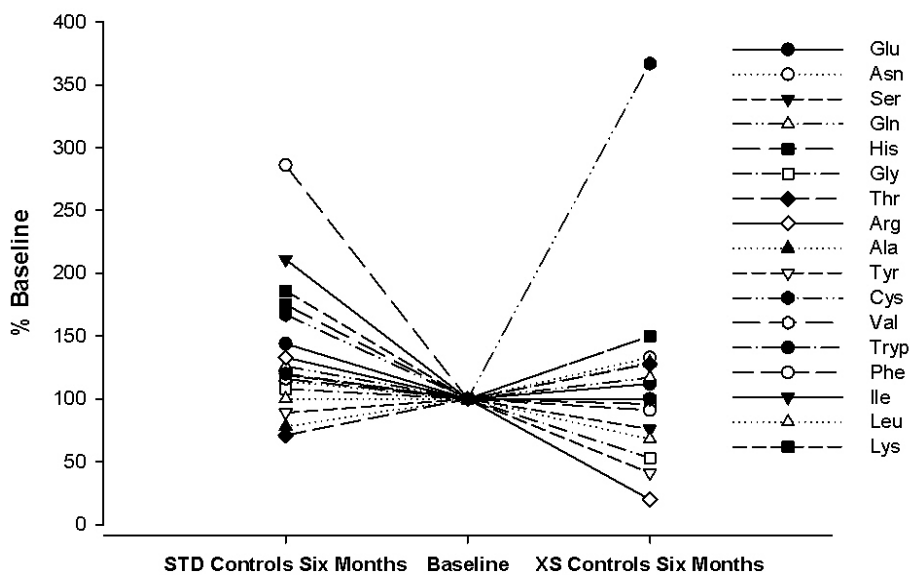


**Figure 3.12. Summary of changes in muscle amino acid concentrations from baseline following six months without exercise with STD or XS bicarbonate therapy.** Values are calculated from median concentrations.

### 3.3.8 Dietary intake

In principle, changes in diet may have contributed to the changes in amino acid concentrations described above. The effect of exercise and bicarbonate on dietary intake was assessed by diet diaries over three days at each time point. Only two patients from the six month exercise group receiving XS bicarbonate and from the non exercise group receiving XS bicarbonate completed and returned these diaries so data on bicarbonate will not be presented.

**Table 3.21. The effect of one month of exercise on dietary intake.**

| | Baseline | | | | One Month | | | |
|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/kg bw) | CHO (g/kg bw) | Protein (g/kg bw) | Fat (g/kg bw) | Energy (kcal/kg bw) | CHO (g/kg bw) | Protein (g/kg bw) | Fat (g/kg bw) |
| Exercise | 23.2 ± 4.2 | 3.0 ± 0.2 | 0.90 ± 0.07 | 0.9 ± 0.1 | 24.5 ± 4.2 | 3.0 ± 0.3 | 0.92 ± 0.07 | 1.0 ± 0.1 |

**Table 3.22. The effect of six months of exercise or without exercise on dietary intake.**
\* denotes significant change from corresponding value at baseline.

| | Baseline | | | | Six Months | | | |
|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/kg bw) | CHO (g/kg bw) | Protein (g/kg bw) | Fat (g/kg bw) | Energy (kcal/kg bw) | CHO (g/kg bw) | Protein (g/kg bw) | Fat (g/kg bw) |
| Exercise | 24.5 ± 3.2 | 3.1 ± 0.3 | 0.97 ± 0.10 | 1.0 ± 0.2 | 19.2 ± 1.2 | 2.5 ± 1.2 | 0.76 ± 0.03* | 0.7 ± 0.1 |
| Controls | 26.6 ± 5.5 | 3.2 ± 0.8 | 1.06 ± 0.15 | 1.0 ± 0.2 | 22.0 ± 1.0 | 2.7 ± 0.6 | 0.84 ± 0.15* | 0.8 ± 0.1 |

Energy intake did not change following one month of exercise (Table 3.21), but was seen to decrease after six months of exercise and a no exercise control period, but this failed to reach significance in both groups ($P = 0.078$ and $P = 0.094$ respectively) (Table 3.22). There was also no change in the carbohydrate (CHO) intake following one month of exercise ($P = 0.800$) or in the non-exercising control group ($P = 0.264$). CHO intake fell after six months of exercise, but

this was not significant (P = 0.084). Protein intake did not change following one month of exercise (P = 0.973), but was seen to fall significantly after six months of both exercise and no exercise (P = 0.028 and P = 0.030 respectively).


### 3.3.9 SNAT2 Expression

Even though the effects on free amino acid concentrations and muscle/plasma concentration gradients described above imply changes in active amino acid transport in muscle, there was no effect of six months of exercise or bicarbonate treatment on the expression of the SNAT2 transporter at the mRNA level (Figure 3.13). The apparent small decrease following one month of exercise with STD bicarbonate was not statistically significant (P = 0.532) (Figure 3.13 inset), nor was the apparent effect of XS bicarbonate in non-exercising subjects (P = 0.742) (Figure 3.13).
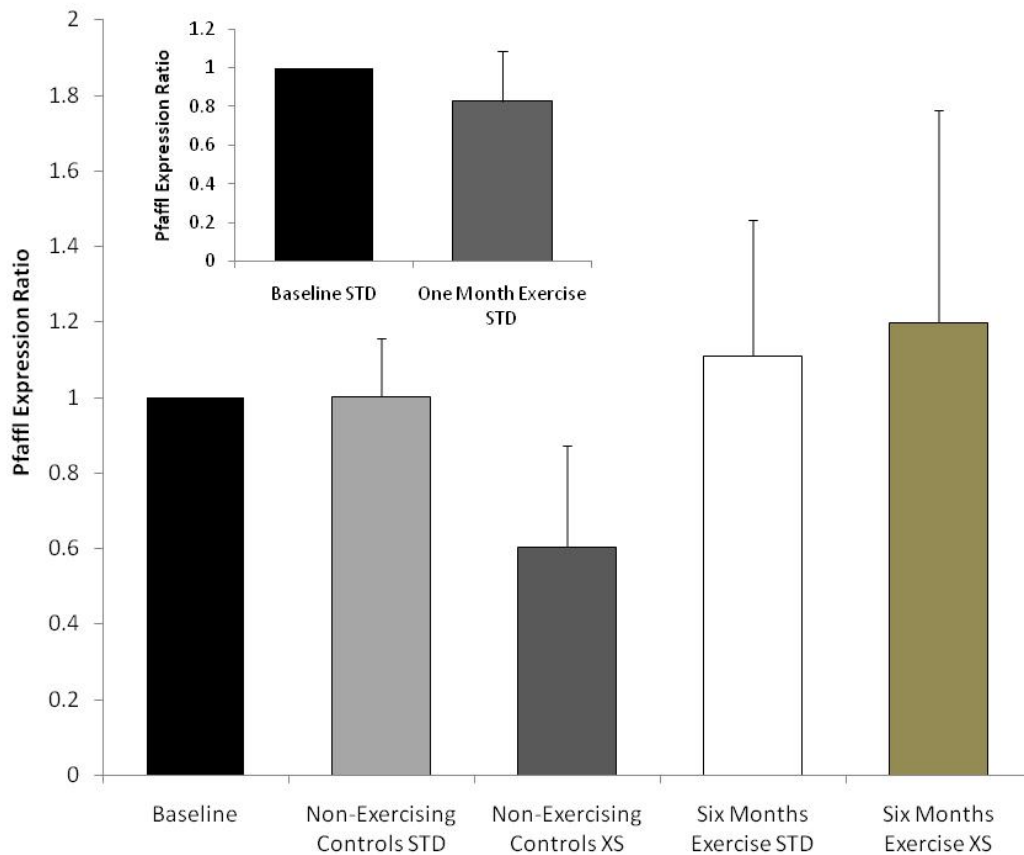
**Figure 3.13. Changes in the expression of the SNAT2 amino acid transporter following six months with or without exercise with STD or XS bicarbonate therapy.** Inset graph displays changes in SNAT2 expression following one month of exercise with STD bicarbonate therapy. Data are presented here as the Pfaffl Expression Ratio (See Chapter 2.6.7) in which, by definition, the baseline value is 1.0.

### 3.3.10 Protein phosphorylation signals

In agreement with earlier studies of the extent of rpS6 phosphorylation in the muscle of healthy fasted subjects (Dreyer et al. 2008), considerable difficulties were experienced in the detection of phosphorylated rpS6 in these wasted patients in the fasted and rested state. The negligible P-rpS6 signal observed was not significantly affected by exercise or alkali treatment even in those subjects whose biopsies gave a quantifiable signal at baseline (Figures 3.14 and 3.15).
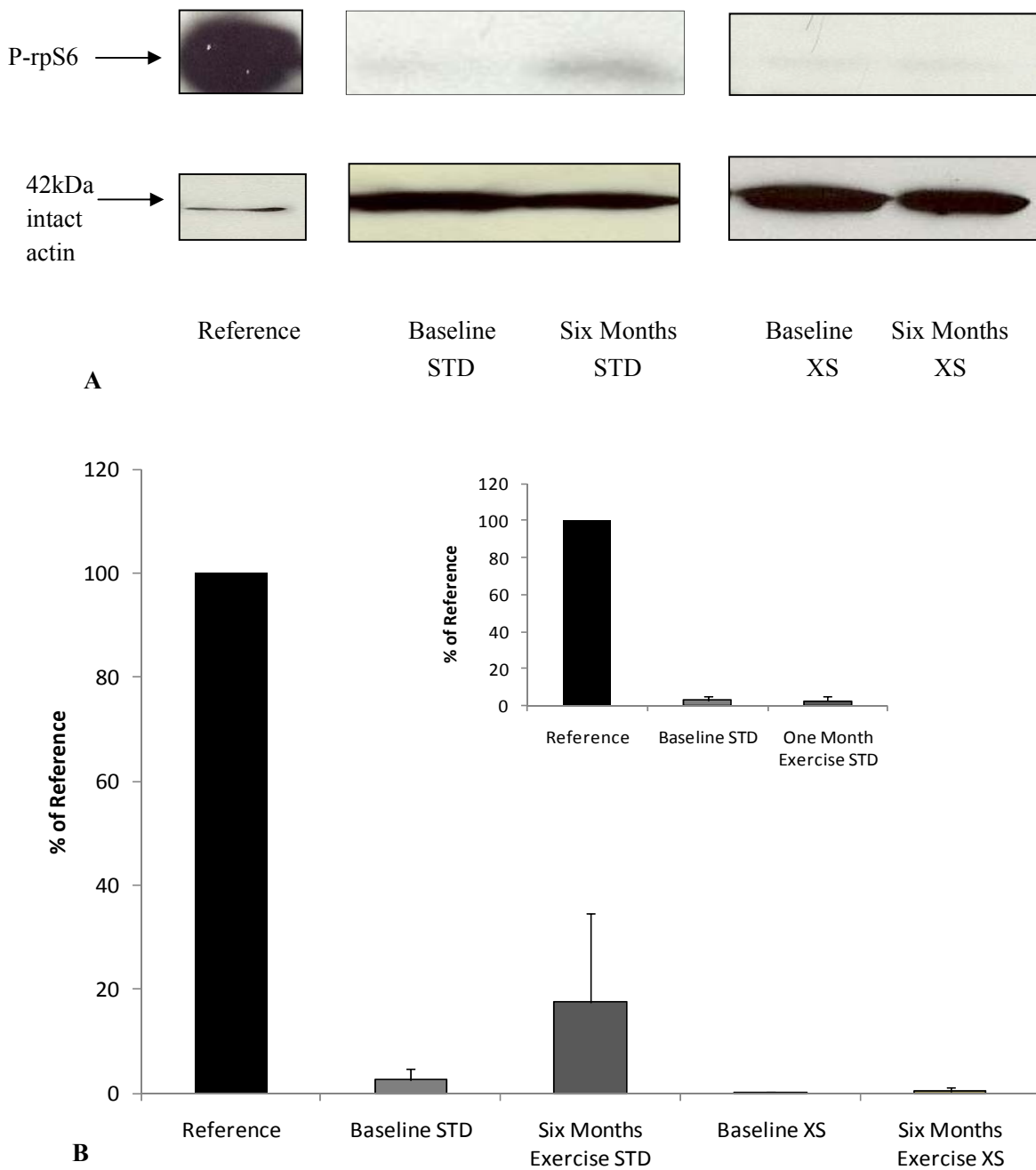
**Figure 3.14. The effect of six months of exercise with STD or XS bicarbonate therapy on rpS6 phosphorylation.** (A) Representative immunoblot showing rpS6 phosphorylation at Ser 235/236. (B) Quantification by densitometry of pooled data from those patients who exercised for six months with STD or XS bicarbonate therapy. Inset shows the quantification by densitometry of the pooled data from those patients who exercised for one month with STD bicarbonate therapy. As a reference against which the patients' samples were expressed a sample of a lysate from insulin-stimulated L6-G8C5 cells was run in parallel with the patients' lysates on every blot.
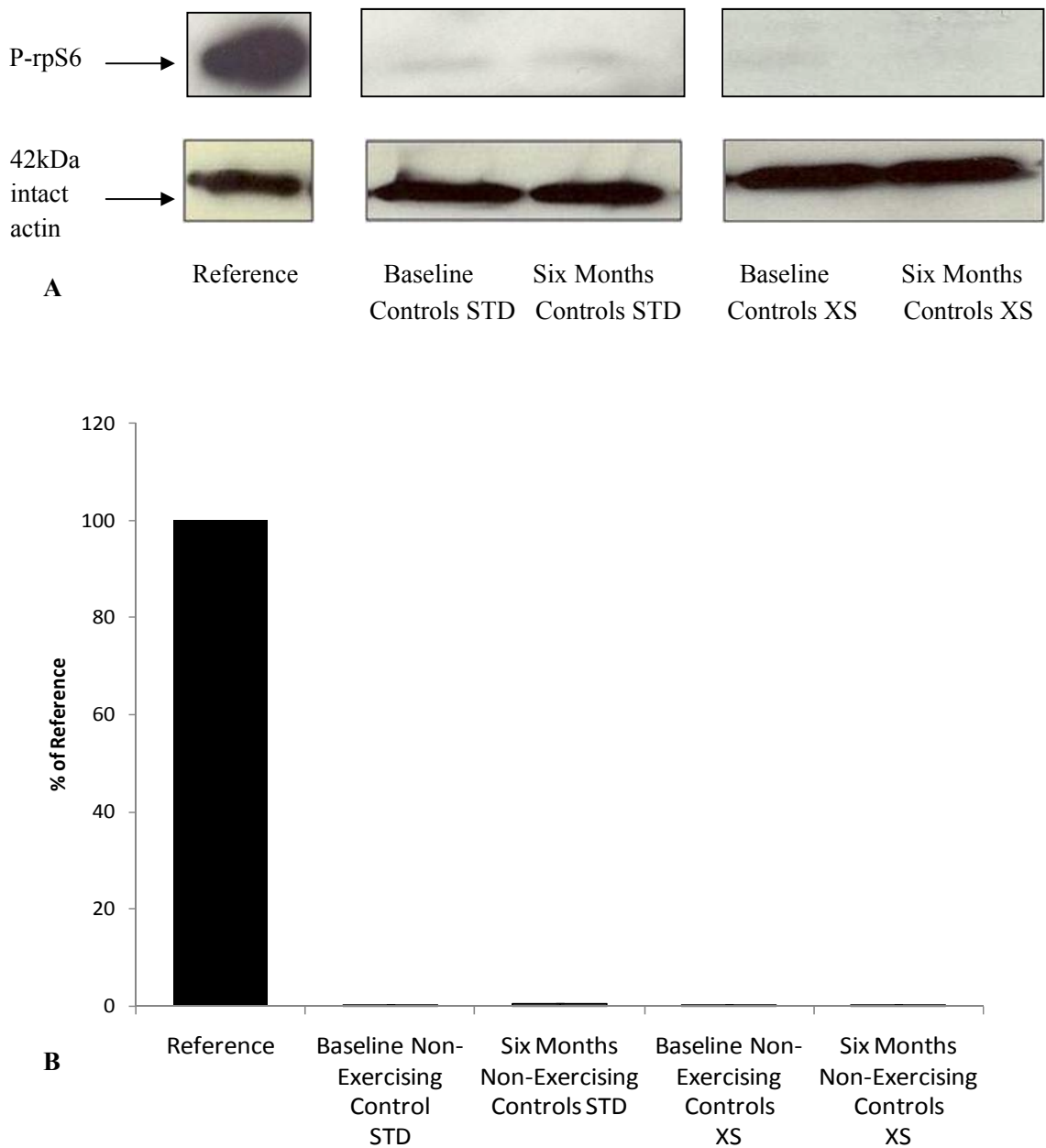
**Figure 3.15. The effect of six months without exercise with STD or XS bicarbonate therapy on rpS6 phosphorylation on the Ser 235/236 residue.** (A) Representative immunoblot showing rpS6 phosphorylation at Ser 235/236. (B) Quantification by densitometry of pooled data from non-exercising controls with STD or XS bicarbonate therapy. As a reference against which the patients' samples were expressed a sample of a lysate from insulin-stimulated L6-G8C5 cells was run in parallel with the patients' lysates on every blot.

Owing to the problems encountered in detecting a P-rpS6 signal, a random sample of biopsies from all patient groups was selected and probed for two further downstream targets of mTOR,

i.e. phosphorylated eukaryotic initiation factor 4E binding protein 1 (P-4E-BP1) and phosphorylated $P^{70}S6$ Kinase (P-$P^{70}$S6K). As with P-rpS6, the P-4E-BP1 and P-$P^{70}$S6K signals were essentially undetectable in these biopsies and were not affected by exercise training or alkali therapy (Figure 3.16).
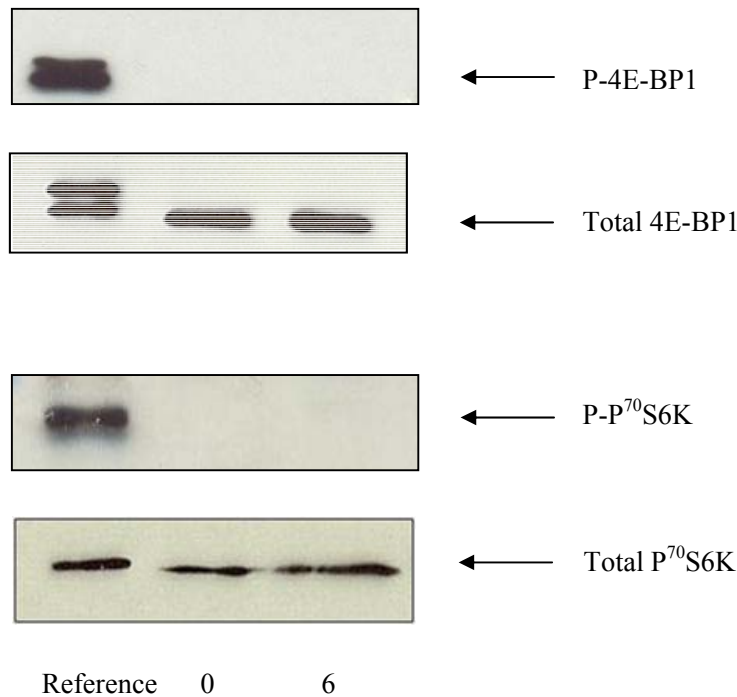


**Figure 3.16. Representative immunblots of 4E-BP1 and $P^{70}$S6K from a non-exercising control patient on STD bicarbonate therapy.** Due to the negligible signal, immunostaining has not been quantified. The reference sample is the sample of lysate from insulin stimulated L6-G8C5 cells presented in Figures 3.13 and 3.14. "0" denotes baseline and "6" six months.

In contrast to the mTOR signals, a readily detectable PKB phosphorylation signal was seen in the biopsies, but no statistically significant changes were detected after one or six months of exercise with STD or XS bicarbonate therapy (Figure 3.17).
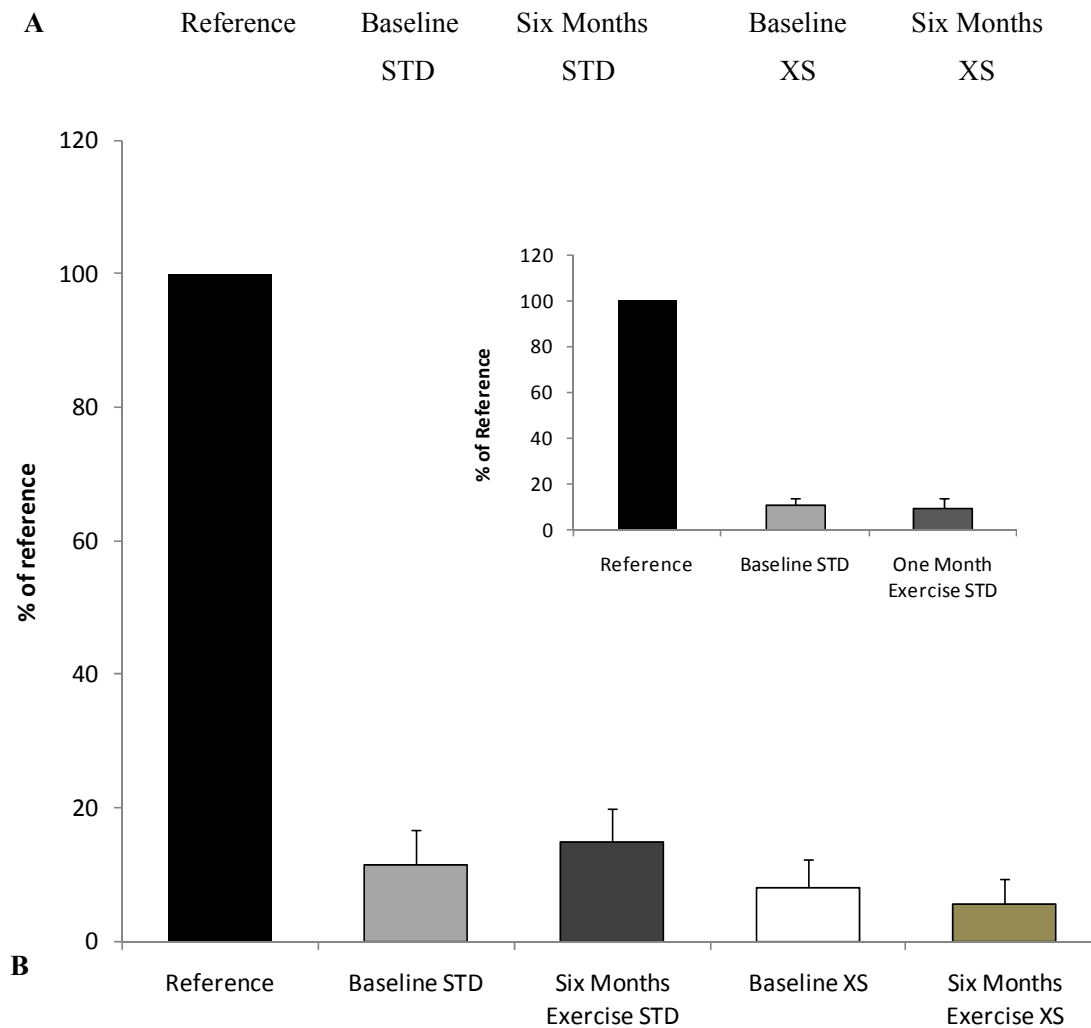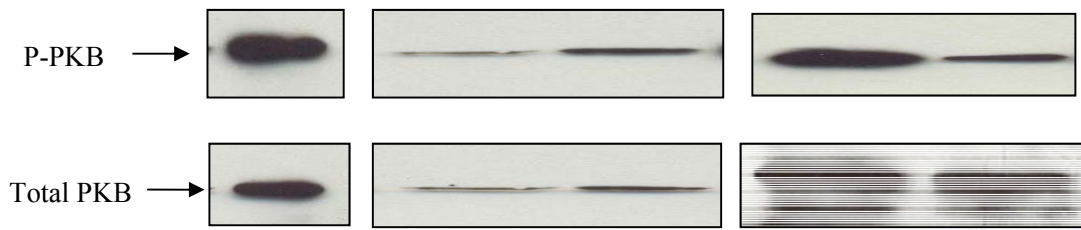
**Figure 3.17. The effect of six months of exercise with STD or XS bicarbonate therapy on PKB phosphorylation on the Ser 473 residue.** (A) Representative immunoblot showing PKB activation at Ser 473. (B) Quantification by densitometry of pooled data from those patients who exercised for six months with STD or XS bicarbonate therapy. Inset shows the quantification by densitometry of the pooled data from those patients who exercised for one month with STD bicarbonate therapy. As a reference against which the patients' samples were expressed a sample of a lysate from insulin-stimulated L6-G8C5 cells was run in parallel with the patients' lysates on every blot.
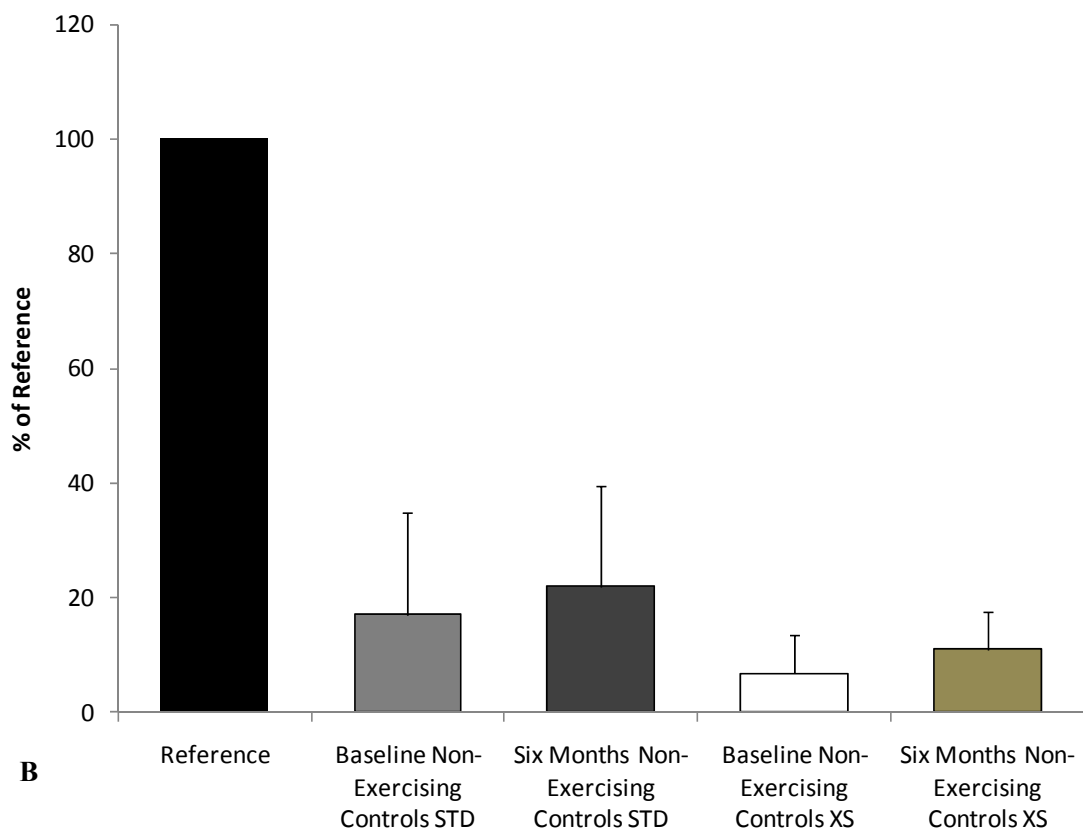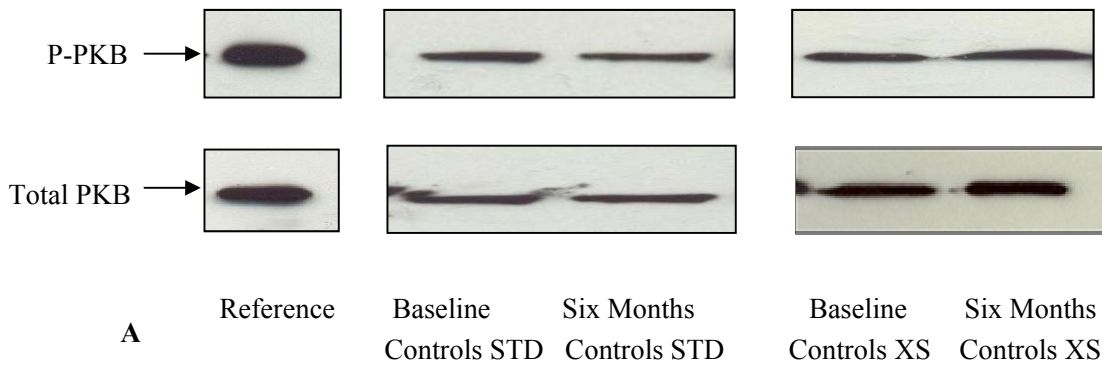
**Figure 3.18. The effect of six months without exercise with STD or XS bicarbonate therapy on PKB phosphorylation on the Ser 473 residue.** (A) Representative immunoblot showing PKB activation at Ser 473. (B) Quantification by densitometry of pooled data from non-exercising controls with STD or XS bicarbonate therapy. As a reference against which the patients' samples were expressed a sample of a lysate from insulin-stimulated L6-G8C5 cells was run in parallel with the patients' lysates on every blot.

### 3.3.11 The 14kDa Actin Fragment

Downstream from PKB, cleavage of actomyosin by caspase-3 to generate the 14kDa proteolytoic fragment was measured as an index of the early steps in myofibrillar protein catabolism (Du et al. 2004; Workeneh et al. 2006) (Figures 3.19 – 3.22). One month or six months of exercise resulted in no statistically significant change in the levels of the 14kDa fragment. Following six months of exercise the apparent decline from baseline in those patients taking STD bicarbonate was not statistically significant (P = 0.096) (Figure 3.21). There was also no detectable change in the non-exercising groups (Figure 3.22).
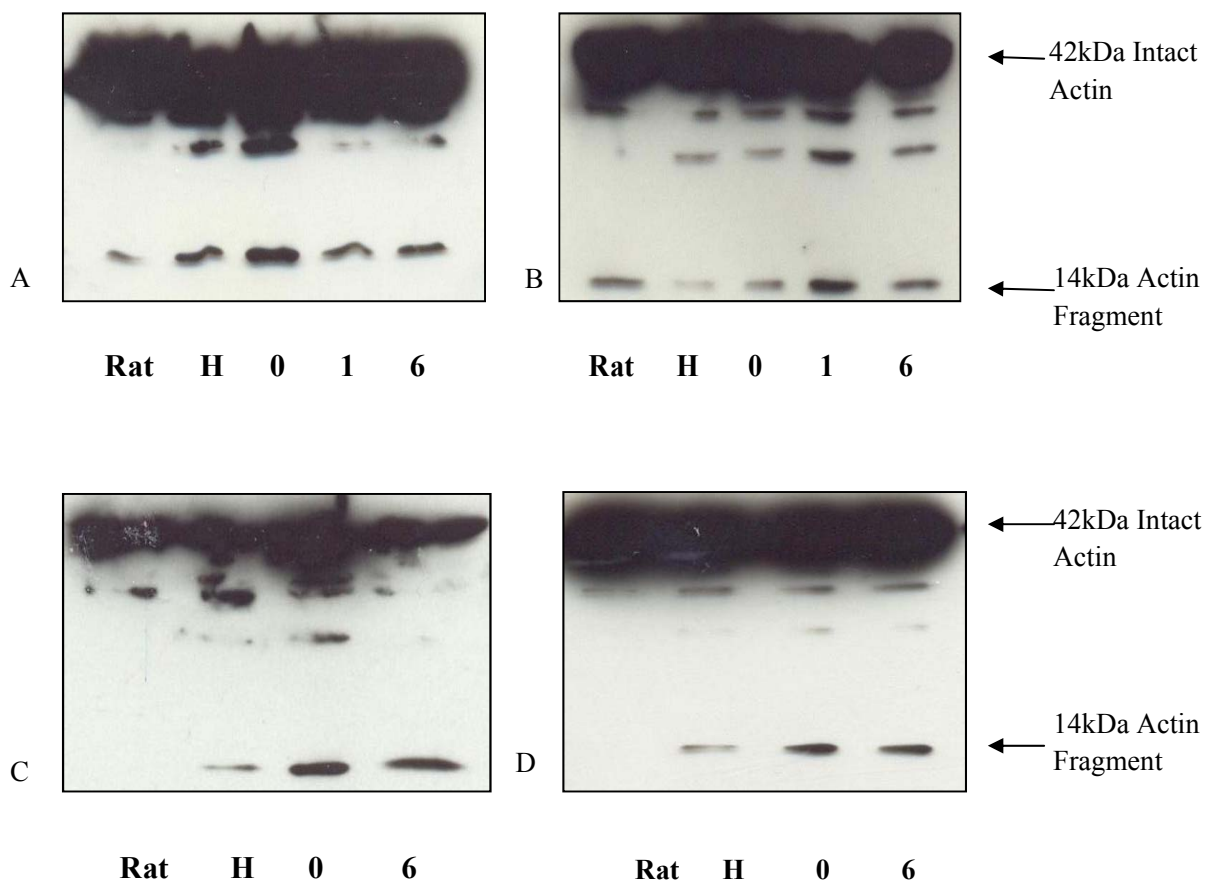


**Figure 3.19. Representative immunoblots to demonstrate the effect of one and six months of exercise with STD (A) or XS (B) bicarbonate therapy and six months without exercise with STD (C) or XS (D) bicarbonate therapy on the level of the 14kDa actin fragment.** Muscle specimens obtained from healthy rat and from a healthy volunteer "H" were run on every blot for comparison with the CKD Patients. "0" denotes baseline, "1" one month and "6" six months.
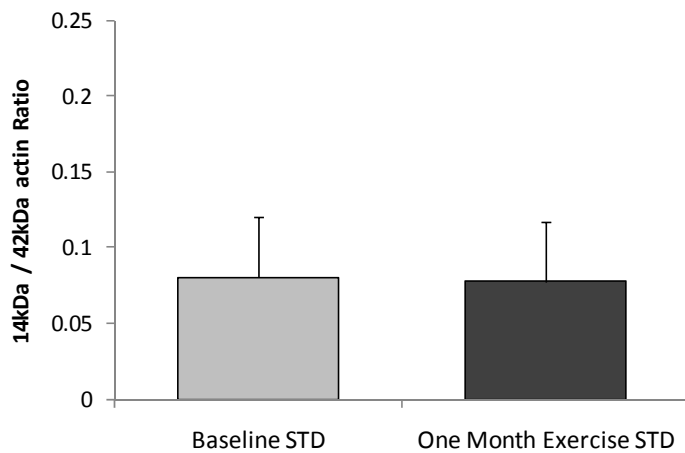
**Figure 3.20. Bar graph denoting pooled quantification by densitometry of the effect of one month of exercise with STD bicarbonate therapy on the level of the 14kDa actin fragment.** Two exposure times were used when developing these results, approximately 10 seconds for 42kDa intact actin and a longer exposure of up to 10 minutes for 14kDa actin fragment (Figure 3.18). These pairs of films were used to quantify 14kDa/42kDa ratio in Figures 3.19 – 3.21).
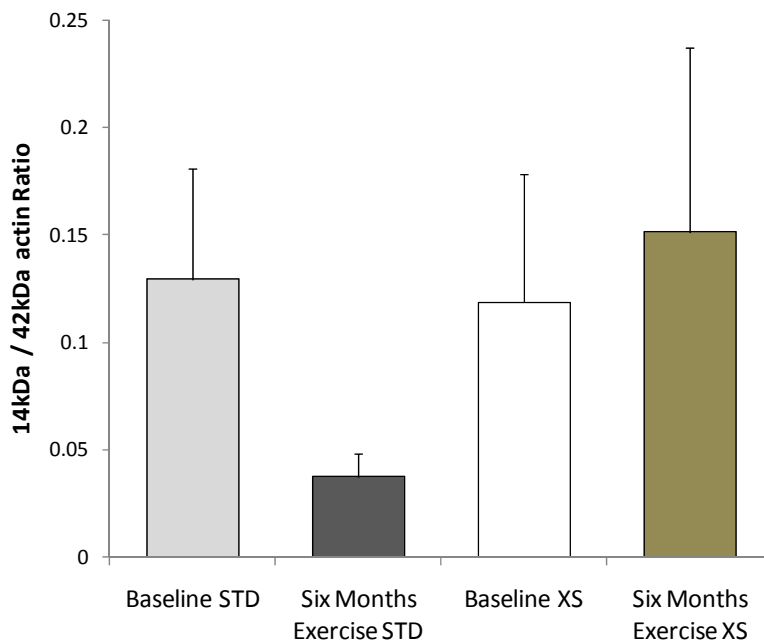


**Figure 3.21. Bar graph denoting pooled quantification by densitometry of the effect of six months of exericse with STD or XS bicarbonate therapy on the level of the 14kDa actin fragment.**
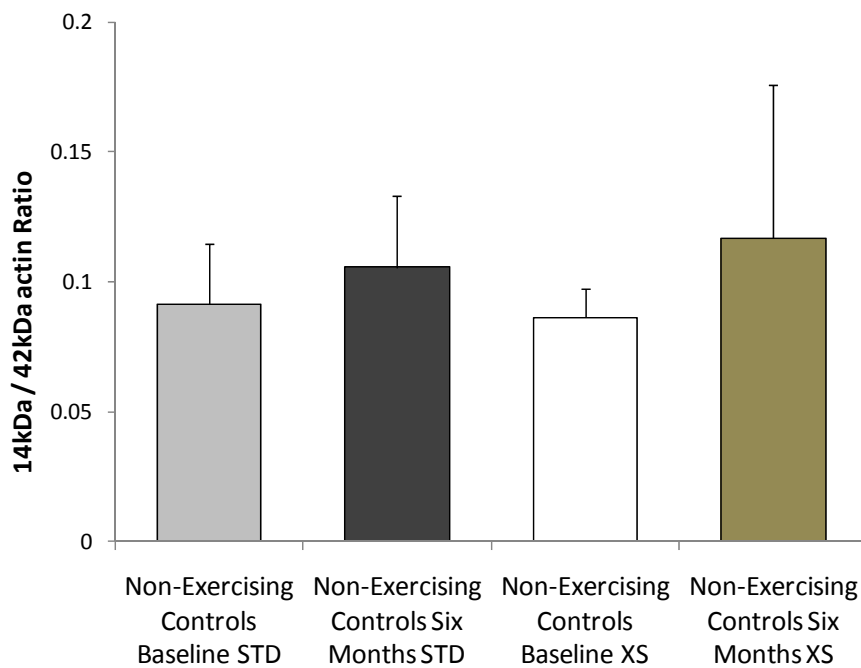
**Figure 3.22. Bar graph denoting pooled quantification of the effect of six months without exercise with STD or XS bicarbonate therapy on the level of the 14kDa actin fragment.**

### 3. 3.12 Ubiquitin E3 ligase mRNA expression

In contrast to the 14kDa actin data, significant changes were observed in the mRNA expression of MuRF-1 and MAFbx. In the one month pilot study, patients receiving exercise therapy with STD bicarbonate treatment showed a detectable decrease in the expression of both of these enzymes (Figures 3.23 and 3.24). In the main six month study, this decrease was only sustained in the exercising patients who also received XS bicarbonate therapy.

One month of exericse with STD bicarbonate therapy resulted in a statistically signifcant reduction in the mRNA expression of both MuRF-1 (P = 0.045) and MAFbx (P = 0.025). Six months of exercise with XS bicarbonate therapy resulted in a decrease in the mRNA expression of MuRF-1 to 41 ± 18% (P = 0.005) and MAFbx to 46 ± 18% (P = 0.04) of baseline, whilst no change was seen in the mRNA expression of either enzyme in the STD bicarbonate group (P = 0.768 and P = 0.664 respectively). Furthermore, the mRNA expression of MuRF-1 was

significantly lower in those patients on XS bicarbonate compared to those on STD bicarbonate treatment (P = 0.049). No such change was observed in the mRNA expression of MuRF-1 or MAFbx in either of the non-exercising control groups, indeed there was a tendency for MAFbx mRNA expression to increase in those non-exercising control patients receiving STD bicarbonate therapy (Figures 3.23 and 3.24).
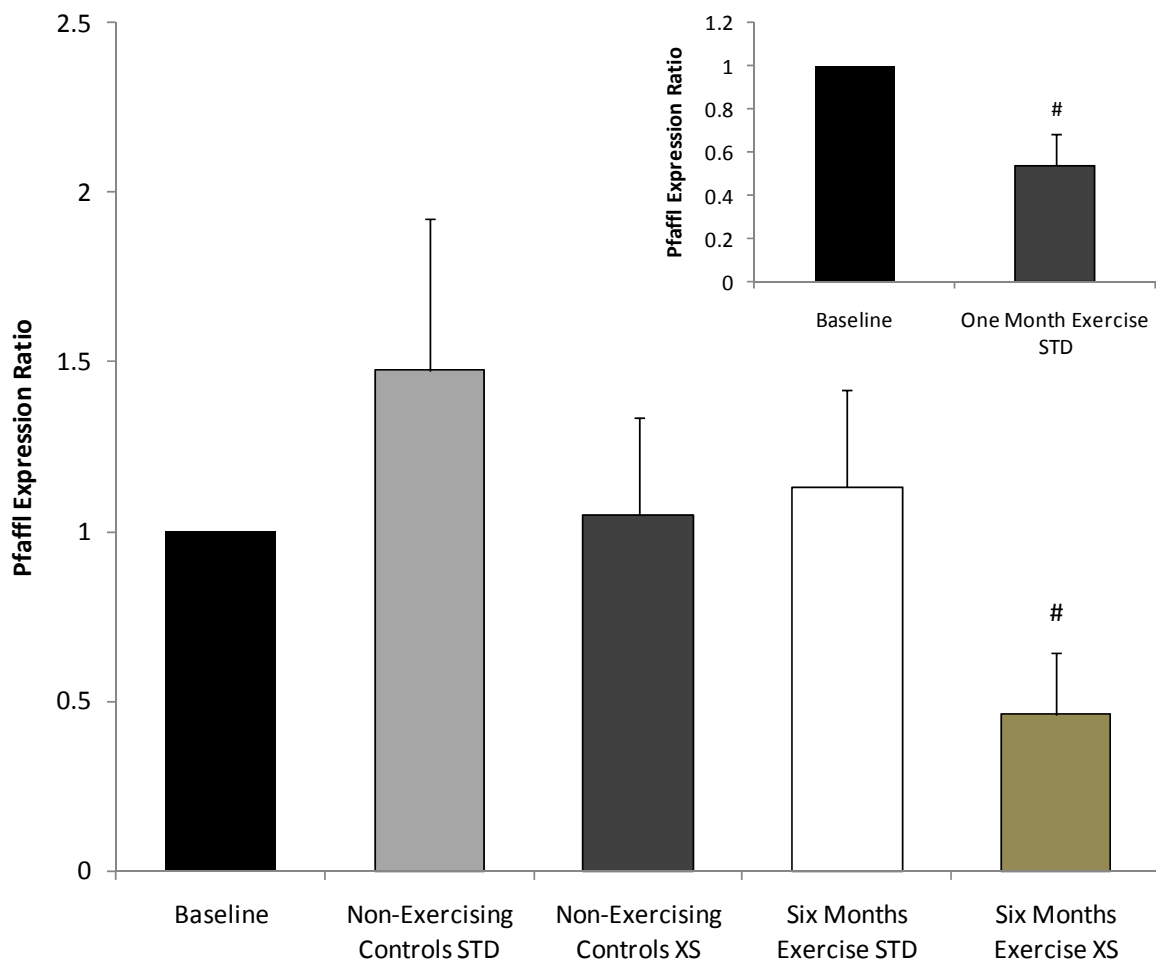


**Figure 3.23. Change in MAFbx mRNA expression with or without exercise and bicarbonate therapy.** Graph inset shows change in MAFbx mRNA expression following one month of exercise with STD bicarbonate therapy. # denotes significant difference from baseline (P<0.05). Data are presented as the Pfaffl Ratio (See Chapter 2.6.7) in which, by definition, the baseline value is 1.0.
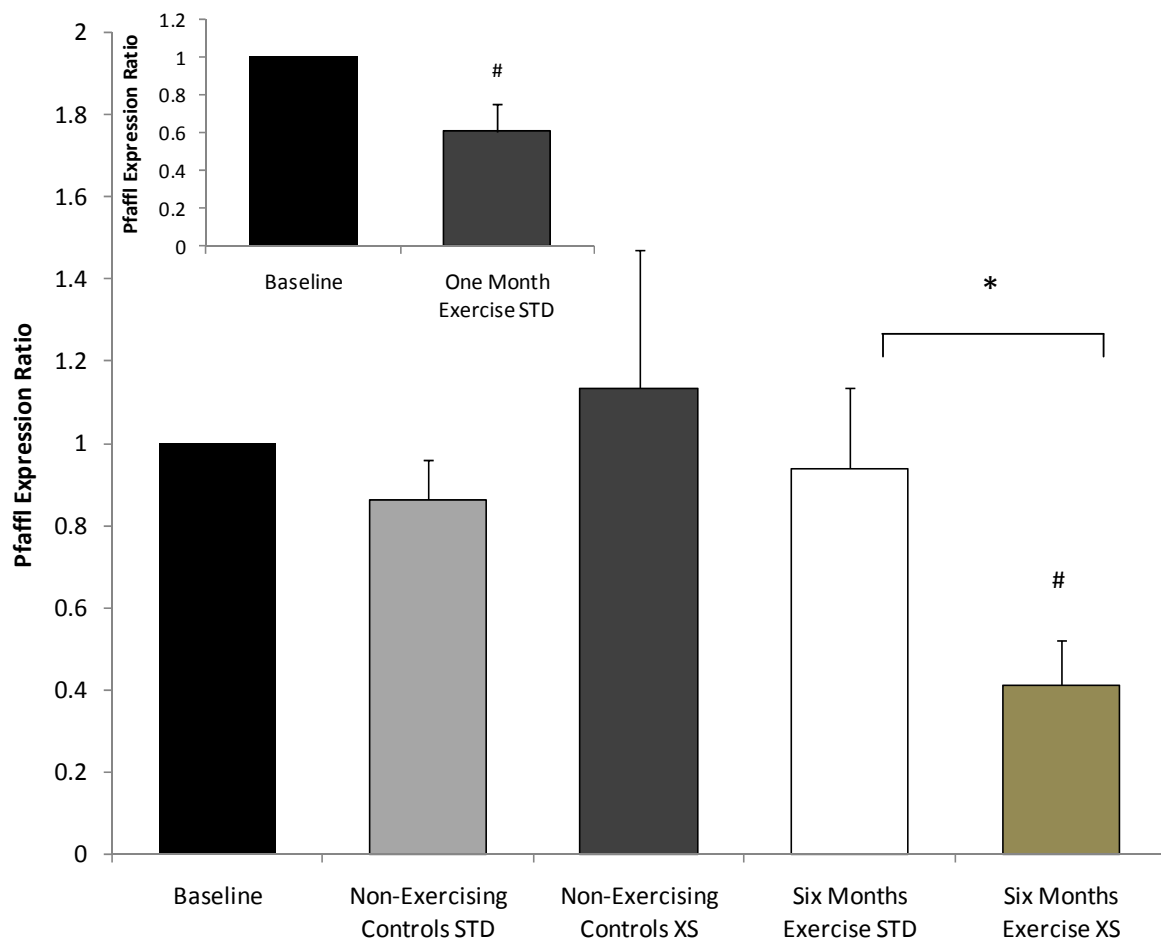
**Figure 3.24. Change in MuRF-1 mRNA expression with or without exercise and bicarbonate therapy.** Graph inset shows change in mRNA MuRF-1 mRNA expression following one month of exercise with STD bicarbonate therapy here # denotes significant difference from baseline ($P<0.05$). In main graph # denotes significant difference from corresponding baseline ($P<0.05$). * denotes significant difference between bicarbonate groups following six months of exercise ($P<0.05$). Data are presented as the Pfaffl Ratio (See Chapter 2.6.7) in which, by definition, the baseline value is 1.0.

### 3.3.13 3-Methyl Histidine (3-MH) excretion

Excretion of 3-MH was measured as a marker of the overall rate of myofibrillar protein degradation, to assess the net effect of the apparent changes that had been examined by measuring the 14kDa actin fragment and E3 ligase mRNA expression. Overall, there was no

change in the rate of myofibrillar protein degradation with or without exercise or additional bicarbonate treatment (Tables 3.23 – 3.24). This was true when the data were corrected for variation in lean body mass by calculating the 3-MH/creatinine excretion as is conventional (Elia et al. 1981), or by expressing 3-MH in relation to lean body mass measured directly using DEXA. Excretion of 3-MH apparently showed a paradoxical increase above baseline after one month of exercise on STD bicarbonate therapy when corrected for creatinine (Table 3.23), indicating an increase in myofibriliar degradation and a worsening of their muscle wasting, but this change was not statistically significant ($P = 0.138$).

**Table 3.23. 3-MH excretion rates corrected for creatinine or for lean body mass (LBM) (DEXA), following one month of exercise with STD bicarbonate therapy.**

|  | Baseline STD | One month Exercise STD |
|---|---|---|
| 3-MH/Creatinine (μmol/mmol crnn/24h) | 33.9 ± 4.6 | 46.9 ± 8.8 |
| 3-MH/LBM (μmol/kg LBM/24h) | 10.4 ± 2.2 | 9.9 ± 1.9 |

**Table 3.24. 3-MH excretion rates corrected for creatinine or for lean body mass (LBM) (DEXA) following six months of exercise with STD or XS bicarbonate therapy.**

|  | Baseline STD | Six Months Exercise STD | Baseline XS | Six Months Exercise XS |
|---|---|---|---|---|
| 3-MH/Creatinine (μmol/mmol crnn/24h) | 33.9 ± 5.9 | 38.8 ± 8.1 | 32.0 ± 8.9 | 35.8 ± 8.0 |
| 3-MH/LBM (μmol/kg LBM/24h) | 10.6 ± 2.6 | 9.4 ± 2.0 | 9.2 ± 1.2 | 12.3 ± 2.7 |

**Table 3.25. 3-MH excretion rates corrected for creatinine or for lean body mass (LBM) (DEXA) following six months without exercise with STD or XS bicarbonate therapy.**

|  | Baseline STD | Six Months STD | Baseline XS | Six Months XS |
|---|---|---|---|---|
| 3-MH/Creatinine (µmol/mmol crnn/24h) | $37.5 \pm 6.9$ | $29.0 \pm 3.9$ | $47.2 \pm 13.9$ | $60.8 \pm 10.1$ |
| 3-MH/LBM (µmol/kg LBM/24h) | $8.6 \pm 1.7$ | $9.0 \pm 1.4$ | $12.4 \pm 4.1$ | $16.3 \pm 3.9$ |

### 3.3.14 Summary

**Table 3.26. Summary of the principal effects observed in this study.** ND denotes not determined

| Parameter | One Month Ex STD | One Month Ex XS | Six Months Ex STD | Six Months Ex XS | Figure / Table |
|---|---|---|---|---|---|
| Exercise Tolerance | ↑ | ↑ | ↑ | ↑ | Figures 3.2 and 3.3 |
| Lactate Response to Exercise | ↔ | ↓ | ↔ | ↓ | Tables 3.6 and 3.7 |
| Hb and Hct Response to Exercise | ↔ | ↔ | ↔ | ↔ | Tables 3.8 and 3.9 |
| Body Weight | ↔ | ↔ | ↔ | ↔ | Table 3.10 |
| BMI | ↔ | ↓ | ↔ | ↓ | Table 3.10 |
| LBM % Total Body Weight | ↑ | ↑ | ↔ | ↑ | Figure 3.7 |
| Fat Mass | ↓ | ↔ | ↔ | ↓ | Table 3.10 |
| Biopsy DNA Content | ND | ND | ↔ | ↔ | Table 3.11 |
| Biopsy RNA Content | ND | ND | ↑ | ↔ | Table 3.11 |
| Biopsy Total Protein | ND | ND | ↔ | ↓ | Table 3.11 |
| Overall Trend for Plasma Amino Acids | ↔ | ND | ↑ | ↔ | Tables 3.12 – 3.14 |
| Overall Trend for Muscle Amino Acids | ↔ | ND | ↓ | ↔ | Tables 3.15 – 3.17 |
| Muscle SNAT2 Expression | ↔ | ND | ↔ | ↔ | Figure 3.13 |
| P-PKB | ↔ | ND | ↔ | ↔ | Figure 3.17 |
| 14kDa | ↔ | ND | ↔ | ↔ | Figures 3.20 and 3.21 |
| MAFbx Expression | ↓ | ND | ↔ | ↓ | Figure 3.23 |
| MuRF-1 Expression | ↓ | ND | ↔ | ↓ | Figure 3.24 |
| 3-MH Excretion | ↔ | ↔ | ↔ | ↔ | Tables 3.23, 3.24 and 3.25 |

## 3.4 Discussion

The main results produced from this study were that exercise tolerance did improve following six months of walking exercise, an effect that was not seen in the non-exercising patients, and that XS sodium bicarbonate did appear to provide the exercisers with some additional benefits. These patients were seen to have larger increases in LBM, greater reductions in the mRNA expression of MuRF-1 compared to those who remained on their standard bicarbonate dose, and were able to maintain amino acid concentration gradients between the plasma and muscle, and therefore were also able to maintain intramuscular amino acid concentrations, which might have significant implications for muscle protein synthesis rates.

### 3.4.1 Exercise tolerance

These results confirm previous reports that exercise tolerance and capacity can be improved in CKD patients (Painter et al. 1986; Koudi et al. 1997; Molsted et al. 2004). One and six months of aerobic exercise training significantly improved the patients' ability to exercise. A small improvement was seen in the exercise tolerance of the non-exercising patients who received STD bicarbonate therapy: this may have arisen through a psychological effect of participating in an exercise training study, or through improper familiarisation with the RPE scale. However, no improvement was observed in the non-exercising group receiving XS bicarbonate in which similar psychological factors could also have led to an improvement, but none was observed. It is possible therefore, that in sedentary CKD patients, XS bicarbonate worsens their exercise tolerance, but there was no clear evidence for such a detrimental effect of bicarbonate alone in the other measurements described in this study. It was decided not to present the HR data from the exercise test in this thesis. In a separate arm of this study that has not been reported here, the effect of exercise on many clinical parameters was investigated, including blood pressure and the dose of anti-hypertensive's the patients were taking. Patients in the exercise group were on greatly reduced doses of their ant-hypertensive medication following six months of walking exercise, which would undoubtedly affect heart rate response in subsequent exercise tests and this in combination with an inherent HR variability in these patients due to cardiac dysfunction lead to the conclusion the HR data was an unreliable measure of exercise tolerance in these patients.

The reduced lactate response to acute exercise that was observed after one and to some extent six months of exercise with XS bicarbonate (Figures 3.4B and 3.5B) has been reported previously in CKD (Kouidi et al. 1998; Akiba et al. 1995) in which 6 and 3 months respectively of a mixed exercise training programme resulted in lower blood lactate concentrations. Kouidi et al. (1998) attributed this to a block in glycolysis resulting in an increase in muscle glycogen levels, similar to that seen in McArdle's disease, but this has not been confirmed and also seems unlikely. It is more likely that this reduced lactate response at one or six months to the same absolute exercise intensity as at baseline, might be attributed to a training effect (MacRae et al. 1992). If so, this implies that the training effect of aerobic exercise was enhanced here by administering extra bicarbonate. A possible factor limiting the ability to exercise in CKD is acidosis: the normal intracellular acidification of skeletal muscle that occurs during exercise, as a result of lactic acid generation, is enhanced in patients with CKD. Nishida et al. (1991) observed using 31-P NMR that there was a greater decline in sarcosolic pH during aerobic exercise compared to healthy controls and a slower rate of its recovery, an observation that has been confirmed by other groups (Durozard et al. 1993; Kemp et al. 2004). In healthy individuals it has also been demonstrated that an induced metabolic acidosis by $NH_4Cl$ administration or through diet manipulation reduces exercise capacity in high intensity exercise (Jones et al. 1977; Greenhaff et al. 1987). Intramuscular acidification during exercise historically was considered to be a major factor in the development of fatigue (Karlsson et al. 1975), which has latterly become a somewhat controversial point (Westerblad et al. 2002). It is possible however, that the administration of extra bicarbonate helped to blunt the onset of acidification and fatigue during exercise, allowing more prolonged and effective exercise in these patients and hence, an enhanced training effect. Indeed this has recently been demonstrated in healthy rats with a chronic alkalosis. Compared to rats on placebo, rats that received sodium bicarbonate before each training session exhibited a longer time to exhaustion following five weeks of interval training, and greater improvements in mitochondrial mass and mitochondrial respiration measured in the soleus (Bishop et al. 2010). This suggests that chronic ingestion of sodium bicarbonate may sufficiently reduce the muscle hydrogen ion accumulation during training allowing for greater adaptations to occur.

Although the plasma lactate levels here that were generated by the exercise test were only 1-2mmol/l above the resting values, it is important to note that this was imposed on an existing metabolic acidosis and these values only represent the appearance of lactate in the blood and the impact of the exercise on intramuscular acidification was unknown. Any attempt to minimise an

additional acid load in these patients may well result in significant beneficial effects, such as those observed here.

### 3.4.2 Body composition

Exercise training resulted in a small but statistically significant increase in LBM of 0.5kg after one month and in LBM as % of total body weight after six months in the XS bicarbonate group only. Going et al. (1993) quoted the precision error in DEXA scanning of lean body mass to be 0.8kg, and so caution is required when interpreting these small apparent effects. The precision error in detecting changes in fat mass by DEXA is generally stated at 1% (Salamone et al. 2000) changes that the results presented here exceed. The decline in fat mass and the rise in LBM/fat mass ratio that were observed in the present study are of functional interest because CKD patients are chronically inflamed (Pereria et al. 1994). Many of the pro-inflammatory cytokines such as TNF-$\alpha$ and IL-6 that are reported to be elevated in these patients (Pereria et al. 1994) are produced by adipose tissue (Coppack, 2001) and can stimulate protein degradation (Goodman, 1991, 1994). By decreasing fat mass, the production of these cytokines may also be reduced and this may help alleviate the patient's pro-inflammatory condition that can result in cardiac disease (Kalantar-Zadeh and Balakrishnan, 2006) which is the biggest cause of mortality in this population (Pecoits-Filho et al. 2002). (The effect of exercise on the inflammatory processes in these patients is described in a PhD thesis by J. Viana, Loughborough University).

 The changes that were observed in body composition are small changes, but they were achieved with aerobic exercise alone. This may be a reflection of how cachectic these patients are; effects being more readily observed because of the initial wasted state of the patients. In future studies, larger gains might be expected with resistance exercise training or a combination of the two.

It was also noted that no body composition changes whatever were detected in the STD bicarbonate group at six months (Table 3.10), the small apparent changes being confined to the XS bicarbonate group as with the lactate response data in Section 3.3.3 above. This may mean that muscle acidification during exercise was a limiting factor in these patients and XS bicarbonate therapy rendered exercise more effective.

### 3.4.3 Free amino acids

A striking decline was observed in the fasting intramuscular amino acid concentrations of those patients who received STD bicarbonate therapy. This was apparent after one month of walking exercise, but a more consistent and significant decline was seen after six months. As there were generally no changes or small increases in the extracellular (plasma) amino acid concentrations, this decline in the fasted steady-state intracellular amino acid concentration seems to have arisen from a collapse of the concentration gradient between the intramuscular and extracellular compartments. This gradient is normally maintained by active transporters, for example SNAT2, which is the major amino acid pump in skeletal muscle cells (Evans et al. 2007, 2008). This decline was not observed in the XS bicarbonate group, suggesting that it was an acidosis-mediated effect. Furthermore, it was not seen in the non-exercising control group receiving STD bicarbonate therapy, indicating that it was not the result of a resting metabolic acidosis, but of an exercise-induced exacerbation of acidosis and therefore may have been a limiting factor for exercise in the exercising patients. The decline in dietary protein intake after six months of exercise on STD bicarbonate therapy may have contributed to the accompanying changes in intramuscular amino acid concentrations, but this seems an inadequate explanation because a similar decline in dietary protein intake was observed in the non-exercising patients with no accompanying effects on muscle free amino acids.

There was no change seen in the gene expression of the SNAT2 transporter, but previous studies *in vitro* have shown that acidified medium significantly decreases the activity of this transporter (Bevington et al. 2002) probably through a direct effect of low pH on the SNAT2 protein (Baird et al. 2006) resulting in a reduction of the intracellular concentration of glutamine and many other amino acids without any detectable decline in SNAT2 expression at mRNA or protein level (Evans et al. 2007, 2008). The reduction in muscle amino acid concentrations and subsequent collapse of gradients in the present study may be the result of a reduced activity of the SNAT2 transporter caused by a local decrease in pH by the production of lactic acid during exercise, an effect that was apparently prevented by the administration of XS bicarbonate.

It has previously been reported that CKD patients show abnormalities in amino acid metabolism (Kopple, 1978) and exhibit lower concentrations of many muscle and plasma amino acids (Bergström et al. 1990; Gulyassy et al. 1968; Young and Parsons, 1970). This is a problem that

appears to be worse in non-dialysed patients and improves upon initiation of dialysis therapy (Bergström et al. 1990). The muscle and plasma amino acid concentrations that are presented here varied widely between individuals, but were similar to those reported by Bergström and colleagues (1990). Further depletion of these concentrations following exercise in these patients has not previously been reported.

### 3.4.4 Protein phosphorylation signals

### 3.4.4.1 Signals through mTOR

Significant changes in free amino acid pools that were observed in response to exercise (Tables 3.12 – 3.17) could in principle affect protein metabolism, acting through amino acid sensors such as mTOR (see Section 1.11.4). However, in the fasted and rested state, under which conditions the muscle biopsies were drawn in the present study, negligible phosphorylation of rpS6, $P^{70}$S6 kinase and 4E-BP1 was observed (Figures 3.14 and 3.16) implying that only a low level of signalling was occurring through mTOR under these conditions. It has been reported that the concentration of most signalling proteins is lower in human skeletal muscle than in rodent muscle (Wackerhage, 2006) and the observation of a negligible P-rpS6 and P-$P^{70}$S6K signal in human muscle in the fasted and rested state is not a new finding (Karlsson et al. 2004; Dreyer et al. 2006, 2008; Fujita et al. 2007). The low intensity of these phosphorylation signals downstream from mTOR suggest that sensing of the changes in free amino acid pool size through mTOR is unlikely to have been a major factor under these conditions. Nevertheless, the weakness of the signals is of practical importance because it confirms the fasted nature of these patients and suggests that the observed amino acid profiles are a true reflection of their endogenous amino acid metabolism and not simply a reflection of the amino acid content of their last meal. The muscle biopsies were taken in the fasted state to allow the amino acid data to be compared to that of Bergström and colleagues (1990) who also studied uraemic patients in the fasted state.

### 3.4.4.2 Signals through PKB

An important stimulus for the muscle wasting that is seen in CKD patients is a reduction in insulin/IGF-1 signalling (Bailey et al. 2006). These authors observed that under basal conditions

IRS-1 associated PI-3K activity was suppressed in rats with induced CKD resulting in a reduced phosphorylation of PKB. In IRS-1 knockout models, mice exhibit abnormal glucose handling, peripheral insulin resistance and growth defects (Araki et al. 1994), problems that are frequently reported in CKD patients, suggesting that this is an important defect, but is yet to be confirmed by data from human studies. As evidence from rodent studies show the PI-3K/PKB signalling pathway is a critical regulator of muscle mass and hypertrophy *in vivo* (Bodine et al. 2001a), activation of this pathway through exercise training could lead to reduced protein degradation through a suppressed expression of E3 ubiquitin ligases, as it is thought that ubiquitin-conjugation by E3 ligases is the rate limiting step in protein degradation (Lecker et al. 1999), and increased protein synthesis through phosphorylation of the downstream effectors of PKB (Lecker et al. 2006).

A recent study by Wang and colleagues (2009) examined the effect of muscle overload or treadmill running in CKD rats on indices of muscle protein synthesis and degradation. They observed an inhibitory effect of both forms of exercise on protein degradation, but less of an effect on protein synthesis with treadmill running compared to overload. This inhibition of protein degradation was accompanied by a small increase in PKB phosphorylation following treadmill running, an effect that was again larger in the rats with muscle overload.

In the present study no overall change was seen in the phosphorylation of PKB in response to exercise (Figure 3.16) apparently contradicting the results by Chen and Colleagues (2008) and Wang and colleagues (2009) who observed in rats, workoverload and treadmill exercise was capable of increasing PKB phosphorylation levels and ultinatley protein synthesis rates. The differences between these studies may stem from a species variation, from the compliance of the patients to perform the required amount of unsupervised exercise or from the fact that PKB phosphorylation in humans was studied here at rest; Differences may have become apparent if the muscle samples had been taken in the period following the exercise bout. Recent evidence from human volunteers has suggested there is a dissociation between anabolic signalling and muscle protein synthesis rates suggesting this pathway is more complex in humans than evidence from rodent studies would suggest, and therefore PKB phosphorylation in humans may simply be reflecting insulin availability (Greenhaff et al. 2008). This would agree with the very low

signals detected in these patients who were studied in the fasted state. However, in spite of this there was a significant reduction in mRNA expression of the E3 ligases, MuRF-1 and MAFbx after one month of exercise with STD bicarbonate and after six months of exercise on XS bicarbonate therapy (Figures 3.23 and 3.24). Previous studies have reported that an increase in PKB phosphorylation causes an increase in the phosphorylation of the FOXO transcription factors resulting in a reduced expression of E3 ubiquitin ligases. The large decline in E3 ligase expression seen here therefore, appears to have occurred via a mechanism that is independent of PKB. It is possible that exercise-induced stimulation of PKB phosphorylation was missed in the present study through some technical artefact. For example, it has previously been suggested that a high phospho-protein phosphatase activity may explain some unexpected results in PKB phosphorylation (Foster et al. 2005) so that exercise and chronic PKB phosphorylation may lead to a compensatory stimulation of protein phosphatases, resulting in artifactual dephosphorylation of PKB in the time taken to draw the biopsy. Such artefacts seem an inadequate explanation, however, because activation of PKB suppresses myofibrillar proteolysis by suppressing caspase-3 activation (Workeneh et al. 2006) (and production of the 14kDa actin fragment) as well as by suppressing E3 ligase expression. In the present study, no statistically significant effects were seen on the 14kDa actin fragment, especially in the patients on XS bicarbonate therapy (Figure 3.20) so suppression of the E3 ligase expression independent of PKB activation would seem a more likely explanation.

The observation here that after six months of exercise, suppression of mRNA E3 ligase expression was confined to the XS bicarbonate group and there was no change in expression in the STD bicarbonate group correlates with marked differences in the accompanying changes in free amino acid pool size between these two patient groups (Figure 3.10). It has been suggested that depletion of amino acids can increase proteolysis (Sadiq et al. 2007) through the ubiquitin proteasome pathway independent of PKB, although these authors attributed this effect largely to amino acid sensing through mTOR. This also seems an inadequate explanation in the present study (Figures 3.13 and 3.15) as no corresponding changes in mTOR signalling were detected. However, regulation of the ubiquitin-proteasome pathway by amino acids independent of mTOR has also been recently described (Eley et al. 2007).

A recent study by Moriscot and colleagues (2010) has shown that following denervation, MuRF-1 is preferentially expressed in type II fibres. It is possible that the reduced expression of this E3 ligase seen in the present study might be explained by a training induced fibre type shift from a type II fast fibre to a type I slow fibre that was not created in the STD group due to the limiting effect of acidosis on exercise training and adaptation (see Section 3.4.1).However, the contribution of a possible fibre type change to this reduced expression in the individual muscle biopsies is unknown as no quantification of fibre type distribution was made.

### 3.4.5 Myofibrillar proteolysis

Complete proteolysis of myofibrillar proteins requires both an initial caspase-3 dependent cleavage step (leading to accumulation of the 14kDa actin fragment) (Workeneh et al. 2006) and a subsequent degradation of the cleavage fragments through the ubiquitin proteasome pathway. Suppression of both of these processes would therefore be expected to bring about overall suppression of myofibrillar proteolysis. In practice in the present study, even through conditions were found which strongly suppressed E3 ligase expression (Figures 3.22 and 3.23), and even though some decline in 14kDa actin fragment may have occurred at six months in the STD bicarbonate group (Figure 3.20), under none of the conditions did they occur simultaneously. Overall suppression of myofibrillar proteolysis (indicated by 3-MH excretion) was not therefore detected in response to the treatments applied in this study (Tables 3.23 and 3.24). In addition to this, muscle protein:DNA ratio has been used to get a sensitive index of muscle protein mass (Crossland et al. 2008). A significant decline in the muscle protein:DNA ratio was observed in those patients that exercised for six months whilst receiving XS bicarbonate therapy. It is unlikely that this reflects muscle wasting, and can probably be attributed to the decline in total protein content in the biopsy (discussed in more detail in Section 3.4.6). There were no other changes observed in the muscle protein:DNA ratio in any of the other groups indicating walking exercise was unable to increase muscle mass in these patients, but similarly, did not cause muscle mass to be reduced.

In spite of this failure to observe net reduction in 3-MH excretion or an increase in muscle protein:DNA ratio, small apparent increases in LBM were detected by DEXA, especially after one month of exercise (Table 3.10). Bearing in mind the technical limitations of DEXA in

detecting small changes (as discussed in Section 3.4.2 above), this may be a reflection of the accompanying suppression of E3 ligase expression which, even though it would be expected to have little effect on overall myofibrillar proteolysis, might still lead to some net accumulation of non-myofibrillar protein whose protein degradation is not thought to require prior processing via caspase-3. Exercise-induced stimulation of muscle synthesis may also have contributed, especially in the fed state.

### 3.4.6 Adverse effects of sodium bicarbonate

Even though exercise therapy in combination with XS bicarbonate may have had beneficial effects on the lactic acid response to acute exercise (Figure 3.5B), body composition (Figures 3.7B and 3.8B), free amino acid pools (Figure 3.11) and E3 ligase expression (Figures 3.23 and 3.24), in principle adverse effects may also occur as a result of this increased bicarbonate load. There are two potential dangers when administering high doses of sodium bicarbonate. Firstly, sodium loading (with secondary fluid loading) may occur, which is of a particular concern in the more hypertensive patients. Secondly, pH overshoot could also occur possibly resulting in a localised alkalosis. These patients did not appear to have been sodium loaded as there were no detectable increases in the water content of the muscle biopsies in the XS bicarbonate group, suggesting that secondary fluid retention in the tissues had not been a major problem. The possibility of alkalosis is more difficult to refute. While blood pH or bicarbonate concentration did not exceed the top end of the normal range, it is difficult to exclude the possibility of transient alkalosis localised to tissue interstitial fluid. Such effects may have contributed to difficulties encountered in obtaining muscle biopsies from those patients randomised to receive XS bicarbonate. While biopsies were routinely obtained without problems from patients receiving STD bicarbonate therapy at all time points, and from XS bicarbonate patients at baseline, significant difficulties were experienced in obtaining subsequent biopsies from this group. It was noted that considerable amounts of fibrous tissue or fascia, were present in these subsequent biopsies, which was particularly a problem after one month of exercise and, for this reason, XS bicarbonate data are not presented at this time point. At six months, fibrous tissue in the muscle biopsies was still observed and is reflected in the significant decline in the soluble protein content of these biopsies relative to baseline (Table 3.11).

This consistent decline in soluble protein of the muscle biopsies and in muscle protein:DNA ratio that was seen in those patients who had exercised for six months with XS bicarbonate is a surprising finding and has not previously been reported. As DEXA measurements indicated that this decline in biopsy soluble protein content was accompanied by gains in LBM, (that were more apparent when expressed as a percentage of total body weight and in relation to fat mass), this protein loss seems to be confined to the biopsy site. The reason for these sampling difficulties is unknown, but there are two possible explanations. Firstly, the distance between biopsies was only 1cm. This may have meant the second and third biopsies were infiltrated by scar tissue altering the biopsy composition and so the close proximity of the multiple biopsy sites might be responsible for the decline in soluble protein. Secondly, as it was only seen in the XS group, it may reflect an adverse pro-fibrotic effect of XS bicarbonate on the tissue regeneration response of the vastus lateralis muscle following the initial biopsy. Recent evidence from mouse models suggests that skeletal muscle regeneration following injury in CKD is significantly impaired and is accompanied by fibrosis (Zhang et al. 2010) and this may have been a problem in the present study. The accompanying amount of protein in the _insoluble_ fraction of the biopsies did not increase, showing that this problem did not occur simply through inadequate homogenisation of the tissue.

In conclusion, six months of aerobic exercise combined with XS bicarbonate therapy resulted in an improved exercise tolerance and associated lactate response, favourable changes in body composition and a reduced mRNA expression of the E3 ubiquitin ligases. This suggests that XS bicarbonate allowed the patients to exercise more effectively by buffering acid that was produced during the exercise bout and demonstrates that the beneficial effects of alkali supplements that have previously been seen in peritoneal dialysis patients (Stein et al. 1997) may also be observed in pre-dialysis patients, but only in combination with exercise. Exercise without XS bicarbonate therapy appeared to disrupt amino acid gradients, resulting in a decline in intramuscular amino acid concentration and perhaps ultimately limited protein synthesis. Overall there was no decrease in myofibrillar protein catabolism (3-MH), possibly resulting from a failure of the treatments to increase PKB phosphorylation sufficiently and decrease casapase-3 mediated cleavage of myofibrils and, in the case of STD bicarbonate therapy, to effectively buffer the additional acid produced during exercise. Whether hypertrophy in CKD can be attained by alkali therapy combined with resistance exercise warrants further investigation. These results suggest

that acidosis is a functionally significant problem in CKD patients attempting an exercise programme, and factors determining the magnitude of this problem are the subject of the next chapter.

Chapter 4


# *In vivo* study of the regulation of sulphuric acid production in CKD

## 4.1 Introduction

The catabolism of sulphur-containing amino acids (as described in detail in Chapter 1.6.1) results in the production of taurine and sulphate (Stipanuk et al. 2004; Coloso and Stipanuk, 1989) and accounts for the majority of the daily titratable acid load in humans. The metabolism of sulphate may be abnormal in CKD, because despite normal plasma methionine concentrations (Gulyassy et al. 1970; Condon and Asatoor, 1971), the concentrations of cysteine and homocysteine, a homologue of cysteine, both in the free and bound forms, are frequently seen to be raised in uraemic patients (Flügel-Link et al. 1983; Robins et al. 1972). Plasma taurine levels, however, in non-dialysed and haemodialysis patients tend to be lower than those seen in healthy individuals (Alvestrand et al. 1982; Qureshi et al. 1989; Suliman et al. 1996) and Bergström and colleagues (1989) reported that in CKD patients the muscle and plasma taurine concentrations were both reduced by the same margin, suggesting that this represents a true depletion of taurine from the cells rather than a decline in the cell/plasma concentration gradient.

The concentrations of cysteinesulphinate (CSA) in uraemic plasma have also been reported to be high (Suliman et al. 1996) and it was speculated that low plasma taurine accompanied by these high levels of CSA arises from a defect in the activity of cysteinesulphinate decarboxylase (CSAD), the rate limiting step in the conversion of CSA to taurine (Suliman et al. 1996). This enzyme requires vitamin B6 as a cofactor, a vitamin in which CKD patients are commonly deficient (Kopple et al. 1981). However, patients in a number of studies by Suliman and colleagues (1999, 2001, 2002) were administered vitamin B6 supplements and so the authors concluded that uraemia itself inhibits CSAD. Anorexia and malnutrition are frequently seen in these patients (Suliman et al. 2002) and it is likely this results in a reduced taurine intake that, when coupled with a reduced taurine synthesis from CSA as proposed by Suliman et al. (1996), results in these reduced plasma taurine levels. Taurine has many important cellular functions including stabilization of the membrane potential, antioxidant properties and a positive inotropic effect on the heart (Suliman et al. 2002; Schaffer et al. 2009) and taurine depletion could possibly result in muscle fatigue and cardiovascular disease (Suliman et al. 2002), common co-morbidities in CKD patients (Nussbaum and Garcia, 2009; Pecoits-Filho et al. 2002).

It was therefore hypothesised that in CKD the flux through this pathway is diverted away from taurine and towards the generation of pyruvate and sulphuric acid ($H_2SO_4$) (Chapter 1, Figure 1.5) thus exacerbating the metabolic acidosis that is already present because of impaired

excretion of acid. Such enhanced catabolism of cysteine to $H_2SO_4$ has been observed previously in cancer and HIV infection - conditions which both show prominent cachexia (Breitkreutz et al. 2000; Hack et al. 1996), but has yet to be demonstrated in CKD patients.

It has been shown previously in peritoneal dialysis patients that alkali therapy results in weight gain and improvements in morbidity (Stein et al. 1997; Szeto et al. 2003), but this was achieved with only small changes in plasma bicarbonate or pH, suggesting that plasma bicarbonate and pH are not sensitive indicators of muscle wasting or clinically significant levels of acidosis in the tissues. It is possible that measures of sulphur amino acid catabolism might be more sensitive clinical biochemical markers of tissue acidification leading to cachexia. Therefore, the aims of this study were

1) To compare sulphate, CSA and taurine excretion in CKD patients (Stage 4-5) who were still producing urine, with a group of healthy control subjects to determine whether these patients do excrete more sulphate and less taurine than the healthy individuals, consistent with a decreased flux through the CSAD enzyme.

2) To examine if sulphate excretion rate correlates with 3-MH excretion (as a measure of myofibrillar protein catabolism) in either group, possibly allowing identification of those patients who produce more sulphate and may therefore be at a greater long-term risk of muscle wasting.

3) To examine the effect of exercise and bicarbonate therapy on sulphur metabolism.

It was hypothesised that compared to healthy controls, CKD patients would exhibit higher excretion rates of sulphate and lower excretion rates of taurine indicating an impairment in the CSAD enzyme, and that the acid generated from the diet would be functionally important in CKD patients resulting in a positive correlation between sulphate excretion and 3-MH excretion rates.

## 4.2 Methods

### 4.2.1 Participant information

This study received ethical approval from the local ethics committee. Prior to the start of the study, all potential participants received written information regarding the nature of the protocol. Following any questions, participants signed a written statement of consent and were made aware of their right to withdraw from the study at any point without the need to give a reason. The healthy volunteers in this study were free of any renal impairment, assessed by serum creatinine and of any known disorder in the metabolism of sulphate. Twenty CKD patients Stage 4-5 who had not yet started dialysis therapy and twenty healthy controls were recruited.

**Table 4.1. Participant characteristics.** Values are presented as median and range.

| | Males | Females | Age (years) | Weight (kg) | Height (m) | Plasma bicarbonate (mmol/l) |
|---|---|---|---|---|---|---|
| CKD Patients | 14 | 6 | 60 (38 – 77) | 80.3 (53.0 – 116.0) | 1.73 (1.48 – 1.92) | 23 (20 – 29) |
| Healthy Controls | 8 | 12 | 39 (31 – 57) | 63.0 (48.0 – 94.0) | 1.68 (1.54 – 1.82) | 26 (22 – 30) |

### 4.2.2 Protocol for urine collection and handling

As described in Chapter 3, the excretion of 3-MH was analyzed as a measure of myofibrillar protein degradation. To do this, patients were required to undergo three days on a vegan diet (i.e. devoid of 3-MH and low in taurine), followed by a complete 24h urine collection on the fourth day, whilst on the same diet and before the initiation of exercise or bicarbonate therapy. These urine samples were also used to perform analyses of sulphate, taurine, CSA and 3-MH excretion (see Chapter 2.3). As part of the exercise study protocol (see Chapter 3.2.3), patients were required to repeat this vegan diet and 24h urine collection after one month and/or after six months to investigate changes in 3-MH excretion, and so the analyses of sulphur metabolites were repeated in these samples to give an indication of the effect of exercise and bicarbonate therapy on excretion rates. The healthy control group gave a 24h urine sample following the vegan diet as for the patients, but the procedure was also repeated six months later whilst on their

normal *ad libitum* diet. Taurine, CSA and 3-MH excretion were analysed by HPLC, (see Chapter 2.3). Sulphate excretion was analysed by turbidimetry (see Section 4.2.2.1).

### 4.2.2.1 Urinary sulphate quantification by turbidimetry

Sulphate precipitated in the form of Barium Sulphate was determined by turbidimetry as described by Ma and Chan (1973). Protein was precipitated from the urine sample by the addition of 5% w/v TCA (200μl) to 50μl of the urine sample or sodium sulphate standard (0-100mM) (50μl), vortexed and incubated on ice for 5 minutes. The tubes were then microcentrifuged at 13,000rpm for 10 minutes at 4°C. 200μl of the resulting supernatant was transferred to a new tube and 1% $BaCl_2$ plus 10% dextran (50μl) was added and immediately vortexed. 200μl from each tube was pipetted into a 96 well plate and incubated at room temperature for 10 minutes to allow barium sulphate turbidity to develop. The plate was then read at an optical density of 660nm on a Titertek Mulitscan spectrophotometer.

### 4.2.3 Blood sampling and handling

On the day of collection, patients arrived at the hospital fasted and a venous and arterial blood sample was taken for the determination of blood bicarbonate concentrations. 7ml venous samples were drawn into tubes containing lithium heparin. Arterial blood samples were drawn from the brachial artery into a blood gas syringe containing heparin. Venous and arterial blood samples were taken to the Pathology Department at the Leicester General Hospital for the determination of blood bicarbonate concentrations. In the case of healthy volunteer samples, a 10ml venous blood sample was taken and dispensed into a plain tube and a tube containing $K_2$ EDTA. No arterial blood sample was taken.

## 4.3 Results

### 4.3.1 Excretion rates

Sulphate excretion rates over 24h on a vegan diet are shown below in Figure 4.1. Although body weight will have an effect upon sulphate excretion, these results are initially presented as absolute excretion rates before a correction for body weight is made.
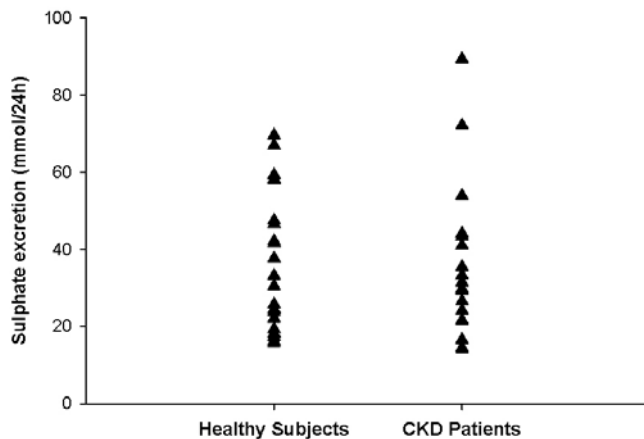


**Figure 4.1. Variation in daily sulphate output in healthy individuals and in CKD patients on a vegan diet.** Healthy controls n = 20; CKD patients n = 20.

Daily sulphate output varied widely, up to five fold, in both the CKD patients (35.5 ± 4.7mmol/24h) and in healthy individuals (35.8 ± 3.9mmol/24h), but there was no significant difference in the sulphate output between the two groups (P = 0.771). There was also no significant difference when these values were corrected for body weight (healthy subjects = 0.56mmol/24h/kg bw; CKD patients = 0.45mmol/24h/kg bw; P = 0.158). These values presented in Figure 4.1 are comparable with those previously reported in CKD (Nakanishi et al. 2002) and to those reported in healthy individuals of approximately 40mmol/day in Documenta Geigy (Diem and Lentner, 1970). The wide variation in the age of the subjects studied showed no detectable correlation with the rate of sulphate excretion (both in the CKD and healthy groups) (r = -1.81; P = 0.431) (data not shown).
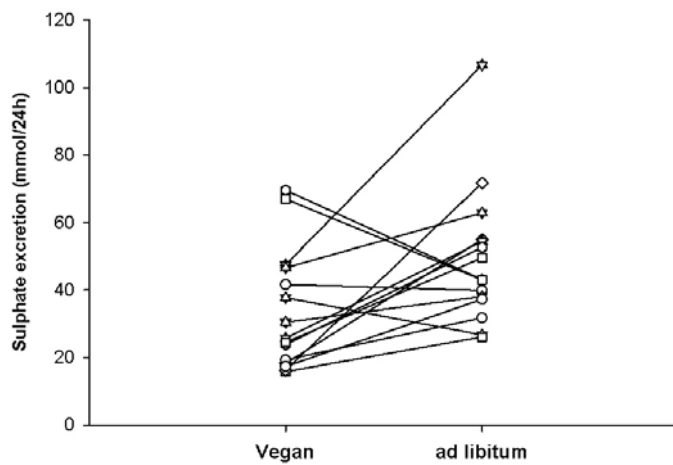
**Figure 4.2. Variation in daily sulphate output in healthy individuals on a vegan compared to an *ad libitum* diet.** Lines represent changes in each individual, n = 16.

In healthy subjects, sulphate excretion was significantly higher on the *ad libitum* diet (49.3 ± 5.3mmol/24h) compared to the vegan diet (33.4 ± 4.6mmol/24h) (P = 0.036), but there was no correlation between sulphate excretion rates measured on the two diets (r = 0.196; P = 0.483).
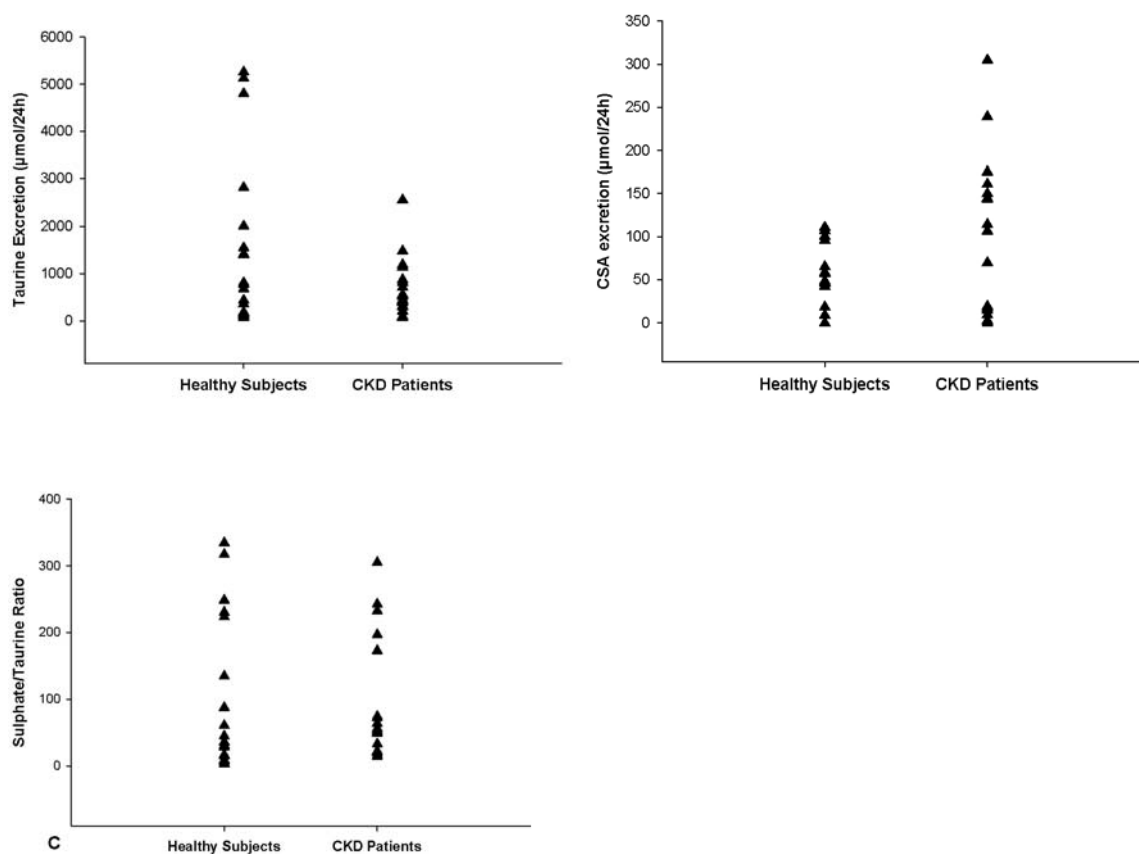
**Figure 4.3. Variation in daily taurine (A) and CSA excretion (B) between healthy subjects and CKD patients on a vegan diet. (C) ratio between sulphate and taurine excretion in healthy subjects and CKD patients.**

Similar to the results for sulphate excretion, there was also a wide variation in the excretion of taurine and CSA in both groups. There was a tendency for taurine excretion to be lower and CSA excretion to be higher in the CKD patients compared to the healthy subjects (Figure 4.3A and 4.3B respectively), but these apparent differences did not achieve statistical significance. There was no statistically significant difference in the sulphate/taurine ratios between the healthy subjects and CKD patients ($P = 0.792$) (Figure 4.3C), nor was there a difference in the taurine/CSA ratios ($P = 0.555$). Consequently, even in the healthy subjects, more than 95 % of the total sulphur excreted was in the form of sulphate.

A strong positive correlation was observed between 3-MH excretion rate and sulphate excretion rate in CKD patients (Figure 4.4A) (r = 0.755; P<0.001). This correlation persisted when 3-MH and sulphate were corrected for body weight (r = 0.761; P<0.001), creatinine (r = 0.529; P = 0.016) and DEXA-derived lean body mass (r = 0.714; P<0.001) (Figures 4.4A, C, E and 4.5), demonstrating that the expected increases in 3-MH and sulphate production with increasing tissue mass were not the cause of the correlation. In contrast, no significant correlation was seen between the excretion rates of 3-MH and sulphate in the healthy control group (r = 0.347; P = 0.133), or when these values were corrected for creatinine (P = 0.061). There was however a significant correlation when corrected for body weight (P = 0.024) (Figures 4.4B, D, F).
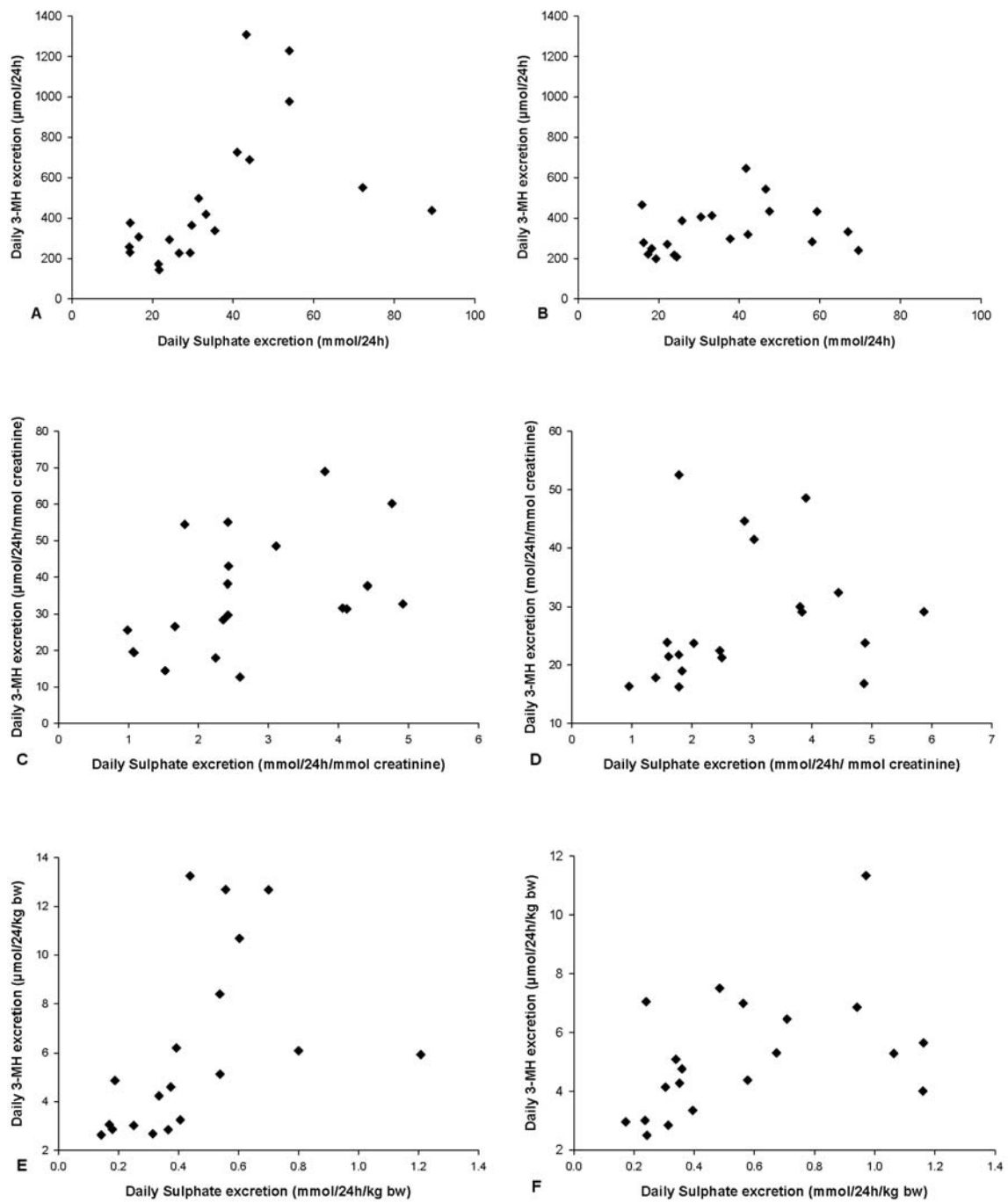
**Figure 4.4. Correlation between daily 3-MH and sulphate excretion in CKD patients (A) corrected for creatinine (C) and body mass (E) and in healthy individuals (B) corrected for creatinine (D) and body mass (F).**
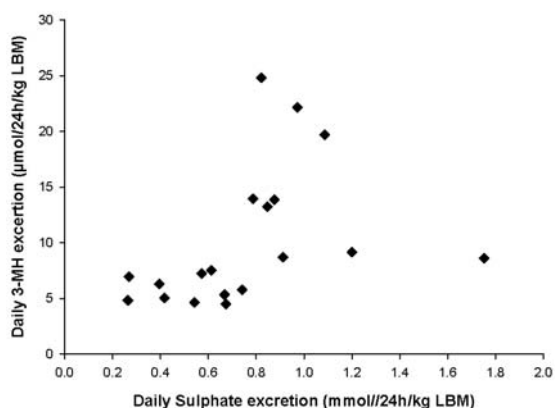
**Figure 4.5. Correlation between daily 3-MH and sulphate excretion corrected for LBM in CKD patients.**

### 4.3.2 Correlations with net tissue wasting (cachexia)

At this stage in the disease progression (CKD 4-5) it was not possible to demonstrate a correlation between elevated rates of 3-MH excretion and net wasting of lean tissue. For example, when the DEXA-derived LBM was expressed as a percentage of total body weight (a measure of cachexia), no correlation was observed with the 3-MH excretion rate (Figure 4.6A). Consequently, no correlation was observed between sulphate excretion rate and severity of wasting (Figure 4.6B).
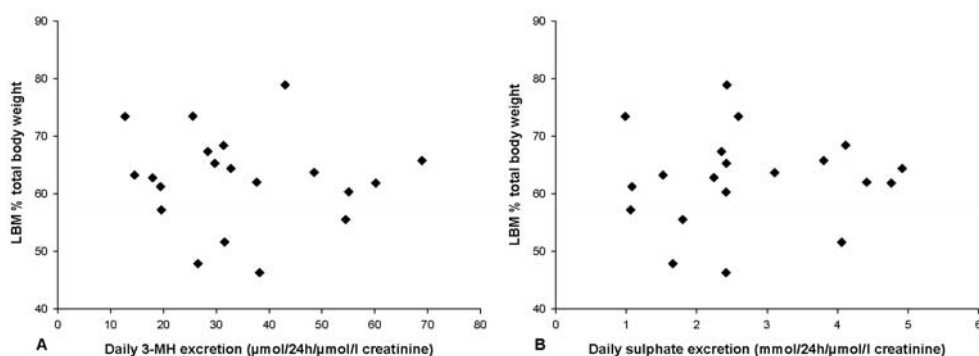


**Figure 4.6. Correlation between LBM expressed as a % of total body weight and (A) daily 3-MH excretion corrected for creatinine and (B) sulphate excretion corrected for creatinine.**

### 4.3.3 Comparison with conventional measures of acid-base status

The proposed catabolic effect of acidosis in CKD (Stein et al. 1997; Szeto et al. 2003) would be expected to lead to a correlation between elevated rates of 3-MH excretion and low blood bicarbonate concentration or pH. In spite of this, no correlation was observed between venous bicarbonate levels and 3-MH excretion rate in either the CKD patients ($r = 0.231$; $P = 0.342$) (Figure 4.7A) or in healthy individuals ($r = 0.196$; $P = 0.407$) (data not shown). There was however, a significant inverse relationship as expected between arterial bicarbonate and 3-MH excretion in CKD patients ($r = -0.680$; $P = 0.011$) (Figure 4.7B).
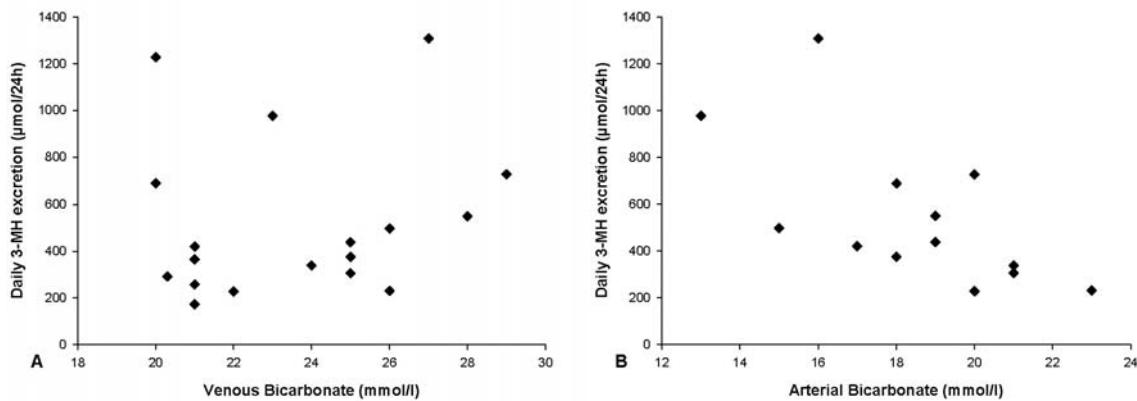


**Figure 4.7. Correlation between 3-MH excretion rate and venous bicarbonate (A) and arterial bicarbonate (B) in CKD patients.** Note, 6 of the 20 patients refused consent for an arterial blood sample.

The accumulation of acid in those CKD patients with higher rates of sulphuric acid production would also be expected to lead to an association between high sulphate excretion and low blood bicarbonate. At least for arterial bicarbonate, the expected trend was observed (Figure 4.8B), but fell short of statistical significance ($P = 0.113$).
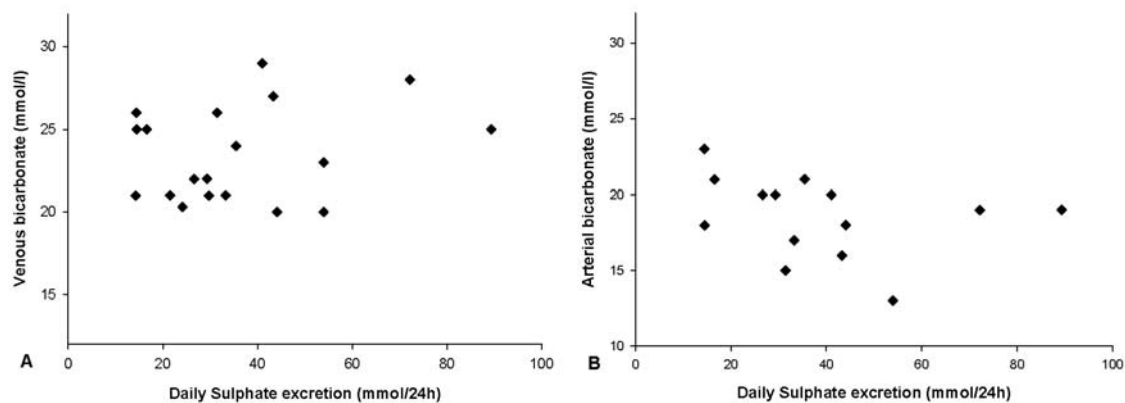
**Figure 4.8. Correlation between blood bicarbonate and daily sulphate excretion in CKD (A) venous blood (B) arterial blood.**

An unexpected observation was that there was a strong positive correlation between venous bicarbonate and sulphate excretion in the healthy controls (r = 0.800; P = 0.001) (Figure 4.9), in marked contrast with that in the CKD patients (Figure 4.8A), presumably indicating that in healthy individuals producing large amounts of sulphuric acid, potent compensatory mechanisms exist to prevent acidosis (Figure 4.9) and prevent increased protein catabolism (Figure 4.4B)
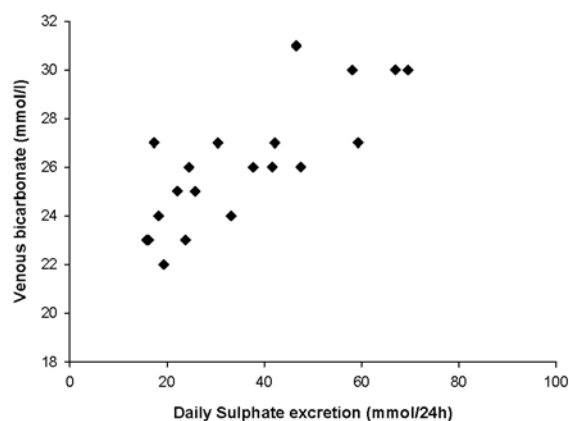


**Figure 4.9. Correlation between venous bicarbonate concentration and daily sulphate excretion in healthy subjects.**

### 4.3.4 Relationship with free amino acid concentrations

No relationship was identified between sulphate excretion and muscle or plasma concentrations of methionine, cysteine, or any other free amino acid (P>0.05) (data not shown).

### 4.3.5 Effects of exercise or bicarbonate therapy on sulphur metabolism

Values in Tables 4.2 and 4.3 show that exercise had no significant effect upon sulphate excretion. After one month, sulphate excretion did appear to have increased, but this change was not statistically significant (P = 0.109) and had disappeared after six months of exercise. XS sodium bicarbonate therapy had no effect on sulphate excretion after one month of exercise (>0.05), however, the excretion rates after six months of exercise from those patients receiving STD and XS bicarbonate were significantly different (P = 0.020). This difference remained when sulphate excretion rates were corrected for body weight (P = 0.012) and LBM (P = 0.028), but not creatinine (P = 0.240). It is important to note however, that most of this apparent difference in sulphate excretion between the STD and XS groups was already present at baseline, but was not statistically significant at that time point (P = 0.125) (Tables 4.2 and 4.3), indicating that the therapy had exerted negligible effect on sulphate output.

**Table 4.2. Effects of one month of exercise and bicarbonate therapy on sulphate excretion (mmol/24h).**

|  | Baseline | One Month |
| --- | --- | --- |
| All Patients | 33.0 ± 4.8 | 41.4 ± 7.4 |
| STD Bicarbonate | 28.3 ± 4.6 | 31.9 ± 4.7 |
| XS Bicarbonate | 40.7 ± 10.0 | 57.3 ± 17.0 |

**Table 4.3. Effects of six months of exercise and bicarbonate therapy on sulphate excretion (mmol/24h).** * denotes a significant difference from corresponding value in STD bicarbonate group (P<0.05).

|  | Baseline | Six Months |
|---|---|---|
| All Patients | 33.0 ± 5.0 | 34.7 ± 5.5 |
| STD Bicarbonate | 26.5 ± 4.8 | 25.2 ± 4.2 |
| XS Bicarbonate | 45.7 ± 11.0 | 51.7 ± 10.1* |

Even though this study was not designed to test the stability of sulphate excretion rate in individual patients, it is interesting to note that differences in the sulphate excretion rate between individuals generally persisted over time. A strong correlation was observed between baseline and six month sulphate excretion rates (R = 0.814; P<0.001) (Figure 4.10C) i.e. those individuals who produced large amounts of sulphate at baseline still produced large amounts six months later, possibly indicating that these individuals are at a higher long-term risk of acidosis-related complications when their ability to excrete this acid is lost.
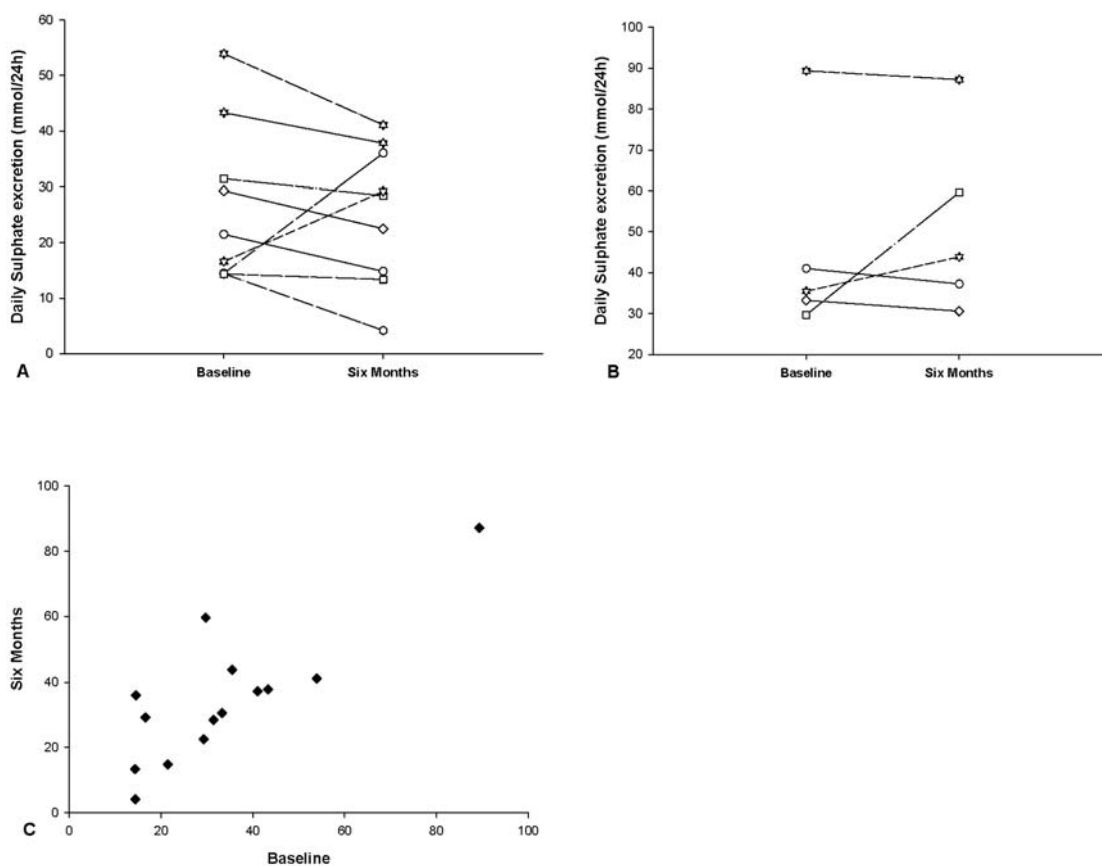
**Figure 4.10. Change in sulphate excretion over time in those CKD patients who received STD bicarbonate (A) and XS bicarbonate (B) therapy. (C) Stability of sulphate excretion over time assessed by correlating baseline and six month data in those patients on STD bicarbonate therapy.**

There was no significant effect of exercise or bicarbonate therapy on the excretion rates of taurine or CSA (Tables 4.4 – 4.7).

**Table 4.4. Effects of one month of exercise and bicarbonate therapy on taurine excretion (μmol/24h).**

|  | Baseline | One Month |
|---|---|---|
| All patients | $816.8 \pm 168.3$ | $879.0 \pm 286.8$ |
| STD Bicarbonate | $862.5 \pm 228.6$ | $882.0 \pm 320.3$ |
| XS Bicarbonate | $734.6 \pm 258.7$ | $873.6 \pm 615.7$ |

**Table 4.5. Effects of six months of exercise and bicarbonate therapy on taurine excretion (µmol/24h).**

|  | Baseline | Six Months |
|---|---|---|
| All patients | 710.4 ± 178.9 | 868.8 ± 262.5 |
| STD Bicarbonate | 748.9 ± 250.3 | 986.9 ± 378.9 |
| XS Bicarbonate | 623.7 ± 191.3 | 603.0 ± 26.7 |

**Table 4.6. Effects of one month of exercise and bicarbonate therapy on CSA excretion (µmol/24h).**

|  | Baseline | One Month |
|---|---|---|
| All patients | 109.4 ± 25.8 | 95.7 ± 26.0 |
| STD Bicarbonate | 122.8 ± 36.6 | 116.4 ± 32.7 |
| XS Bicarbonate | 85.3 ± 31.8 | 58.5 ± 41.8 |

**Table 4.7. Effects of six months of exercise and bicarbonate therapy on CSA excretion (µmol/24h).**

|  | Baseline | Six Months |
|---|---|---|
| All patients | 97.6 ± 26.0 | 109.3 ± 27.3 |
| STD Bicarbonate | 114.9 ± 36.1 | 125.4 ± 34.6 |
| XS Bicarbonate | 66.3 ± 33.2 | 80.3 ± 46.5 |

## 4.4 Discussion

### 4.4.1 Uraemic defects in sulphur metabolism

It has previously been reported that CKD patients have abnormal plasma concentrations of the sulphur containing amino acid cysteine and their catabolites (Flügel-Link et al. 1983; Alvestrand et al. 1982; Suliman et al. 1996) indicating a defect in their metabolism. In particular Suliman and colleagues (1996) proposed that a defect may be present in the activity of the CSAD enzyme which may result in cysteine metabolism being diverted away from non-acidic products such as taurine and towards sulphuric acid. By making the assumption that dietary sulphur intake and urinary sulphur excretion are at steady state, the present study attempted to investigate fluxes through these sulphur amino acid catabolic pathways by measuring excretion rates on a vegan diet. This failed to detect statistically significant changes in these fluxes (Figure 4.1) when compared with healthy individuals, although the tendency towards decreased taurine excretion, increased CSA excretion (Figures 4.3A and B) may be consistent with the proposed impairment of flux through CSAD in CKD. Nevertheless, it should be emphasised that, even in healthy people, 95% of the total sulphur excreted is in the form of sulphuric acid rather than taurine (Section 4.3.1). Therefore, even if CSAD is strongly inhibited in CKD, this would probably have little additional impact on acid production in these patients.

It is frequently reported that CKD patients have low muscle and plasma taurine levels (Qureshi et al. 1989; Bergström et al. 1989) which, in the presence of high cysteine and CSA concentrations, led to the suggestion of a defect in the activity of the enzyme CSAD in uraemia. The median taurine excretion rate in the patients in the present study was less than half of that seen in the healthy control group (Figure 4.3), but this apparent difference fell short of statistical significance. The patients studied by Suliman and colleagues (1996), leading them to the conclusion of a defective CSAD flux, were established haemodialysis and peritoneal dialysis patients, unlike the pre-dialysis patients in the current study. It may be that there is a disorder in sulphur metabolism in end-stage renal disease that is not yet fully developed in pre-dialysis patients of the type studied here. It has also been observed that the dialysis procedure itself results in a loss of taurine (Loftberg et al. 2000), which may also explain why clearer depletion of taurine levels was observed in the earlier studies.

**4.4.2 Correlation with protein catabolism**

No significant increase was observed in the sulphate excretion rate in the present CKD patients, when compared with healthy control subjects. This contrasts with the results from other catabolic diseases, such as cancer and HIV infection (Breitkreutz et al. 2000; Hack et al. 1996), in which large sulphur losses have been reported. However, an important finding in the present study was that there was very wide variation in the sulphate excretion rate between individuals (both in the healthy control group and in the CKD patients (Figure 4.1)) and this wide variation showed a strong positive correlation with 3-MH excretion, but only in the CKD patients. Two possible interpretations of this observation are that:

a) Increased sulphuric acid production causes increased protein catabolism if excretion of the acid is impaired, OR
b) Increased protein catabolism liberates L-Met and L-Cys from the degraded proteins which are then further degraded, leading to increased sulphuric acid production.

*In vivo* both effects may occur: (a) may contribute to the lethal effects of the acidosis generated in chicks receiving supraphysiological doses of L-Cysteine (Dilger & Baker, 2008) whereas (b) may contribute to the strongly increased L-Cysteine catabolism and sulphate excretion reported in severe cachexia (Breitkreutz et al. 2000; Hack et al. 1996). In the present study (a) seems to be the dominant factor because this interpretation predicts that sulphuric acid production should not lead to acid accumulation and hence protein catabolism if acid excretion is normal. This was indeed the case in healthy individuals (Figure 4.5B) and the 3-MH/sulphate correlation was not seen in this group, presumably because in non-uraemic individuals there is a significant ability to buffer and excrete the sulphuric acid.

This suggests that in the patient population, sulphate excretion may be a good indicator of myofibrillar protein degradation, and those patients who consistently produce large amounts of sulphate in their urine may be at a greater long-term risk of muscle wasting. The rate of sulphate production in each individual did appear to remain relatively stable over time (Figure 4.10C): those individuals who were seen to produce large amounts of sulphate (sulphuric acid) at baseline generally still produced large amounts six months later, which has not been shown previously. This needs to be confirmed in a larger long-term study but, if verified, is potentially important because it might allow a single 24h urine sample, early in the progression of the

disease, to be used to screen for those patients at greatest risk from metabolic acidosis and muscle wasting.

As expected, the rate of sulphate excretion was higher in healthy individuals on the *ad libitum* diet compared to the vegan diet, presumably because of a greater intake of foods high in sulphur amino acids, for example meat (Nakamura et al. 2002) and therefore a greater flux through the sulphuric acid pathway. If this also applies to CKD patients on their usual diet, this implies that the severity of the apparent effect on protein catabolism observed on the vegan diet in Figure 4.5 is probably an under-estimate.

### 4.4.3 Correlation with cachexia

There was no detectable relationship in this study between LBM and 3-MH excretion (Figure 4.6A), contrary to what might have been expected. If indices of myofibrillar degradation increase as in Figure 4.5, this might be expected to be reflected in whole body measures of LBM. Several factors probably contributed to the failure to observe this. The inability of DEXA to detect small changes in muscle mass was discussed in the previous chapter (Section 3.4.2). At this stage in the disease progression (CKD 4-5), it may also be too early for significant wasting to be demonstrated, and the patients may have achieved a steady-state in which increased protein catabolism is compensated by increased protein synthesis (Reaich et al. 1995). Further long-term studies may therefore be required to demonstrate the clinical significance of elevated sulphuric acid production rate in cachexia.

### 4.4.4 Conventional measures of acid-base status

More conventional means of assessing a patient's acid base status are venous and arterial bicarbonate measurements and pH measurements (Robertson, 1989). Venous bicarbonate measurements are routinely used by clinicians in the treatment of CKD, but as this is prone to influence by many factors for example diet and environment, it is a less desirable measure than arterial bicarbonate, which is usually lower than the venous bicarbonate levels. Unlike the relationship observed between sulphate and 3-MH excretion in the present CKD patients, no relationship was found between venous bicarbonate and either sulphate or 3-MH excretion (Figures 4.7A and 4.8A). There was however, a significant inverse relationship between arterial

bicarbonate and 3-MH excretion (Figure 4.7B), suggesting that arterial bicarbonate rather than venous measurements may be a more reliable indicator of acidosis leading to protein catabolism. The reason for this observed correlation with arterial but not venous bicarbonate is unknown, but may reflect the variable nature of venous bicarbonate levels as described above. As arterial blood sampling involves considerable discomfort to the patients, further investigation of urinary sulphate excretion as a surrogate, non-invasive measure of acid-base status in CKD may be worth pursuing.

### 4.4.5 Alkali therapy and 3-MH excretion

Even though the positive correlation between sulphate excretion and 3-MH excretion demonstrated in Figure 4.5 and the inverse correlation between arterial bicarbonate and 3-MH excretion demonstrated in Figure 4.7B suggest that acid accumulation in CKD is driving myofibrillar protein degradation, six months of XS bicarbonate therapy with or without exercise therapy in Chapter 3 (see Section 3.3.12) failed to show the expected suppression of 3-MH excretion in these patients. This failure might be attributable to three factors:

1) The XS bicarbonate therapy may have had insufficient impact on pH/bicarbonate concentration in the tissue interstitial fluid and indeed there was no significant change in arterial pH following XS bicarbonate therapy (P = 0.110; see Chapter 3, Table 3.3). However, clear effects were observed in Chapter 3.3.11 on muscle E3 ligase expression suggesting that this is an inadequate explanation.

2) This discrepancy might also have arisen from a stimulatory effect of bicarbonate therapy on appetite and feeding. Metabolic acidosis is a known appetite suppressant (Koch & Kaske, 2008) and increased dietary protein intake (and hence sulphur intake) leads to the generation of sulphuric acid (Trilock and Draper, 1989; Leman and Relman, 1959). However, no significant increase in sulphate excretion was found to accompany the XS bicarbonate therapy: sulphate excretion was indeed higher in the XS bicarbonate group than in the STD group after 6 months (Table 4.3) but this seemed to arise mainly from a pre-existing elevation in sulphate output in the XS bicarbonate patients which was present at baseline (Table 4.3). This observation of a higher rate of sulphuric acid production at baseline and after six months in the XS bicarbonate patients may partly

explain however the low muscle amino acid concentrations and amino acid gradients that were observed in Chapter 3 in these patients (Tables 3.13 and 3.16). This may mean that in future studies of this type it will be necessary to match the different patient groups for sulphur intake/sulphate excretion at the start of the study.

3) Failure of XS bicarbonate to suppress 3-MH excretion in spite of an apparent link between sulphuric acid production and 3-MH (Figure 4.5A) may mean that some toxic product of the sulphuric acid pathway *other than acid* is contributing to myofibrillar protein catabolism. In addition to acid, this pathway also generates the toxic intermediate sulphite ($SO_3^{2-}$) from sulphurous acid ($H_2SO_3$) (See Chapter 1, Figure 1.5). Sulphite accumulates in patients with advanced CKD, attaining concentrations of approximately 5 µmol/L (Kajiyama et al. 2000) - concentrations which are known to induce oxidative stress (Vincent et al. 2004) and hence inflammation – a known contributor to cachexia. This accumulation is made possible by the fact that the detoxifying enzyme sulphite oxidase (Chapter 1, Figure 1.5) which converts sulphite to sulphate ($SO_4^{2-}$) has much lower activity in humans than in other mammals such as rat (Kucukatay et al. 2006).

### 4.4.6 Conclusions

In conclusion, no significant difference was observed between healthy subjects and CKD patients in their excretion rates for sulphate, taurine or CSA. The observation of a lack of a relationship between venous bicarbonate and 3-MH excretion, and the identification of a strong correlation between sulphate and 3-MH excretion, suggests that sulphate excretion might be a better clinical indicator of acidotic patients at risk of cachexia than conventional measures such as venous bicarbonate. This relationship between sulphate and 3-MH excretion also implies that sulphuric acid production is functionally important in these patients, and therapy aimed at slowing sulphur catabolism or diverting it away from sulphuric acid, for example towards the non-acidic end product taurine, might be worth investigating in high-risk acid-generating patients. A possible way in which this might be achieved is examined in the next chapter.

Chapter 5

*In Vitro* modelling of the factors that generate acidosis, and the effects of stress signals on sensing of acidosis through the SNAT2 transporter

## 5.1 Introduction

In the previous two chapters it was concluded that metabolic acidosis may be an important influence on muscle metabolism in CKD, particularly when exercise, resulting in generation of lactic acid, is imposed on a background of uraemic metabolic acidosis. Two important observations were:

- **Acid generation** A strong positive correlation between the rates of sulphuric acid production and myofibrillar protein catabolism (3-MH excretion) in these patients. At the end of Chapter 4, it was suggested from this correlation that patients with a high rate of sulphate generation may be at a higher risk of acidosis-induced muscle wasting.
- **Acid sensing** Collapse of the intramuscular/extracellular amino acid concentration gradients after six months of exercise – a defect which was concluded to be a consequence of acidosis because it was prevented by administering additional oral bicarbonate (Chapter 3).

These observations lead to two important questions:

**1) Acid generation** Is the higher rate of sulphuric acid generation seen in some patients a <u>cause</u> of increased protein catabolism (through worsening acidosis) or a <u>consequence</u> of protein catabolism (through liberation of sulphur amino acids from protein)?

**2) Acid sensing** Inhibition of pH sensitive System A amino acid transporters (especially SNAT2/slc38a2) by acidosis could account for the observed collapse of amino acid gradients, but this inhibition is thought to be a direct, rapidly reversible, effect of low pH on the SNAT2 protein (Bevington et al. 2002; Baird et al. 2006) implying that collapse of the amino acid gradients would only occur while acidosis was at its most severe during lactic acid generation accompanying exercise, but in practice, the effects on amino acids were detected in the resting state. This leads to the question of whether the metabolic stress of acidosis can also exert <u>long-term</u> inhibitory effects on SNAT2 over a time scale of hours.

For technical reasons these problems were difficult to investigate directly in the patients *in vivo*. In order to measure SNAT2 activity directly *in vivo*, ^{11}C-MeAIB must be used and the analysis of which requires a cyclotron adjacent to the clinical research facility, therefore the effect of

exercise on SNAT2 activity could therefore not be measured directly *in vivo*. In order to address the question on acid generation above, rates of protein metabolism would need to be manipulated using cycloheximide and MG132. This would be unsafe and unethical to do *in vivo* and therefore in this chapter *in vitro* experiments using cultured cell models are described which attempt to answer the questions raised above. The chapter is divided into three parts:

**Part A** investigates *in vitro* the factors controlling the production of sulphuric acid in cultured L6-G8C5 skeletal muscle cells and HepG2 hepatocytes. The L6-G8C5 myotube model has been used extensively in this laboratory to study metabolic effects of acidosis (Evans et al. 2008; Evans et al. 2007; Pickering et al. 2003; Bevington et al. 2002; Bevington et al. 2001; Bevington et al. 1998). The HepG2 cell line has been used previously as an *in vitro* model to study regulation of sulphur amino acid catabolism in liver (Dominy et al. 2007) which is the dominant organ responsible for sulphuric acid production in mammals (Stipanuk, 2004). Muscle makes a smaller contribution to whole-body sulphuric acid production (Ensunsa et al. 1993) but it was also felt to be important to study a muscle model here because locally generated sulphuric acid may be the pool with the most direct access to the myocytes *in vivo* and hence a greater possible influence on muscle catabolism.

Initial experiments in this laboratory using the turbidimetric sulphate assay that was applied to the patients' urine in Chapter 3 showed that sulphate production by HepG2 and L6-G8C5 cells was detectable *in vitro* and that a confluent 35mm culture well of HepG2 cells produced approximately 20nmoles over three days (A. Eltagouri and J.R. Brown, unpublished observations). However, this technique was too insensitive for routine use in investigating effects on sulphate metabolism. A more sensitive technique was therefore developed, measuring $^{35}$S-sulphate generation from catabolism of $^{35}$S-L-Methionine, followed by selective harvesting of the $^{35}$S-sulphate by precipitation with Barium as described in Chapter 2.10.2.

This $^{35}$S assay system was also used to test the feasibility of manoeuvres aimed at diverting sulphur metabolism away from sulphate (sulphuric acid) towards non-acidic end products such as taurine. Boelens et al. (2003) observed that trauma patients frequently had low plasma glutamine and taurine concentrations and, following previous reports on the beneficial effect of glutamine supplementation in decreasing infections (Houdijk et al. 1998) and restoring body-fluid distribution (Scheltinga et al. 1991), they hypothesised that glutamine supplementation may

help to increase plasma taurine concentrations, an amino acid that is involved in these two processes. They found that enteral glutamine supplementation resulted in increased plasma taurine levels, a finding that they also confirmed in surgically stressed rats (Boelens et al. 2003). In the current study, it was hypothesised that glutamine supplementation (possibly by trapping sulphur in the form of taurine) might decrease sulphuric acid generation in both L6 and HepG2 cells.

**Parts B and C** investigate *in vitro* (using L6-G8C5 myotubes) whether stress signals generated by acidosis or exercise might induce longer term inhibition of the SNAT2 transporter than would be obtained by the direct inhibitory effect of low pH on the SNAT2 protein (Baird et al. 2006). Two potential stress signals were investigated:

## Part B: The effect of glucocorticoid

It is widely accepted that acidosis is an important contributor to muscle wasting and impaired growth (Mitch, 2006; Reaich et al. 1993; Kalhoff et al. 1993; McSherry and Morris, 1978). However, *in vivo* low pH is not the only catabolic signal generated by acidosis (Chapter 1.7.2): metabolic acidosis results in an increase in glucocorticoid secretion (May et al. 1986; Pickering 2003) and a positive correlation has been identified between muscle proteolysis and plasma cortisol levels (Garibotto et al. 1994).

As glucocorticoid and acid appear to work in a coordinated manner *in vivo* (Chapter 1.7.2), it was hypothesised that like acid, glucocorticoid might also be acting through an inhibitory effect upon SNAT2.

At present this is difficult to test directly *in vivo*. System A/SNAT2 activity has been measured in human skeletal muscle *in vivo* and has been shown to be inhibited in CKD (Asola et al. 2001), but this involves measuring uptake of ^{11}C-MeAIB which requires a cyclotron adjacent to the clinical research facility. Plasma glucocorticoid concentration (cortisol) is reported to be elevated in CKD patients (McDonald et al. 1979; Rosman et al. 1982), but the assays for cortisol are prone to possible interference by glucuronide conjugates which circulate at high concentrations in patients with CKD (Nolan et al. 1981). For these reasons, the effects exerted by glucocorticoid on SNAT2 were investigated *in vitro*. Initial experiments in this laboratory (H. Butler,

unpublished observations) had indicated that SNAT2 activity in L6-G8C5 myotubes is inhibited after 4 – 24h of exposure to Dexamethasone. The experiments described below extend and characterise this observation. It was therefore hypothesised that glucocorticoids like acidosis, would cause a decrease in SNAT2 transport activity and would also result in similar reductions in intracellular glutamine concentrations. If the action of glucocorticoids and acidosis is through distinct mechanisms, the effects of the two combined would be expected to be additive.

**Part C: The effect of mechanical stretch upon this transporter.**

It is well known that mechanical stimuli play a key role in the maintenance of skeletal muscle mass *in vivo* and *in vitro* (reviewed in Chapter 1.17). Such signals arising from mechanical stress during exercise could potentially modulate System A/SNAT2 activity. Henriksen et al. (1992) found that an acute exercise bout in rats increased the System A transporter activity in skeletal muscle in the presence of a submaximal concentration of IGF-1 and two other studies have also reported an acute stimulatory effect of exercise on System A activity in rat (Henriksen et al. 1993; King, 1994). This is of interest because of recent evidence that SNAT2 can activate PI3-K and PKB and increase phosphorylation of proteins downstream of mTOR (Evans et al. 2007; Evans et al. 2008) leading to the hypothesis that this transporter may be a sensor of mechanical stretch and may mediate the anabolic signals reviewed above.

Clearly such acute stimulatory effects on System A transporters cannot explain the collapse of muscle/ECF amino acid gradients observed in the exercising patients in Chapter 3. However, long-term effects of mechanical stress on System A activity in skeletal muscle cells (as in the patients) have not been investigated. The final part of this chapter therefore investigated the effect of passive continuous stretch and cyclic stretch (up to 48h duration) on the activity and expression of the SNAT2 transporter in L6-G8C5 myotubes and it was hypothesised that both passive and cyclic stretch would cause an up-regulation of SNAT2 transport activity through an increase in transcriptional regulation.

## 5.2 Methods

## 5.2.1 Cell culture

Cells used in this thesis were L6-G8C5 rat skeletal muscle from the European Collection of Animal Cultures (ref. 92121114) and human Hep G2 hepatoma (ref.85011430).

### 5.2.1.1 Maintenance of cell lines

L6-G8C5 cells were routinely passaged and were grown to confluence in Growth Medium containing Dulbecco's Modified Essential Medium (DMEM) (Invitrogen, UK, 11880-) supplemented with 10% v/v heat inactivated foetal bovine serum (FBS) (Invitrogen, UK, 10270) (heat inactivated by incubation at 56°C for 30 minutes), 2mM glutamine (Invitrogen, UK 25030) 1% v/v penicillin-strepomycin (Invitrogen, UK 15140) and 10mg/L phenol red (Sigma, Dorset, UK, P5530).

Hep G2 cells were grown to confluence in Growth Medium containing Minimum Essential Medium (MEM) (Invitrogen, UK, 21090) supplemented with 10% v/v FBS, 2mM glutamine, 1% v/v penicillin-strepomycin (Invitrogen, UK, 15140) and 1% v/v non-essential amino acids (Invitrogen, UK, 11140).

### 5.2.1.2 Routine cell passaging

L6-G8C5 cells on 9cm diameter Petri dishes were routinely sub-cultured every 4 days to prevent them becoming more than 75% confluent. Growth Medium was aspirated and cells were rinsed with 5ml Hanks Balanced Salt Solution (HBSS) (Invitrogen, UK, 24020) to remove serum. Trypsin- EDTA (2ml) (Invitrogen, UK, 25300) was added to the cells which were then incubated at 37°C for 10 minutes until detached from the bottom of the plate. Growth medium was added to inactivate the Trypsin-EDTA and this cell suspension was centrifuged at 200g for 5 minutes at room temperature. The cells were resuspended in Growth Medium seeded at a density of 16 X $10^4$ per 9cm Petri and cultured at 37°C under humidified 95% air and 5% $CO_2$.

Hep G2 cells in 25cm$^2$ flasks were routinely sub-cultured every 4 days to prevent becoming more than 75% confluent. Growth Medium was aspirated and cells were rinsed with 5ml HBSS (Invitrogen, UK, 24020) to remove serum. Trypsin- EDTA (2ml) (Invitrogen, UK, 25300) was added to the cells which were then incubated at 37°C for 10 minutes until detached from the bottom of the plate. Growth medium was added to inactivate the Trypsin-EDTA and this cell suspension was centrifuged at 200g for 5 minutes at room temperature, The cells were resuspended in 6ml Growth Medium. 1ml of cell suspension and 4ml Growth Medium was added to a 25cm$^2$ flask and cultured at 37°C under humidified 95% air and 5% $CO_2$.

## 5.2.1.3 Differentiation of myoblasts into myotubes for experiments

Cells were seeded on 35mm diameter 6-well plates at 43 X 10$^4$ cells/well in Growth Medium. When the L6-G8C5 cells had reached confluence, which occurred after 3 days, the Growth Medium was replaced with MEM (Invitrogen, UK, 21090) supplemented with 2% v/v heat inactivated FBS (Invitrogen, UK, 10270), 2mM glutamine, and 1% v/v penicillin-streptomycin (Invitrogen, UK, 15140), which was replenished every 2 days. By 8 days after seeding, the cells had differentiated into fused myotubes suitable for experimental incubations.

## 5.2.1.4 Experimentation

Confluent myotubes were rinsed with HBSS (Invitrogen, UK, 24020) and incubated at 37°C, under humidified 95% air / 5% $CO_2$ in the relevant test medium. Test medium consisted of MEM supplemented with 2%v/v Dialysed Foetal Bovine Serum (DFBS) (Invitrogen, UK, 26400) and 1% v/v Penicillin-Streptomycin (Invitrogen, UK, 15140) at pH 7.4 unless otherwise stated. Other components of the test media varied depending upon the experimental conditions and are detailed in figure legends in this chapter. Test media for amino acid free conditions contained Earls Balanced Salts Solution (Sigma, Dorset, UK, E2888) supplemented with 1% v/v Penicillin-Streptomycin with MEM vitamins (Invitrogen, UK, 11120037) at pH 7.4. unless otherwise stated.

**5.2.1.5 Passive stretch technique**

Adapted from that described in Rauch and Loughna, (2008). L6-G8C5 cells were seeded on type I collagen coated Bio-flex six well plates (Flexcell International Corporation, NC, USA, BF-3001C). Once the cells were confluent and fused, a glass marble (15mm diameter) was placed centrally under each well with a 3kg weight on top of the culture plate to create approximately an 18% linear stretch of the culture surface. Myotubes were stretched for 17 or 48h in media containing 2mM glutamine. The % linear stretch was calculated using trigonometry from the measured vertical movement shown in Figure 2.1A as described by Rauch and Loughna (2008).

**5.2.1.6 Cyclic stretch technique**

L6-G8C5 cells were seeded on type I collagen coated Bio-flex six well Flexcell plates and stretched using Flexercell Strain Unit Fx-3000 (Flexcell International Coproration, NC, USA). Cells were cyclically stretched using the same protocol as Goto et al. (2003), a 2 second sine wave stretch with a 4 second release that resulted in an 18% maximum stretch. In each experiment a second plate of cells was cultured under the same conditions, but was not stretched and so acted as a control. Stretch pattern is depicted in Figure 2.2.
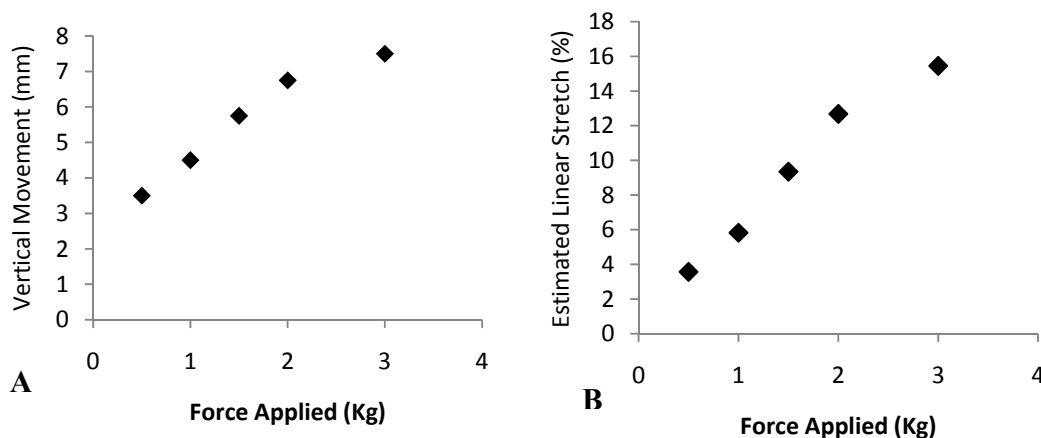


**Figure 5.1. Graphs indicating (A) the measured vertical displacement of the highest point on the culture surface on applying various loads to the top of the culture plate. (B) the accompanying calculated linear stretch of the L6-G8C5 myotubes.**
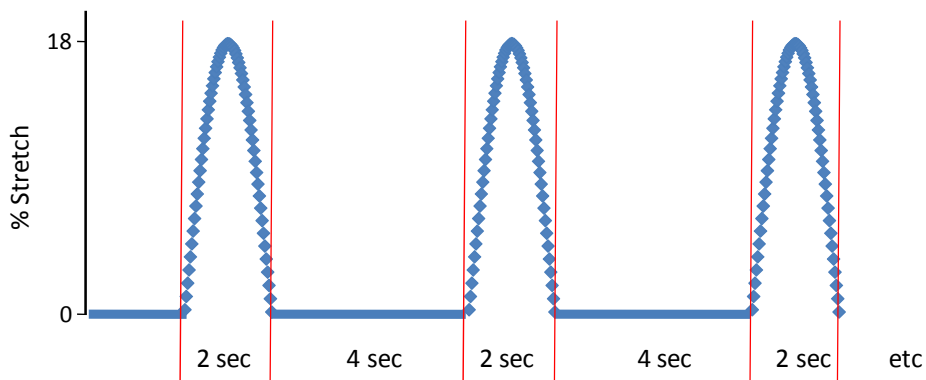
**Figure 5.2. Stretch applied to L6-G8C5 myotubes during cyclic stretch, a 2 second sine wave stretch with a 4 second release resulting in an 18% stretch.**

## 5.2.2 Radio-isotope techniques

### 5.2.2.1 Detection of [35]Sulphate production by L6 and Hep G2 cells by ^{35}S-methionine labelling

Confluent cultures on 35mm wells were incubated in sulphate-free MEM (Invitrogen, UK, 51200) with L-^{35}S-Met at 10.25μCi/ml; 1175μCi/μmol for 24h. Following incubation, 800μl of medium was sampled and placed into a 1.5ml Eppendorf tube on ice. 20% w/v TCA (267μl) was added to the samples, vortexed and incubated on ice for 10 minutes to precipitate the protein. The tubes were microcentrifuged at 13,000rpm for 10 minutes at 4˚C and 700μl of the supernatant was transferred to a new tube. To this, 100mM $Na_2SO_4$ (100μl) was added followed by 1% w/v $BaCl_2$ (200μl) and vortexed. The tubes were microcentrifuged at 13,000rpm for 10 minutes at 4˚C to spin down the ^{35}S-labelled barium sulphate. The supernatant was aspirated and the pellet was vortexed to help break it up and washed twice in 100mM $Na_2SO_4$ (1ml), by vortexing and microcentrifugation at 13,000rpm for 10 minutes at 4˚C. To the pellet, 1ml Ecoscint A Scintillant (National Diagnostics, Hesssle, UK) was added and the tubes vortexed thoroughly and immediately transferred to scintillantion vials containing 3ml of Ecoscint A Scintillant and counted on an LKB 1219 liquid scintillation counter.

Recovery measurements using fresh culture medium spiked with $^{35}SO_4^{2-}$ demonstrated that approximately 70% of the radioactive sulphate was detected in the scintillation vial and this recovery was essentially constant over a wide range of sulphate concentrations (Table 2.6).

**Table 5.1. $^{35}SO_4^{2-}$ Recovery Measurements**

(Pooled data from two experiments each performed in triplicate)

| | Nominal Dilution Factor | | | | | |
|---|---|---|---|---|---|---|
| | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ |
| **Mean added $^{35}SO_4^{2-}$ (cpm)** | 1330000 | 135000 | 16200 | 1773 | 380 | 124 |
| **% Recovery** | $67 \pm 3$ | $70 \pm 4$ | $66 \pm 5$ | $71 \pm 4$ | $73 \pm 4$ | $71 \pm 4$ |

When culture medium was incubated with L-$^{35}$S-Met in the absence of cells, $^{35}SO_4^{2-}$ radioactivity was almost indistinguishable from background in the final Barium Sulphate pellet (data not shown).


### 5.2.2.2 $^{14}$C MeAIB uptake measurements

SNAT2 (System A) transport activity was measured by the incorporation of $^{14}$C MeAIB from extracellular medium into the intracellular space of L6 cells cultured on 22mm diameter plates.

Following incubation of cultures on 35mm wells with test media, medium was aspirated and the cells were washed twice with 2ml Hepes Buffered Saline (HBS) (see Appendix A). HBS (1ml) was added to all the wells, and to the wells to be used as transport blanks, unlabelled MeAIB was added (10μl) to give a final concentration of 10mM. $^{14}$C-MeAIB (20μl) containing 0.5mCi (18.5MBq/L) was added to all the wells and incubated for 5 minutes at room temperature. The $^{14}$C –labelled medium was aspirated and the cells washed 3 times with 2ml ice cold 0.9% NaCl followed by storage at -20˚C.


### 5.2.2.3 Processing plates to assess $^{14}$C-MeAIB incorporation into cells

Plates were thawed and each well scraped in 0.05M NaOH (400μl) and transferred to a 1.5ml Eppendorf tube. The tubes were incubated at 70˚C for 30 minutes in a water bath to digest the cells. Cell digests (110μl) were mixed with 4ml Ecoscint A Scintillant (National Diagnostics, Hessle, UK) in a scintillation vial and left in the dark overnight to allow chemiluminesence to decay before counting on a LKB 1219 liquid scintillation counter with quench correction.

**5.2.2.4 Northern Blotting**

For cell culture experiments in which plentiful RNA was available, expression of SNAT2 mRNA was quantified by Northern blotting. RNA (30μg) extracted following the procedure described in Section 2.9.1 was resolved by denaturing gel electrophoresis on a 1% w/v agarose 1.9% formaldehyde gel in MOPS/EDTA/sodium acetate. RNA was loaded using RNA gel loading sample buffer (see Appendix A) with Ethidium Bromide (0.5μg/ml) to visualise the bands and the gel was run at 80V for approximately 1 hour. The integrity of the RNA was checked using a transilluminator and the gel was washed in DEPC water to remove the formaldehyde. The gel was blotted overnight onto nylon membrane (GE Health Care Life Sciences, Little Chalfont, UK), which was then cross linked by UV illumination the following morning. The membranes were then incubated with pre-hybridisation solution (see Appendix A) for 4 hours at 42˚C. Membranes were hybridised overnight with $^{32}$P labelled cDNA probes using a Prime-a-gene kit, (Promega, Hampshire, UK, TB049) for SNAT2 and cyclophilin. The probe templates were a full length cDNA for rat SNAT2 (a kind gift from Professor J. Erickson as described in Yao et al. 2000) and for cyclophilin (a kind gift from Dr Izabella Pawluczyk from SmithKlein, Beecham, UK). After the incubation, the membrane was washed twice in a low stringency wash containing 2X SSPE and 0.2% SDS for 10 minutes at room temperature followed by two more washes in high stringency wash containing 0.2X SSPE and 0.2% SDS for 30 minutes at 65˚C. The hybridisation signal was visualised using autoradiography. The membrane was placed on top of a Kodak X-OMAT XAR-5 film and a Kodak X-OMAT LS film (Sigma, Dorset, UK) and exposed for 8-24h. The LS film was developed first, and the exposure time for the XAR-5 film was adjusted according to the signal obtained on the LS film.

## 5.3 Results

## Part A

### *In vitro* modelling of the factors controlling the production of sulphuric acid ($^{35}SO_4^{2-}$) in cultured L6 skeletal muscle cells and HepG2 hepatocytes

### 5.3.1 Characterisation of $^{35}SO_4^{2-}$ output

The time course of the accumulation of ($^{35}SO_4^{2-}$) in the culture medium when the cells were incubated with $^{35}$S-L-Methionine is shown for L6-G8C5 myotubes in Figure 5.1 and for HepG2 cells in Figure 5.2. As a positive control, flux through the pathway from L-Methionine to sulphuric acid was stimulated in some cultures by increasing the L-Methionine concentration in the medium from 100 to 1100µM (- the specific radio-activity of the $^{35}$S-L-Methionine in the medium in those cultures was kept constant by also increasing the amount of $^{35}$S-L-Methionine added).

As expected, $^{35}SO_4^{2-}$ production was strongly stimulated in the positive control cultures in both L6-G8C5 and HepG2 cells ($P<0.001$). The basal rate of production of sulphate was approximately 10 times faster in HepG2 cells than in L6-G8C5 myotubes, consistent with the more rapid rate of sulphuric acid production reported in liver compared with skeletal muscle (Garcia and Stipanuk, 1992). Both cell types exhibited a time-dependent generation of $^{35}SO_4^{2-}$ from $^{35}$S-L-Methionine. Sulphate generated from L6-G8C5 cells continued to accumulate over 72h ($P<0.05$) (Figure 5.1). In HepG2 cells a paradoxical fall in the concentration of $^{35}SO_4^{2-}$ was observed in the medium at 72h (Figure 5.2) (implying re-incorporation of the $^{35}SO_4^{2-}$ into a pool not precipitated by barium) and this was accompanied by loss of cell viability owing to exhaustion of the medium. For that reason all incubations in subsequent experiments were restricted to 24h or less. It would be important in future experiments to perform measurements of protein breakdown to see if sulphate generation does actually cause increases in protein degradation that is implied from the correlations seen in Chapter 4.
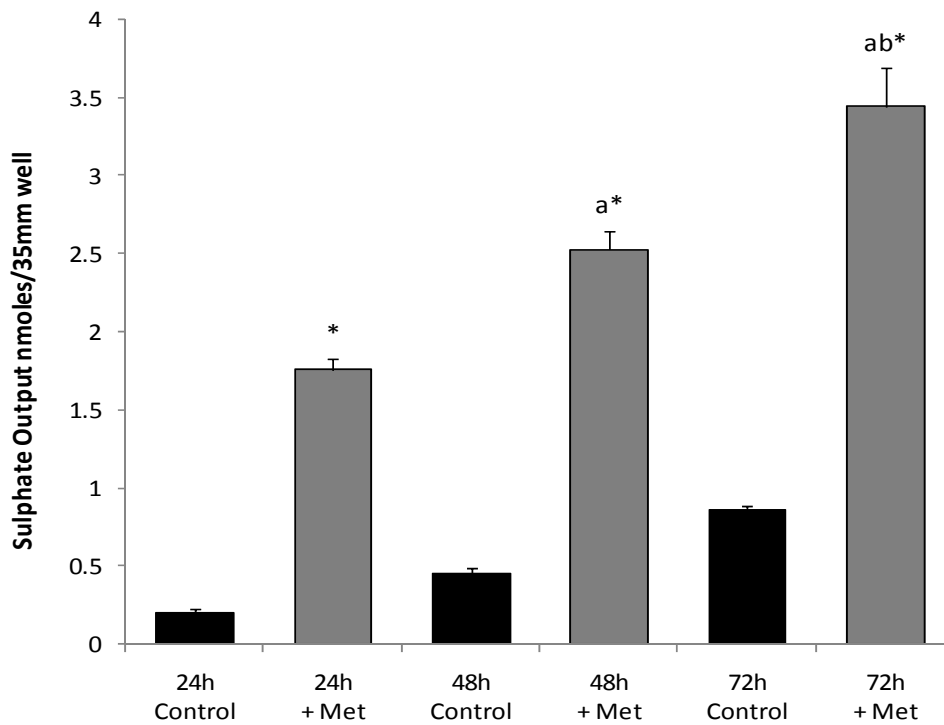
**Figure 5.3. Time course of $^{35}SO_4^{2-}$ production from L6-G8C5 myotubes and the effect of L-Methionine (Met) loading of the medium.** Media contained sulphate-free MEM with 100μM L-Methionine, 2mM L-Gln and 2% dialysed foetal bovine serum. "+ Met" cultures were incubated with 1100μM L-Methionine. Data are from a representative experiment (with at least 3 replicate culture wells per treatment). *denotes significant difference from corresponding control condition for that time point (P<0.001), [a]denotes significant difference from 24h + Met condition (P<0.05) and [b]denotes significant difference from 48h + Met condition (P<0.05).
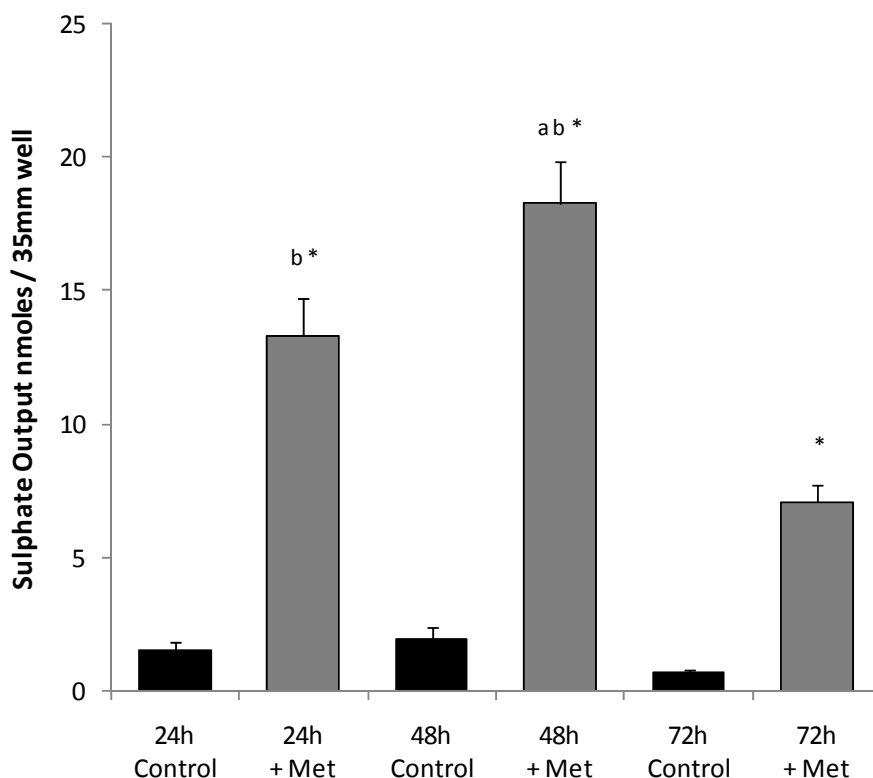
**Figure 5.4. Time course of $^{35}SO_4^{2-}$ production from HepG2 cells and the effect of L-Methionine (Met) loading of the medium.** Media contained sulphate-free MEM with 100µM L-Methionine, 2mM L-Gln and 2% dialysed foetal bovine serum. "+ Met" cultures were incubated with 1100µM L-Methionine. Data are from a representative experiment (with at least 3 replicate culture wells per treatment). *denotes a significant difference from corresponding control condition (P<0.001), [a]denotes significant difference from 24h + Met condition (P<0.05) and [b] denotes significant difference from 72h + Met condition (P<0.05).

## 5.3.2 Effect of inhibitors of protein metabolism on $^{35}SO_4^{2-}$ output

Incubation with the proteasome inhibitor MG132 (to block protein degradation) and with cycloheximide (CYC) (to inhibit protein synthesis) had no significant effect on sulphate generation in L6-G8C5 myotubes (Figure 5.3). However, with these treatments some effect was observed in HepG2 cells. MG132 appeared to reduce sulphate generation when used at a concentration of 10µM, but this fell just short of statistical significance (P = 0.064; Figure 5.4). When CYC was administered at a concentration of 50µM there was also a significant reduction in sulphate production compared to the control condition (P = 0.023).
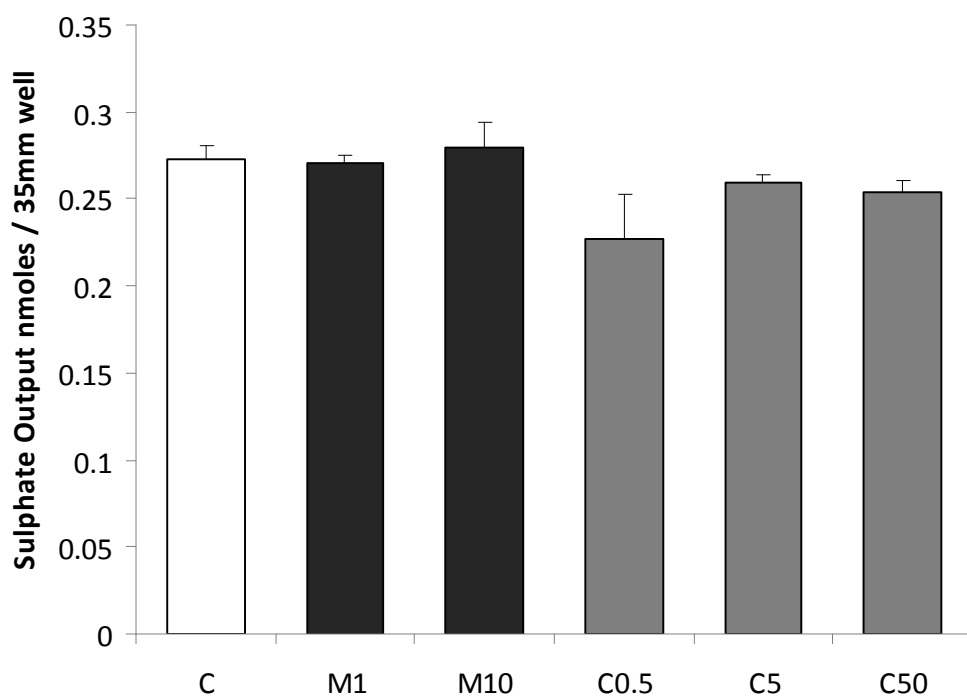
**Figure 5.5. The effect of incubation with MG132 or Cycloheximide on $^{35}SO_4^{2-}$ production in L6-G8C5 myotubes.** To maintain cell viability, incubations were restricted to 7h. Media contained sulphate-free MEM with 100µM L-Methionine, 2mM L-Gln and 2% dialysed foetal bovine serum. Condition C served as the control, whilst M1 contained MG132 (1µM) M10, MG132 (10µM), C0.5, Cycloheximide (0.5µM), C5, Cycloheximide (5µM) and C50 contained Cycloheximide (50µM). Data are from a representative experiment (with at least 3 replicate culture wells per treatment).
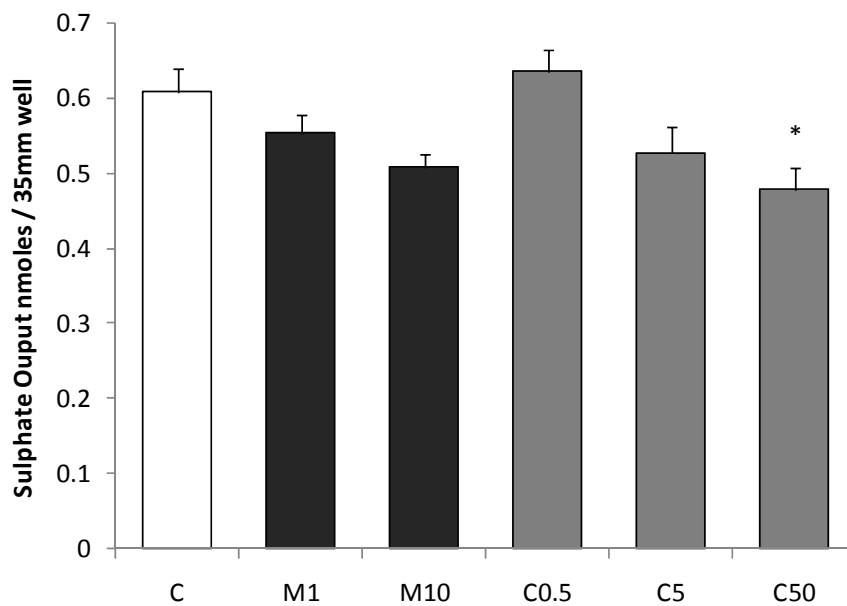
**Figure 5.6. The effect of incubation with MG132 or Cycloheximide on $^{35}SO_4^{2-}$ production in HepG2 cells.** To maintain cell viability, incubations were restricted to 7h. Media contained sulphate-free MEM with 100µM L-Methionine, 2mM L-Gln and 2% dialysed foetal bovine serum. Condition C served as the control, whilst M1 contained MG132 (1µM) M10, MG132 (10µM), C0.5, Cycloheximide (0.5µM), C5, Cycloheximide (5µM) and C50 contained Cycloheximide (50µM). Data are from a representative experiment (with at least 3 replicate culture wells per treatment). *denotes a significant difference from condition C (P<0.05).

## 5.3.3 Effect of non-sulphur amino acids on $^{35}SO_4^{2-}$ output

Both in L6-G8C5 myotubes and HepG2 cells, L-Glutamine starvation significantly increased sulphate production compared with control cultures with 2mM L-Glutamine, but this effect was less marked in HepG2 (Figures 5.5 and 5.6). Increasing L-Glutamine to a supraphysiological concentration (8mM) had no further suppressive effect. Addition of L-Glutamate (a potential method for reversing the aspartate aminotransferase step in the sulphuric acid production pathway (Chapter 1, Figure 1.5); or competitive inhibition of System A transporters with a saturating dose of their non-metabolisable substrate MeAIB (a potential method for blocking L-Methionine influx) also had no further suppressive effect in the presence of 2mM L-Glutamine.
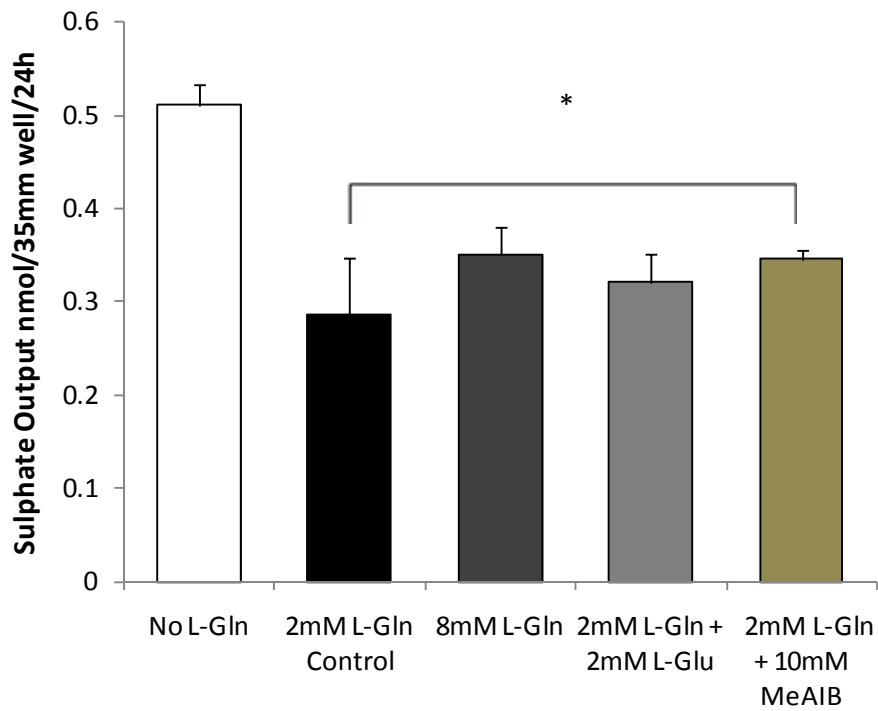
**Figure 5.7. The effect of possible therapeutic agents on $^{35}SO_4{}^{2-}$ production in L6-G8C5 myotubes.** Media contained sulphate-free MEM with 100μM L-Methionine, 2% dialysed foetal bovine serum, and the specified amino acid concentrations. Data are from a representative experiment (with at least 3 replicate culture wells per treatment). Cells were incubated for 24h with these media. *denotes a significant difference from L-Glutamine starved condition (P<0.05).
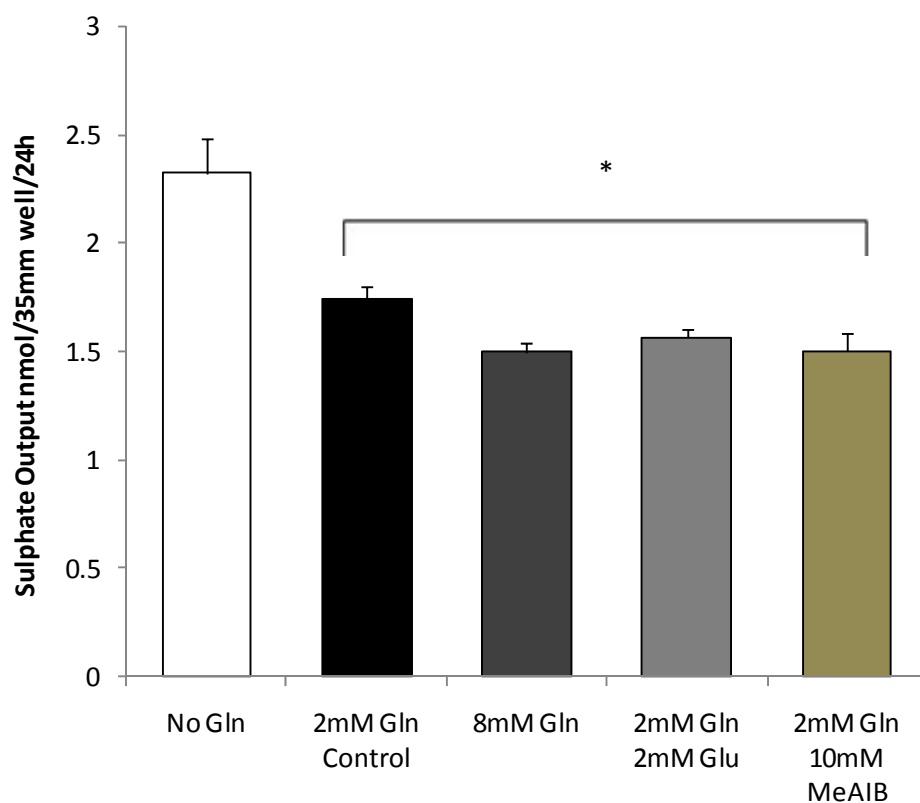
**Figure 5.8. The effect of possible therapeutic agents on $^{35}SO_4^{2-}$ production in HepG2 cells.** Media contained sulphate-free MEM with 100μM L-Methionine, 2% dialysed foetal bovine serum, and the specified amino acid concentrations. Data are pooled from 2 experiments (with at least 3 replicate culture wells in each experiment). Cells were incubated for 24h with these media. *denotes a significant difference from L-Glutamine starved condition (P<0.05).

## Part B

### 5.3.4 The effect of glucocorticoid on the activity and expression of the SNAT2 amino acid transporter

As previously demonstrated by Evans et al. (2008), these results show acidosis (pH 7.1) significantly reduced SNAT2 transport activity in L6-G8C5 myotubes compared to that in control incubations at pH 7.4 (by approximately 42%, P<0.001; Figure 5.7). Following a 7 hour incubation with 500nM dexamethasone at pH 7.4, SNAT2 transport activity was also reduced by approximately 40% (P<0.001). The effects of a reduction in pH and the addition of 500nM dexamethasone on transport activity were found to be approximately additive (Figure 5.7).
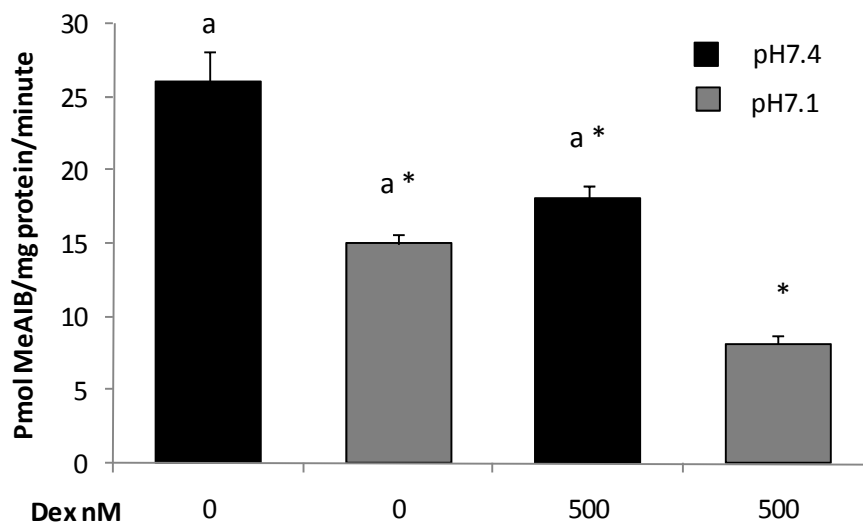
**Figure 5.9. Effect on SNAT2 transport activity of 7h incubations at the specified pH with and without 500nM dexamethasone (Dex) in L6-G8C5 myotubes.** Media were based on MEM and contained 2mM L-Gln with 2% dialysed foetal bovine serum. Pooled data from 3 experiments are shown with 4 replicate culture wells in each experiment. The pH of the medium during the transport assay (Chapter 2.11.1) was maintained at the same value as that in the preceding 7h incubation in culture medium. *denotes a significant difference from the pH 7.4 control value without Dex (P<0.05). [a]denotes a significant difference from 500mM Dex pH 7.1 (P<0.05).

The time course of the effect of glucocorticoid on SNAT2 transporter activity was biphasic (Figure 5.8). A significant reduction in transport activity was observed in the first 7h of incubation with Dexamethasone but, following this initial decrease, a gradual recovery was observed, with net stimulation of the transport activity relative to the control condition after 48h, (P = 0.009).
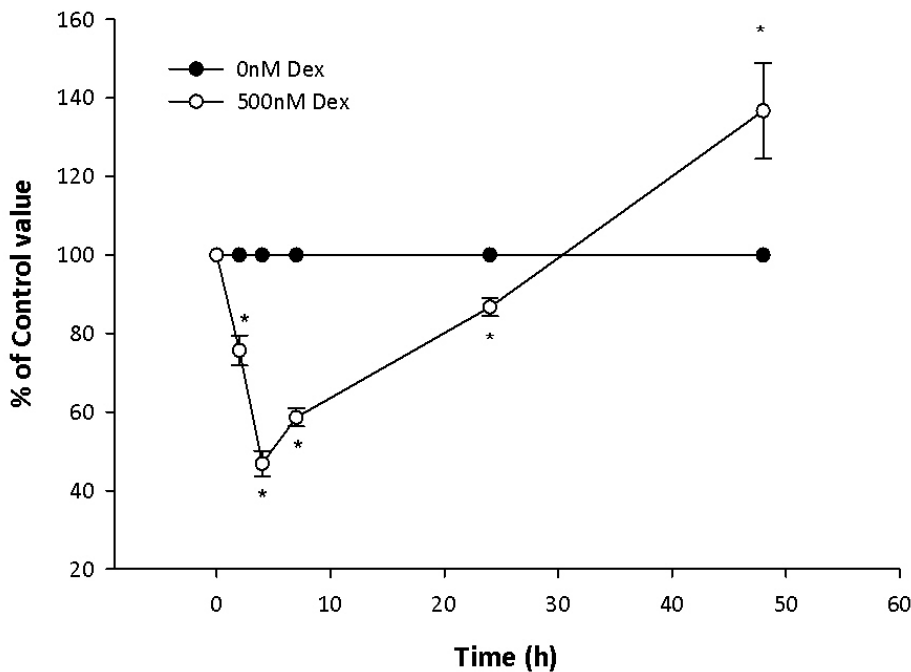
**Figure 5.10. Time course of the effect of 500nM dexamethasone (Dex) on SNAT2 transport activity at pH 7.4 expressed as a percentage of the corresponding control value measured without Dex.** Media were based on MEM and contained 2mM L-Gln with 2% dialysed foetal bovine serum. Pooled data from at least 3 independent experiments are shown. *denotes a significant difference relative to the control value at the corresponding time point (P<0.05).

Consistent with changes in transport rates, there were also changes observed in the intracellular amino acid concentrations, especially L-Glutamine a major SNAT2 substrate. After 7h incubation with 500nM Dexamethasone at pH 7.4, the intracellular L-Glutamine concentration had declined by approximately 22%, just short of statistical significance (P = 0.053). Lowering the pH of the media from 7.4 to 7.1 also reduced L-Glutamine concentrations (P = 0.044) and, as observed in the effects on transport activity, the effects of acidosis and glucocorticoid on intracellular L-Glutamine concentration were additive (Figure 5.9A). However, after 48h (Figure 5.9B), the effect of Dex was no longer detectable, consistent with the recovery in SNAT2 transport activity that was seen at this time point (Figure 5.8).
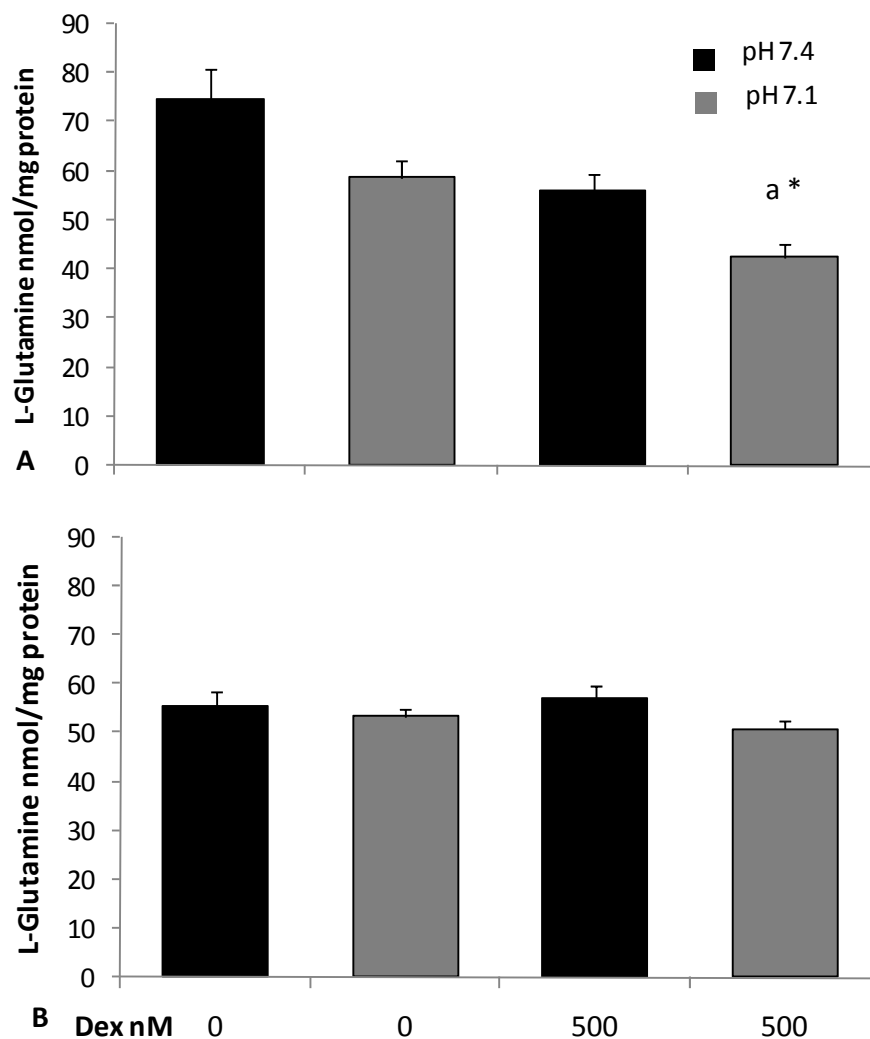
**Figure 5.11. Effect of pH and 500nM dexamethasone (Dex) on intracellular L-Glutamine concentration in L6-G8C5 myotubes after 4h (A) and 48h (B) incubation.** Pooled data from 3 experiments. *denotes significant difference from pH 7.4 control condition without Dex (P<0.05). [a] denotes significant difference from pH 7.1 condition without Dex (P<0.05).

The inhibition of transporter activity following incubation with Dexamethasone was abolished both by RU38486, a specific glucocorticoid receptor antagonist; and by Actinomycin D, an inhibitor of transcription (Figure 5.10), indicating that Dexamethasone was acting through a classical transcription-dependent pathway mediated by glucocorticoid receptors. However, by Northern blotting no change was detected in the expression of the SNAT2 transporter in response to Dexamethasone even after 17h (Figure 5.11). An apparent increase in SNAT2 expression was observed followed incubation with acidified media, but due to the wide scatter in the results, this failed to reach statistical significance (P = 0.151).
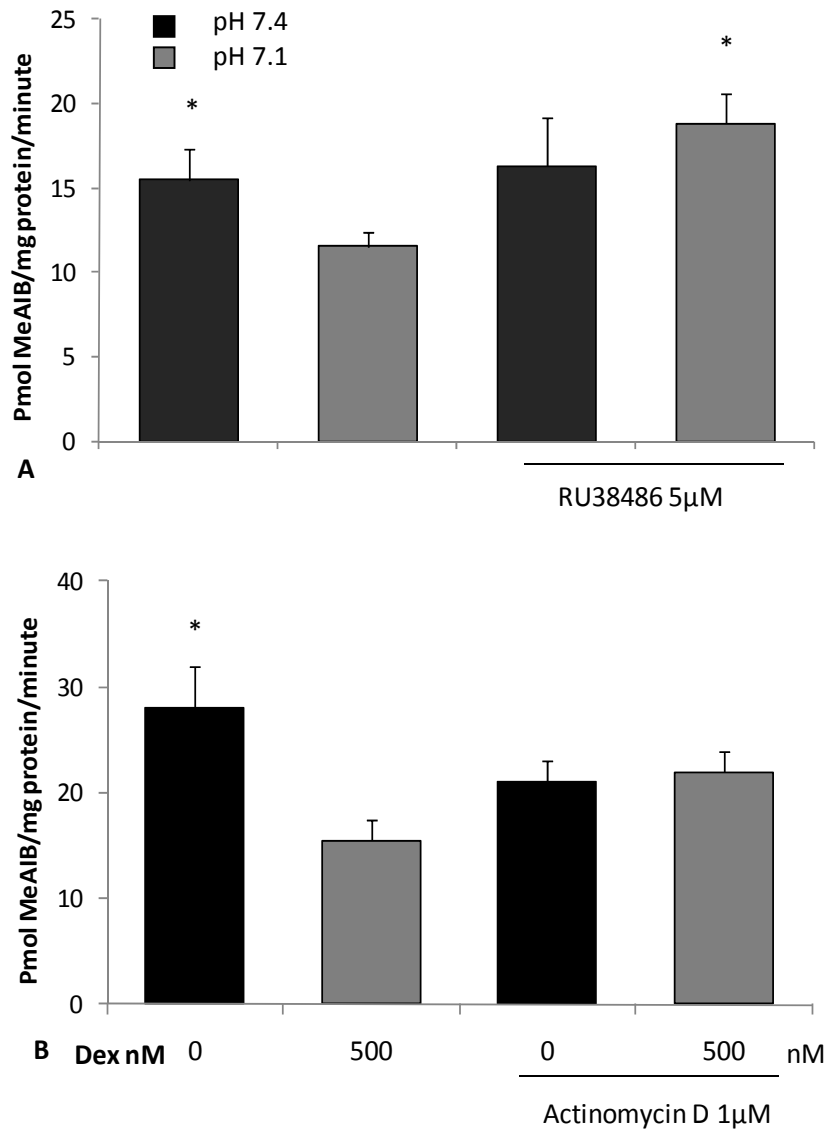
**Figure 5.12. Effect of inhibition of the glucocorticoid receptor with RU38486 (A) and of transcription with Actinomycin D (B) on SNAT2 transport activity in L6-G8C5 myotubes with or without 500mM Dexamethasone (Dex).** Media were based on MEM and contained 2mM L-Gln with 2% dialysed foetal bovine serum. Pooled data from 2 experiments are shown with 4 replicate culture wells for each treatment. Cultures were incubated with relevant test media for 4h. *denotes significant difference from treatment with 500nM Dex alone (P<0.05).
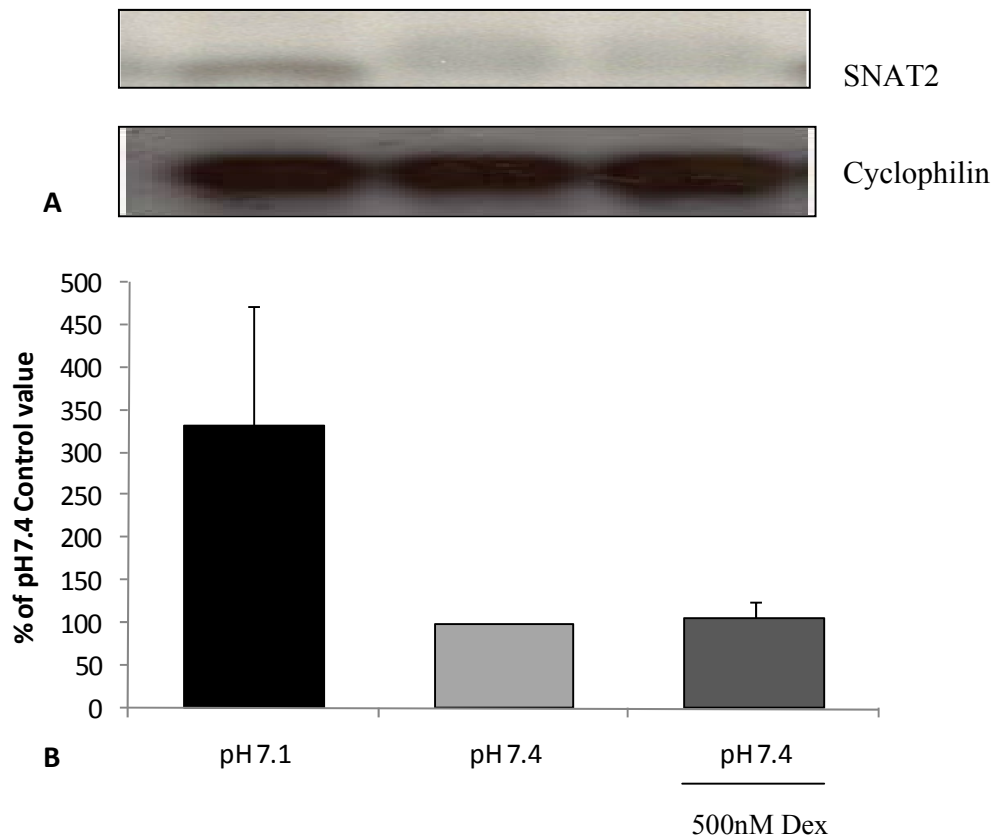
**Figure 5.13. Effect on SNAT2 expression in L6-G8C5 myotubes of 17h incubations at the specified pH with or without 500mM dexamethasone (dex) measured by Northern blotting.** Media were based on MEM and contained 2mM glutamine with 2% dialysed foetal bovine serum. (A) Representative Northern blot autoradiograph. (B) Pooled quantification data by densitometry from three experiments are shown with at least four replicate cultures for each treatment.

In contrast, assessment of SNAT2 protein expression in membrane preparations obtained by ultracentrifugation from L6-G8C5 myotubes showed a clear down-regulation of the protein in response to 4h of incubation with 500nM Dexamethasone (Figure 5.12A) and this was confirmed by quantification using densitometry ($P = 0.005$; Figure 5.12B). No corresponding effects were seen with the $\alpha$-Na, K ATPase ($P = 0.509$) or Annexin II ($P = 0.080$) that acted as control proteins. (Figures 5.12C and D). As reported previously (Evans et al. 2008) low pH had no reproducible effect on SNAT2 protein expression.
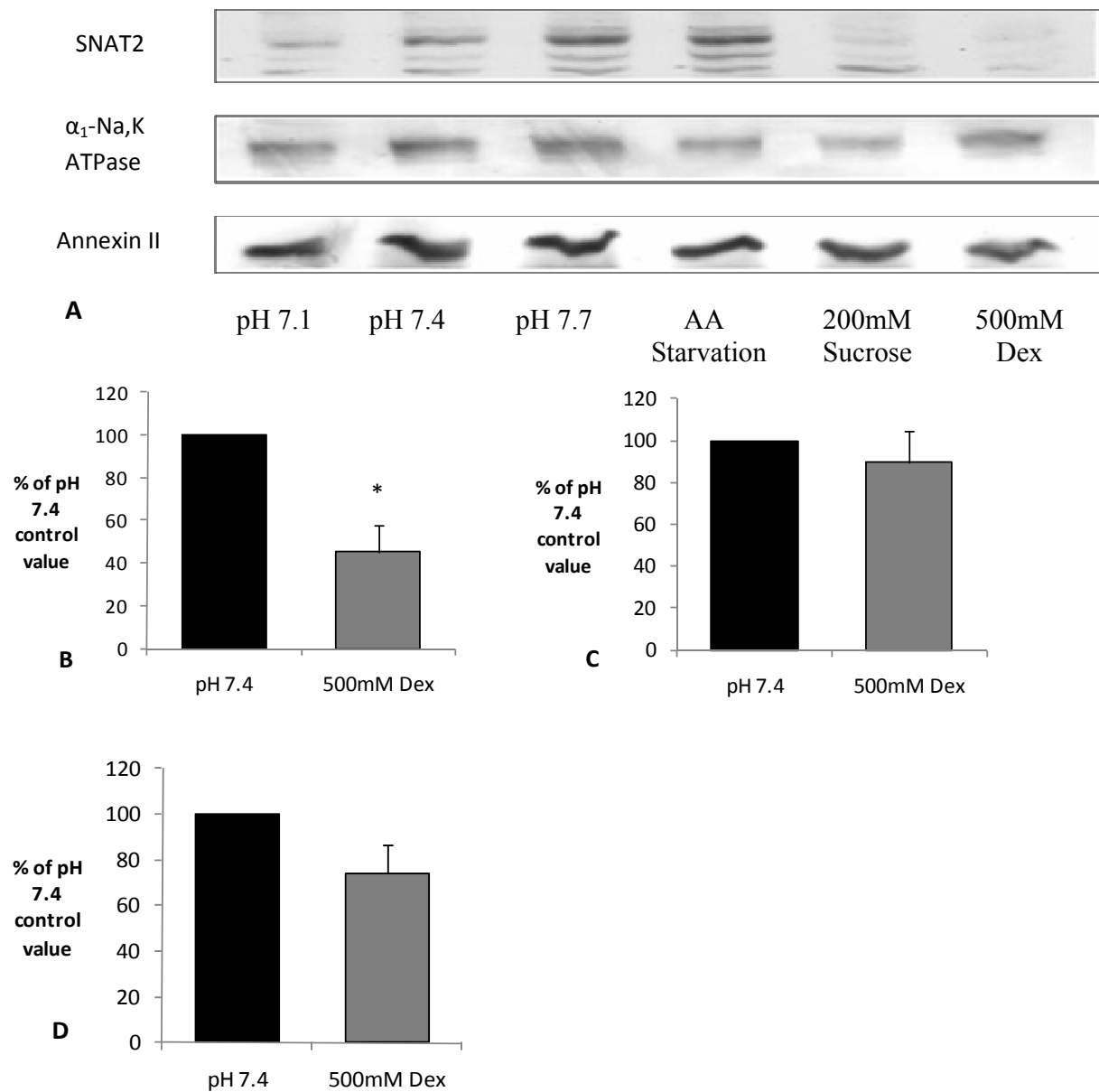
**Figure 5.14. Effect of pH, 500nM Dexamethasone and positive controls (amino acid starvation and hyperosmolality (200mM Sucrose)) on the expression of SNAT2 protein in cell membranes prepared as described in Chapter 2.5.1.** Media were based on MEM and contained 2mM L-Gln with 2% dialysed foetal bovine serum. L6-G8C5 myotubes were incubated with the relevant test media for 4h. In the amino acid starved condition myotubes were incubated in amino acid free media, as described in Section 5.2.1.4. Immunoblot (A) shows proteins that were separated by SDS-PAGE from a 170,000g membrane preparation, probed with antibodies specific for SNAT2 or $\alpha_1$-Na,K-ATPase or Annexin II as loading controls. Densitometry results for (B) SNAT2 (C) $\alpha_1$-Na,K-ATPase and (D) Annexin II are results of 5 pooled experiments. *denotes significant difference from pH 7.4 control value ($P<0.01$).

## Part C

### 5.3.5 The effect of mechanical stretch on SNAT2 transporter activity

It was observed that 48h of continuous passive stretch resulted in a significant increase in the transport activity of SNAT2 (P = 0.028), but only a small and statistically insignificant effect was obtained after 17h (P>0.05; Figure 5.13). However, under conditions of cyclic intermittent stretch (Figure 5.14), 17h was sufficient to observe an up-regulation in activity and this effect was even more apparent after 48h (P<0.05) (Figure 5.14). This was accompanied by an apparent increase in the expression of the transporter after both 17h and 48h (Figure 5.15), but at both time points, this failed to reach statistical significance.
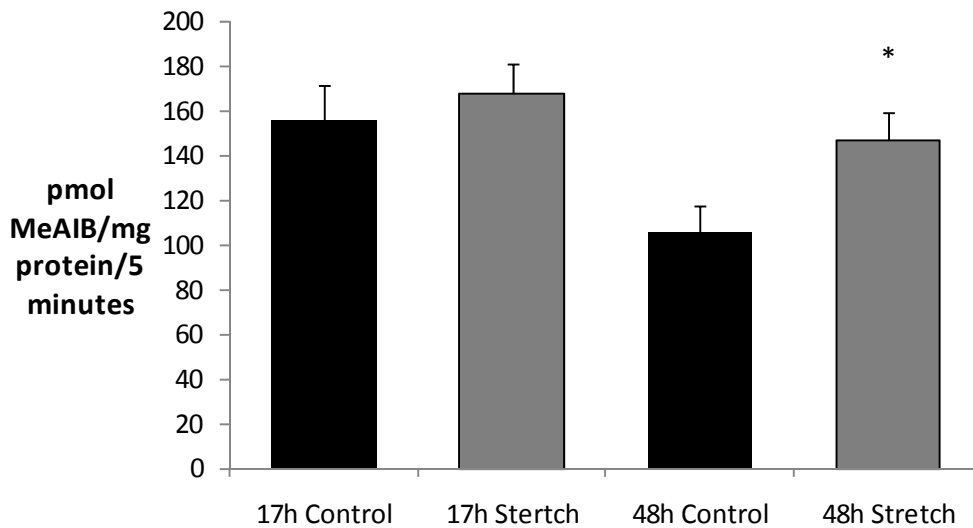


**Figure 5.15. Effect of 17h or 48h of passive continuous stretch on SNAT2 transporter activity.** Media were based on MEM and contained 2mM L-Gln with 2% dialysed foetal bovine serum. Pooled data from 2 experiments are shown with at least 4 replicate culture wells in each experiment. L6-G8C5 myotubes were cultured on Collagen-coated Flexcell silicon-based culture wells that, upon initiation of the experiment, were placed on glass spheres and stretched by loading with a 3kg weight placed on the top of the culture plate as described in Chapter 2.1.5 to maintain a constant passive stretch (Rauch and Loughna, 2008). *denotes significant difference compared with 48h control condition (P<0.05).
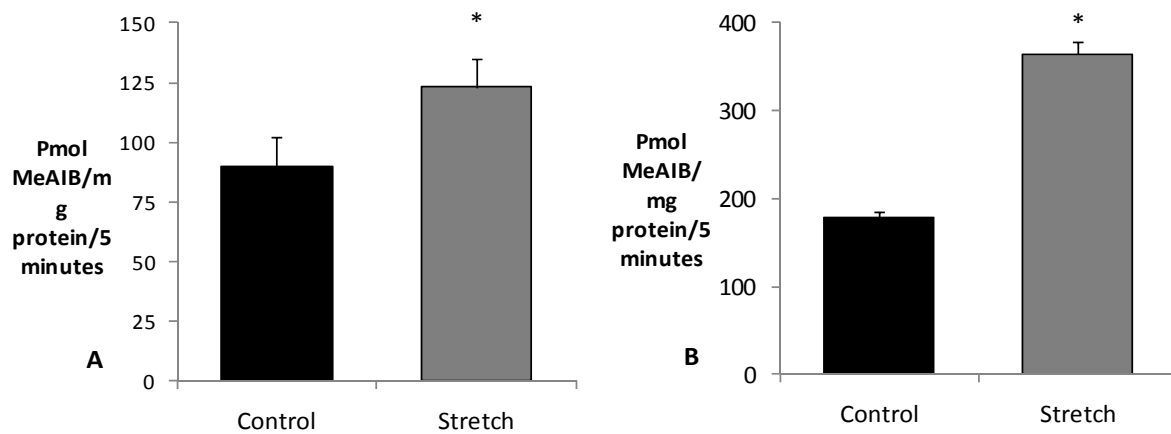
**Figure 5.16. Effect of 17h (A) and 48h (B) of cyclic stretch on SNAT2 transport activity in L6-G8C5 myotubes.** The culture medium was based on MEM at pH 7.4 and contained 2mM glutamine with 2% dialysed foetal bovine serum. Myotubes were cultured on Collagen-coated Flexcell silicon-based culture wells stretched using a Flexcell Fx3000 system set to 2 second sine wave stretch with 4 second release as described in Section 2.1.5. * denotes significant difference from control value ($P<0.05$). Pooled data from 2 experiments.
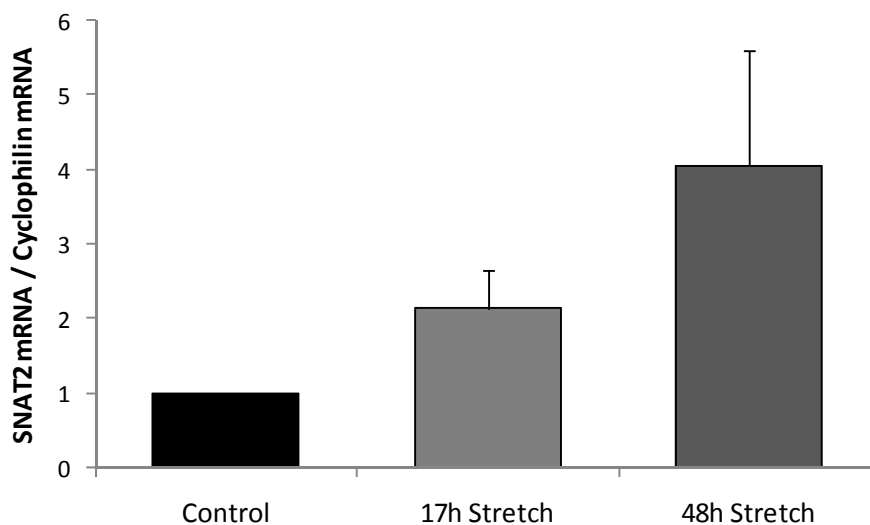


**Figure 5.17. Effect of cyclic stretch on expression of SNAT2 in L6-G8C5 myotubes assessed by Q-PCR as described in Chapter 2.9.7.** SNAT2 expression was related to Cyclophilin expression using the Pfaffl equation (Pfaffl, 2001). Myotubes were cultured and stretched exactly as described in Figure 5.14. The culture medium was based on MEM at pH 7.4 and contained 2mM glutamine with 2% dialysed foetal bovine serum. Pooled data from 2 experiments are shown with 3 replicate culture wells in each experiment.

## 5.4 Discussion

### 5.4.1 *In vitro* modelling of the factors controlling the production of sulphuric acid ($^{35}SO_4^{2-}$) in cultured L6 skeletal muscle cells and HepG2 hepatocytes

Characterisation of $^{35}SO_4^{2-}$ output from cells incubated with $^{35}$S-L-Methionine (Figures 5.1 to 5.6) indicate that this is a sensitive assay for sulphuric acid production *in vitro*, which detects (as expected) a high production rate in a hepatocyte cell line, a significantly lower rate in a skeletal muscle cell line, stimulation by L-Methionine loading, and no output in the absence of cells (Chapter 2.10.2). Recent data from this laboratory (L. Tooley and J.R. Brown, unpublished observations) have also confirmed that it is inhibited by amino-oxyacetate an inhibitor of aspartate aminotransferase in the sulphuric acid pathway (Figure 1.5). The detection here of sulphur amino acid catabolism to sulphuric acid in the L6-G8C5 *skeletal muscle* cell line is unlikely to be simply a cell culture artefact: sulphuric acid production has not been extensively documented in muscle, but has nevertheless been clearly demonstrated in rat skeletal muscle (Garcia and Stipanuk, 1992) and there are reports that it is stimulated under conditions of cachexia *in vivo* (Hack et al. 1996). It would be important in future experiments to perform measurements of proteolysis alongside measurements of sulphate production to investigate if sulphate generation is causally related to 3-MH excretion that is implied from the correlations seen in Chapter 4.

### 5.4.2 Effect of inhibitors of protein metabolism on $^{35}SO_4^{2-}$ output

The correlation between sulphate excretion and 3-MH excretion in Chapter 4 (Figure 4.4A) could have arisen as a result of stimulation of protein degradation by acidosis, or through the release of sulphur amino acids by protein degradation. If the second explanation is true, near complete inhibition of proteolysis *in vitro* with a high concentration of the proteasome inhibitor MG132 should strongly impair sulphate output. Conversely abolishing protein synthesis with a high dose of cycloheximide (an inhibitor of the elongation process in translation) should result in enhanced generation of sulphate due to increased availability of L-Cysteine and L-Methionine (Stipanuk, 2004). In practice, little effect of either of these treatments was seen in the L6-G8C5 myotubes and only modest effects in HepG2 cells, suggesting that, at least *in vitro*, sulphuric acid production is not strongly responsive to changes in protein metabolism. Ideally this result should be confirmed by a non-radio-isotopic method to guard against the possibility that sulphate

output from degraded protein has been missed because of incomplete labelling of sulphur in the protein pools.

## 5.4.3 Effect of non-sulphur amino acids on $^{35}SO_4^{2-}$ output

The observation that feeding L-Glutamine *in vivo* may divert sulphur catabolism from sulphuric acid towards taurine (Boelens et al. 2003) implies that L-Glutamine might be of therapeutic value in suppressing sulphuric acid production. The data from L6-G8C5 myotubes and HepG2 hepatocytes in Figures 5.5 and 5.6 suggest that this may be the case, but as intracellular taurine concentrations were not measured in the present study, the mechanism of the suppression of sulphate output by L-Glutamine is currently unknown. With the relatively high L-Glutamine concentrations tested here, it is possible that L-Glutamine also acted partly by competing with L-Methionine and L-Cysteine at plasma membrane transporters such as SNAT2, resulting in a lower intracellular availability of these substrates for sulphate metabolism. L-Methionine is an effective substrate for SNAT2 (Yao et al. 2000) and inhibition or silencing of SNAT2 in L6-G8C5 myotubes significantly depletes intracellular L-Methionine (Evans et al. 2007). However, competitive inhibition of SNAT2 with a saturating concentration of its substrate MeAIB (Figures 5.5 and 5.6) failed to suppress sulphate output any further than was the case with 2mM L-Glutamine, suggesting that competitive effects of L-Glutamine on SNAT2 are not the full explanation.

The effect of L-Glutamate  (L-Glu) was also tested in Figures 5.5 and 5.6. In principle L-Glu could exert a mass-action effect on the aspartate aminotransferase equilibrium:

L-Glu + beta-sulphinylpyruvate (BSP) <--> alphaketoglutarate + cysteinsulphinate (CSA);
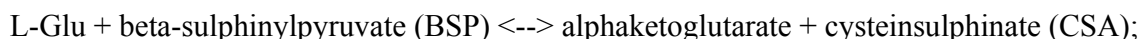
thus depleting BSP (the precursor of sulphuric acid) (Yang and Brunengraber, 2000), and expanding the CSA pool (the main determinant of Taurine production through the regulatory enzyme CSA decarboxylase). In practice this exerted no effect beyond that observed with 2mM L-Glutamine, possibly because L-Glutamate generated from L-Glutamine was already exerting a maximal effect on this equilibrium.

**5.4.4 The effect of glucocorticoid on the activity and expression of the SNAT2 amino acid transporter**

Metabolic acidosis is reported to increase circulating glucocorticoid concentrations (May et al. 1986) and elevated concentrations are seen in CKD (Rosman et al. 1982; McDonald et al. 1979). Therefore the aim of the second part of this chapter was to investigate whether glucocorticoid might mediate a persistent inhibitory effect of acidosis on SNAT2 which could have contributed to the sustained collapse of muscle amino acid gradients in the acidotic exercising patients in Chapter 3.

Dexamethasone administered at a concentration of 500nM did significantly reduce the transport activity of SNAT2 by approximately 40% and, in combination with acidified media, resulted in an additive inhibitory effect of approximately 70%. The effect of glucocorticoid persisted for several hours suggesting that this is indeed a feasible mediator of longer term inhibitory effects on active transport of amino acids into muscle in acidotic CKD patients and warrants further investigation. Although a high dose of dexamethasone was used here this was because it appears this cell line is relatively insensitive. In order for effects of insulin on protein degradation to be seen *in vitro*, an insulin concentration that is approximately 100 times greater than that seen to produce an effect *in vivo* has to be used. This problem was also encountered in selecting a glucocorticoid dose to be used, doses lower than 500nM failed to produce an effect and so a relatively high non-physiological dose had to be used.

These changes in transport activity were mirrored by changes in the intracellular L-Glutamine concentration, one of the main substrates for this transporter (Hyde et al. 2003; Yao et al. 2000). It has previously been reported that reducing the pH of the medium to 7.1 causes significant decreases in intracellular L-Glutamine concentrations (Evans et al. 2007). Incubation at pH 7.1 for 4h in the present study resulted in a 22% reduction in the intracellular L-Glutamine concentration confirming this earlier report. Glucocorticoids were also seen to produce a comparable effect: 4h incubation with 500nM dexamethasone decreased glutamine levels by 25% and when dexamethasone and acid were combined, the effect was additive, resulting in a 43% decline, comparable with the decline in the L-Glutamine gradient observed in the STD bicarbonate group in Chapter 3, Table 3.16.

The effects on transport activity and intracellular L-Glutamine depletion appeared to show a biphasic time course, with inhibition in the first 7h, but after this time there was a rebound of SNAT2 activity which was actually significantly above baseline after 48h with Dexamethasone. Biphasic effects of glucocorticoid have been reported previously by Pickering et al. (2003) who observed a biphasic effect of Dexamethasone on the time course of protein degradation in L6-G8C5 myotubes. Kayali et al. (1990) also observed that in normal and diabetic rats, the daily administration of corticosterone resulted in an increase in protein degradation rates measured by 3-MH release from the hindquarters in the first four days. After this time, a fall in degradation rate back to baseline was seen, an effect that appeared to be independent of insulin and could not be explained by the authors.

The additive effect of glucocorticoids and acid that was observed on SNAT2 transport activity, and subsequent changes on the intracellular L-Glutamine concentration, suggests that these two effects are occurring through distinct mechanisms. Previous reports of the effect of low pH on SNAT2 transport activity (Bevington et al. 2002) have shown that this effect is rapidly reversed when the acidified medium is washed away consistent with acid exerting a direct effect upon the SNAT2 transporter protein (reviewed in Chapter 1.14.1) Consequently, to observe the effect of low pH on SNAT2 activity, the transport assay must also be performed at a low pH. This is not the case for Dexamethasone, which exerts a more persistent effect on SNAT2 activity that is detectable even when the Dexamethasone has been removed from the medium. This persisting effect is consistent with glucocorticoid acting upon gene expression through a classical steroid receptor-mediated transcription dependent pathway (Figure 5.10). This pathway is activated by ligand binding to the glucocorticoid receptor resulting in the ligand-receptor complex translocating to the nucleus and binding to glucocorticoid response elements that are found on the promoter region of target genes (Granner, 1996).

The data in Figure 5.10 suggest that Dexamethasone inhibits SNAT2 through effects on gene expression, but not on expression of the SNAT2 gene. Northern blotting detected no change in the expression of the transporter after 17h incubation with Dexamethasone (although a short-lived effect may have been missed). It is possible that glucocorticoid acts by increasing the expression of a protein which leads to the down-regulation of the SNAT2 protein observed in Figure 5.12.

**5.4.5 The effect of mechanical stretch on SNAT2 transporter activity**

The principal conclusion from this part of the study was that prolonged mechanical stress for up to 48h stimulates SNAT2 activity in L6-G8C5 myotubes, consistent with the stimulation of System A activity reported in rats following acute exercise (Henriksen at al 1992, 1993; King, 1994). Under none of the conditions tested did mechanical stress <u>inhibit</u> SNAT2 activity. Therefore there is no evidence at this stage that the patients' exercise regime in Chapter 3 led directly to the observed collapse of the muscle amino acid gradients.

However, the observations that both continuous and cyclic stretch were able to activate SNAT2, and that SNAT2 is apparently able to regulate phosphorylation of rpS6 and 4E-BP1 and hence translation initiation (Evans et al. 2007) are of interest. Atherton et al. (2009) have shown that cyclic stretch of L6-G8C5 myotubes also increases the phosphorylation of these proteins, and previous research by Vandenburgh (1987) found that passive stretching of cells *in vitro* resulted in a large increase in protein synthesis and a small decrease in protein degradation. This may mean that SNAT2 serves as a sensor of mechanical stretch, mediating anabolic signals to protein metabolism and increasing protein synthesis rates (Vandenburgh, 1987). It will now be interesting to determine whether inhibition or silencing of SNAT2 leads to inhibition of stretch-induced increases in phosphorylation of proteins downstream of mTOR.

**5.4.6 Conclusions**

Part A of this Chapter confirms that skeletal muscle does play a role in sulphate metabolism as previously suggested (Garcia and Stipanuk, 1992). Although its involvement is to a lesser degree than hepatocytes, it may be of importance as this source of sulphuric acid will be the most readily available one to myocytes. At least *in vitro*, the potential for L-Glutamine to be used as a therapy to reduce sulphuric acid production looks promising, and warrants further investigation.

The results from part B of this chapter demonstrate a chronic inhibition by glucocorticoids of the SNAT2/slc38a2 transporter that appears to act via a separate mechanism to that of acidosis. This presents a possible line of enquiry to be investigated that may explain the collapse of the intracellular/extracellular amino acid gradients observed in the exercising patients in Chapter 3, but as these results were obtained *in vitro*, caution is needed in translating these conclusions directly to the effects seen in humans.

The results from part C of this chapter lead to the hypothesis that SNAT2/slc38a2 may be a sensor of mechanical stress, which may be able to up-regulate transport activity rates and, making use of the results from Evans et al. (2007), increase signalling through mTOR. Therefore, if acidosis and raised glucocorticoid levels in CKD are capable of inhibiting this transporter, this implies a role for SNAT2 in the regulation of protein metabolism under conditions relevant to uraemia. Whether this inhibition can be relieved by resistance exercise (i.e. mechanical stress) remains to be seen.

/

Chapter 6


General Discussion

## 6.1 Aerobic exercise training in CKD patients and the effect of sodium bicarbonate

There are many reports now published that demonstrate exercise in haemodialysis patients, both aerobic and resistance, improves exercise capacity (Painter et al. 1986; Kouidi et al. 1997; Molsted et al. 2004; Sakkas et al. 2003) and the results from Chapter 3 show this is also the case following aerobic exercise training in pre-dialysis patients. One of the most important findings from this chapter was the effect that additional sodium bicarbonate imposed on adaptations to the exercise training. A reduced lactate response to the same absolute workload was seen with modest increases in lean body mass (LBM) and reduced expression of the ubiquitin E3 ligases, none of which were seen in the exercising patients who received only their standard bicarbonate treatment. A possible interpretation is that XS bicarbonate allowed patients to exercise for longer by buffering acid that was produced during the exercise bout. The results presented in Chapter 3 suggest that in these patients acidosis is not beneficial, but actually appears to be detrimental preventing the exercise-induced adaptations seen in the patients in the XS bicarbonate group. Acidification of skeletal muscle may be a limiting factor to exercise in this patient population, Nishida et al. (1991) observed a greater decline in sarcosolic pH during exercise in uraemic patients compared to healthy controls, and it is possible that the addition of XS bicarbonate helped to prevent such a fall. This beneficial effect of bicarbonate is in line with results from Stein et al. (1997) who observed that additional alkali therapy resulted in an increase in body weight and mid-arm circumference and reduced morbidity, and Szeto et al. (2003) who saw increases in free fat mass and LBM with reduced morbidity in peritoneal dialysis patients.

Surprisingly, those patients who exercised while receiving only their standard bicarbonate therapy exhibited striking declines in intramuscular amino acid pools and a collapse of the intracellular/extracellular amino acid gradients. This was an effect that was apparent after one month of exercise, but was much larger after six months, and may have the potential to limit protein synthesis. Importantly, the administration of extra bicarbonate to exercising patients was able to prevent this collapse and maintain intramuscular amino acid concentrations. Amino acid gradients are usually maintained by active transporters, with the pH sensitive SNAT2/slc38a transporter apparently being the main active amino acid transporter in skeletal muscle cells. Previous evidence from our laboratory has shown that inhibition of this transporter by acidosis *in vitro* results in the depletion of L-Glutamine and other amino acids whose intracellular concentrations are maintained by the L-Glutamine concentration gradient set up by SNAT2

(Evans et al. 2007). Therefore, it was hypothesised that as this effect on amino acids was only seen in the exercising patients on STD bicarbonate therapy and not in the non-exercising controls also receiving only their STD bicarbonate therapy, it was an effect that was mediated by the exacerbation of acidosis by exercise. However, this gradient disruption was observed in the resting state, after any exercise-induced acidosis and therefore amino acid disruption may have dissipated.

The results from the *in* vitro experiments in chapter 5 provide interesting speculation on a second factor that might be involved in the observed collapse in amino acid gradients. Acidosis results in an increase in circulating glucocorticoid levels (May et al. 1986) which have been reported in CKD (Rosman et al. 1982; McDonald et al. 1979) and appear to be a permissive factor for the catabolic effect of acid. For this reason, it seemed plausible that, like acid (Evans et al. 2007), glucocorticoids may also be exerting an effect on protein metabolism through SNAT2. *In vitro*, glucocorticoids (Dexamethasone) did suppress SNAT2 transport activity and reduce intracellular glutamine concentrations and, when applied in conjunction with acid, the effect was additive suggesting that these factors are working through distinct mechanisms. Caution is required when applying conclusions drawn from *in vitro* experiements to *in vivo* data, however, it is possible that glucocorticoids mediate a persistent inhibitory effect of acidosis on SNAT2 which could have contributed to the sustained collapse of muscle amino acid gradients seen in the acidotic exercising patients in Chapter 3. Whether the circulating glucocorticoid concentration in acidotic CKD patients is sufficient to exert such an effect *in vivo*, and whether exercise further increases this concentration, remains to be determined.

Owing to the positive results seen in this aerobic training pilot study, further investigation is warranted into the effects of a resistance training programme, where much larger effects on net muscle mass might be expected. It would be important to continue to compare the effect of extra bicarbonate therapy, which if the current results are correct, may be a crucial supplement to make to exercise therapy in pre-dialysis CKD patients.

## 6.2 Exercise and measures of myofibrillar proteolysis

Despite the observation that exercise and XS bicarbonate therapy reduced E3 ligase expression, other markers of myofibrillar proteolysis showed no consistent beneficial effect and reduced

levels of the 14kDa actin fragment and 3-MH excretion were not observed together under the same condition that suppressed E3 ligase expression. There was no statistically significant decline in 14kDa actin levels in any group, contrary to the decline previously reported in combined aerobic and resistance exercise in haemodialysis patients (Workeneh et al. 2006). Similarly no change was observed in 3-MH excretion rates in any group, and although a decrease was seen in the muscle protein:DNA ratio in the XS group which appeared to be induced through technical error, no changes were seen in muscle: DNA ratio in the remaining groups, and this resulted in only very small effects on LBM observed by DEXA. This inability to reduce 3-MH excretion and therefore muscle proteolysis possibly resulted from a failure of the treatments to increase PKB phosphorylation sufficiently (although the importance of this is human muscle now seems to be unkown) and reduce caspase-3 activity, and, in the case of STD bicarbonate therapy, to effectively buffer the acid generated by exercise. Endurance exercise traditionally does not result in the accretion of skeletal muscle protein and the fact some small increases in LBM following aerobic exercise were seen here may be a reflection of how cachectic these patients were. In future studies, larger gains might be expected with resistance exercise training.

When interpreting the results from Chapter 3, there are some limitations that should be taken into account. Firstly, sample sizes were not equal in all the groups and some groups were left with small numbers owing to a significant drop-out rate which has previously been reported in exercise studies with CKD patients (Koufaki et al. 2000; Malagoni et al. 2008). Such problems are difficult to avoid when performing a six month exercise training study in a disease population. Secondly, as the exercise programme was unsupervised, there was no way to quantify exactly how much exercise the patients actually performed. They were asked to fill in exercise diaries, but a digitalized method of assessing this would have been more accurate, for example an accelerometer. Finally, the allocation of patients to either an exercise or control group was not randomized. This means that the result may have been influenced by comparing an intervention group who were willing to exercise, with a control group who were unwilling. In future studies it would be helpful to recruit a large number of patients all willing to exercise, and then randomly allocate them to exercising and non-exercising groups, but the large patient population required would probably need a multi-centre study. Unfortunately, due to logistical problems, it was necessary in the present study to limit recruitment to Leicester General Hospital.

## 6.3 Production of sulphuric acid and links to protein breakdown

As the results from Chapter 3 showed the importance of limiting acidosis, sources of acid generation were investigated in Chapter 4. Sulphur containing amino acids that are consumed in the diet are ultimately catabolised to either sulphuric acid (sulphate plus hydrogen ions) or the non-acidic end product taurine. Interestingly, there was no relationship observed between venous bicarbonate concentration (the conventional measure of acidosis) and 3-MH excretion. There was however, a strong correlation between sulphate excretion and 3-MH excretion in the patients, but not in healthy controls. This suggests that in this patient population, sulphuric acid production is functionally significant and might be a better indicator of those patients at risk of acid-induced muscle wasting. However, it is important to bear in mind this conclusion has been made from only a correlation and is required to be confirmed using more robust experiments. Whether glutamine supplementation, possibly by trapping sulphur in the form of taurine (Boelens et al. 2003), can significantly limit the production of sulphuric acid *in vivo* and exert beneficial effects under conditions of uraemia warrants further investigation.

## 6.4 Summary

In conclusion, aerobic exercise in pre-dialysis CKD patients was a relatively un-researched area and this study has demonstrated that aerobic exercise is also able to confer some benefits to pre-dialysis patients, as well as the haemodialysis population as previously documented, but only when applied in combination with additional bicarbonate therapy. Indeed, the evidence presented suggests that exercise with standard bicarbonate therapy, and therefore lower serum bicarbonate levels, might even be detrimental in the long-term. The conventional measure of acidosis, serum bicarbonate seems to be an insensitive marker of protein wasting in this population and this study tentatively proposes sulphate excretion rates as a putative indicator of those patients at high risk of skeletal muscle wasting. Glutamine therapy has been observed *in vitro* as a potential means by which to decrease sulphuric acid production. Finally, based on evidence *in vitro*, the pH sensitive SNAT2 transporter has been identified as a possible sensor of acidosis and mechanical stress, but much more work is required to demonstrate that it has an important role in protein metabolism *in vivo* following exercise and under conditions relevant to uraemia.

# Appendix A

## Solutions and Reagents

Folin Reagent A (1 Litre)
20g Anhydrous $Na_2CO_3$
4g NaOH
0.2g K,Na-tartrate. $4H_2O$
Ultra pure water up to one litre

Folin Reagent B (1 Litre)
5g $CuSO_4.5H_2O$
Ultra pure water up to one litre

UIC3 Protease Inhibitor Cocktail (100ml)
8.55g Sucrose
0.47g Hepes
0.032g Sodium Azide
0.076g EGTA
Adjust to pH 7.4 with 0.5M NaOH
Ultra pure water up to 100ml
20 µl PMSF (25mg)
14.2µl E64 (5mg)
13.7µl Pepstatin A (5mg)
9.5µl Leupeptin (10mg)

SDS-PAGE Muscle Lysis Buffer (10ml)
0.08g NaCl
0.002g KCl
0.002g $MgCl_2$
500µl Tris 1M pH 7.5
20µl Ethylenediaminetetraacetic acid (EDTA) 0.5M pH 8
250µl Ethylene glycol tetraacetic acid (EGTA) 40mM
800µl Glycerol
1ml 10% Triton X-100

100μl β-Glycerophosphate 1M

1.18ml Sodium Orthovanadate 8.5mM

1ml Sodium Fluoride 500mM

10μ β-Mercaptoethanol

5.09ml ultra pure water


<u>SDS-PAGE L6 Lysis Buffer (10ml)</u>

500μl Tris 1M pH 7.5

20μl Ethylenediaminetetraacetic acid (EDTA) 0.5M pH 8

250μl Ethylene glycol tetraacetic acid (EGTA) 40mM

1ml 10% Triton X-100

100μl β-Glycerophosphate 1M

1.18ml Sodium Orthovanadate 8.5mM

1ml Sodium Fluoride 500mM

10μ β-Mercaptoethanol

5.89ml ultra pure water


<u>Laemelli Reducing Sample Buffer</u>

4ml Ultra pure $H_2O$

1ml 0.5M Tric HCL pH 6.8

0.8ml Glycerol

1.6ml 10% w/v Sodium Dodecyl Sulphate (SDS)

0.4ml β-Mercaptoethanol

0.2ml 0.05% w/v Bromophenol Blue


<u>10X Running Buffer (1 Litre)</u>

30.3g Trizma Base

144g Glycine

100ml 20% SDS

Ultra pure water to one litre


<u>1X Running Buffer (1 Litre)</u>

100ml 10X Running buffer

900ml Ultra pure water


<u>10X Transfer Buffer (1 Litre)</u>

30.3g Tris-Base

144g Glycine

Ultra pure water to one litre


1X Transfer Buffer (1 Litre)

100ml 10X Transfer Buffer

200ml Methanol

100ml 10% SDS

Ultra pure water to one litre

Blue


10X TBS

60.55g Trizma base

87.66g NaCl

Ultra pure water to 700ml

pH to 7.6 using 6M HCl

Ultra pure water to one Litre


1X TTBS + 0.1% tween (1 Litre)

100ml 10X TBS

Ultra pure water to one litre

1ml Tween 20


RNA Gel Loading Sample Buffer

50% Glycerol

10mM Tris

1mM EDTA

Bromophenol blue 0.05% w/v to give a readily detectable colour

DNA Sample Buffer

0.25% Bromophenol Blue

15% Ficoll (Type 400) in Water


HBS

140mM BaCl

20mM Hepes Acid

2.5mM $MgSO_4. 7H_2O$

5mM KCl

1mM CaCl$_2$. 2H$_2$O

10mg/l Phenol red

Adjust to pH 7.4 with 0.5M NaOH


Pre-hybridisation Solution

5 ml Formamide

2.5ml 20X SSPE

1ml 50X Denhardt's solution

0.5ml 20% SDS w/v

1ml DEPC Water

200μg/ml Salmon sperm DNA

Warm to 42˚C prior to use.

# Appendix B

## Methods Development

There were no protocols were set up in the Laboratory for working with skeletal muscle, and for western blotting in particular, a great deal of methods development had to be done to get the assay working effectively. Below is a step by step description of this process.

1) I started by practicing the protocol described by Karlsoon et al. (2004) on rat skeletal muscle to determine the phosphorylation status of P-rpS6, P-PKB, P-P$^{70}$S6K, and P-4E-BP1. Westerns were run with 30μg protein, the amount of protein routinely loaded onto the gels in our Laboratory when running cell culture lysates. These Westerns worked well.

2) Professor Ron Maughan kindly taught Dr Kosmadakis the biopsy technique and in the process provided myself with some human skeletal muscle to continue methods development with. Initially, all Westerns were run for P-rpS6 with the aim of setting up this western first. I repeated the protocol that had obtained good results in rodent muscle, but there was only a very faint signal detectable in healthy human skeletal muscle.

3) The amount of protein loaded onto the gel was increased to 100μg protein and gave a better signal than 30μg. This was then increased to 150μg, which gave no improvement over 100μg.

4) Two patients withdrew very early on in the study, but allowed their baseline samples to be used in methods development. The protocol was repeated with 100μg and then 150μg protein, but no signal was detectable. As a result of this, 150μg protein was routinely loaded onto the gel to maximise the possibility of detecting a signal with further modifications.

5) It was decided to always run a positive control on the same gel as the muscle samples to ensure that the lack of a signal was not due to a technical failure of the Western. L6-G8C5 cells stimulated with insulin served as the positive control and a volume containing 10μg protein was loaded onto the gel. (A reduced amount of protein was used here because the length of time the membrane was exposed to the film meant that anything more than 10μg resulted in a very large overexposed band.)

6) The effect of using a signal enhancer reagent and an antibody extender from Thermo Scientific was tested. Due to cost reasons it was decided to use the signal enhancer

reagent, but this was later removed from the protocol as it made little difference and prevented the membranes from being re-probed with other antibodies.

7) I tried varying the length of time the membrane was exposed to the film, finally increasing to 24h exposures, which did give stronger signals than shorter exposures and this exposure time was then routinely used.

8) I switched from using nitrocellulose to PVDF membranes, as these are sometimes suggested to be more sensitive. However, they seemed to make little difference, but again, in order to maximise the possibly of detecting a signal, they were continued to be used.

9) I tried increasing the length of time proteins were transferred from the gel to the membrane using the wet transfer system from 2 to 3 hours and finally up to 4 hours to make sure all the protein was transferred out of the gel successfully. However, coomassie blue staining showed there was difference in the amount of protein that remained in the gel between the three different transfer times, and no difference in the signals detected, so the transfer time remained at 2 hours.

10) I started to compare the lysis buffer recipe I was using (which was the same as that used in cell culture experiments in our Laboratory) with those in the literature. It was essentially the same, except some research groups add salts to help dissolve the protein and glycerol to maintain a uniform concentration of the lysate when frozen. Salts and glycerol were then routinely added to the lysis buffer.

11) I tried using sonication following homogenisation to ensure the biopsy had completely gone into solution. This made no difference and was removed from the protocol.

12) The length of time the muscle homogenate was incubated in the lysis buffer was increased from 30 to 60 and finally to 90 minutes, and instead of incubating it on ice, the tubes were rotated in the cold room. After this modification and the improved lysis buffer recipe, the lysates did appear to be more translucent and to contain less insoluble material.

13) Some research groups use the semi-dry transfer method, and this was tried following the modifications made in point 12. This method did seem to give a better transfer and the wet transfer system was no longer used.

14) I changed the blocking agent from 5% milk powder to 5% Bovine Serum Albumin (BSA), but this resulted in a high background and was no longer used.

15) I then applied to the Loughborough University Ethical Advisory Committee to run a healthy volunteer study to obtain muscle biopsies from healthy individuals, in which phosphorylation signals should be detectable. If I could see a signal, then I could be sure that the lack of a detectable signal in the biopsies from CKD patients was the correct result and it was not simply the result of technical difficulties. A signal was detectable, so it was concluded that there is simply very little phosphorylation of rpS6 in these patients.

This low phosphorylation signal was also found to be the case for P-4E-BP1 and P-P$^{70}$SK6, but phosphorylation of PKB could be seen.

The protocol described in Section 2.5 was used in the Western blotting experiments for phosphorylated proteins described in this thesis.

# References

Abe, H. (2000). Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. *Biochemistry*, *65(7)*, 757-765.

Acker, M.G. & Lorsch, J.R. (2008). Mechanism of ribosomal subunit joining during eukaryotic translation initiation. *Biochemical Society transactions, 36(4)*, 653-657.

Akiba, T., Matsui, N., Shinohara, S., Fujiwara, H., Nomura, T. & Marumo, F. (1995). Effects of recombinant human erythropoietin and exercise training on exercise capacity in hemodialysis patients. *Artificial Organs, 19(12),* 1262-1268.

Allen, D.G., Lamb, G.D. & Westerblad, H. (2008). Skeletal muscle fatigue: cellular mechanisms. *Physiological Reviews, 88(1),* 287-332.

Alvestrand, A., Furst, P. & Bergstrom, J. (1982). Plasma and muscle free amino acids in uremia: influence of nutrition with amino acids. *Clinical nephrology, 18(6),* 297-305.

Alvestrand, A., Bergstrom, J., Furst, P., Germanis, G. & Widstam, U. (1978). Effect of essential amino acid supplementation on muscle and plasma free amino acids in chronic uremia. *Kidney international, 14(4),* 323-329.

Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B.,3rd, Johnson, R.S. & Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature, 372(6502),* 186-190.

Asola, M.R., Virtanen, K.A., Peltoniemi, P., Nagren, K., Jyrkkios, S., Knutti, J., Nuutila, P.R. & Metsarinne, K.P. (2001). Amino acid transport into skeletal muscle is impaired in chronic renal failure. *Journal of the American Society of Nephrology, 12*, 64A.

Atherton, P.J., Szewczyk, N.J., Selby, A., Rankin, D., Hillier, K., Smith, K., Rennie, M.J. & Loughna, P.T. (2009). Cyclic stretch reduces myofibrillar protein synthesis despite increases in FAK and anabolic signalling in L6 cells. *The Journal of physiology, 587(Pt 14),* 3719-3727.

Baar, K. (2006). Training for endurance and strength: lessons from cell signaling. *Medicine and science in sports and exercise, 38(11),* 1939-1944.

Baar, K. & Esser, K. (1999). Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *The American Journal of Physiology, 276(1 Pt 1),* C120-7.

Bai, X. & Jiang, Y. (2010). Key factors in mTOR regulation. *Cellular and molecular life sciences, (2),* 239-253.

Bailey, J.L., Zheng, B., Hu, Z., Price, S.R. & Mitch, W.E. (2006). Chronic kidney disease causes defects in signaling through the insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway: implications for muscle atrophy. *Journal of the American Society of Nephrology, 17(5),* 1388-1394.

Bailey, J.L., Wang, X., England, B.K., Price, S.R., Ding, X. & Mitch, W.E. (1996). The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin-proteasome pathway. *The Journal of clinical investigation, 97(6),* 1447-1453.

Baird, F.E., Pinilla-Tenas, J.J., Ogilvie, W.L., Ganapathy, V., Hundal, H.S. & Taylor, P.M. (2006). Evidence for allosteric regulation of pH-sensitive System A (SNAT2) and System N (SNAT5) amino acid transporter activity involving a conserved histidine residue. *The Biochemical journal, 397(2),* 369-375.

Ball, D., Greenhaff, P.L. & Maughan, R.J. (1996). The acute reversal of a diet-induced metabolic acidosis does not restore endurance capacity during high-intensity exercise in man. *European journal of applied physiology and occupational physiology, 73(1-2),* 105-112.

Ballard, F.J. & Tomas, F.M. (1983). 3-Methylhistidine as a measure of skeletal muscle protein breakdown in human subjects: the case for its continued use. *Clinical science, 65(3),* 209-215.

Barnea, N., Drory, Y., Iaina, A., Lapidot, C., Reisin, E., Eliahou, H. & Kellermann, J.J. (1980). Exercise tolerance in patients on chronic hemodialysis. *Israel journal of medical sciences, 16(1),* 17-21.

Bella, D.L. & Stipanuk, M.H. (1995). Effects of protein, methionine, or chloride on acid-base balance and on cysteine catabolism. *The American Journal of Physiology, 269(5 Pt 1),* E910-7.

Bergström, J., Alvestrand, A. & Furst, P. (1990). Plasma and muscle free amino acids in maintenance hemodialysis patients without protein malnutrition. *Kidney international, 38(1),* 108-114.

Bergström, J., Alvestrand, A., Furst, P. & Lindholm, B. (1989). Sulphur amino acids in plasma and muscle in patients with chronic renal failure: evidence for taurine depletion. *Journal of internal medicine, 226(3),* 189-194.

Bergström, J., Furst, P., Noree, L.O. & Vinnars, E. (1974). Intracellular free amino acid concentration in human muscle tissue. *Journal of applied physiology, 36(6),* 693-697.

Bergström, J., Hermansen, L., Hultman, E. & Saltin, B. (1967). Diet, muscle glycogen and physical performance. *Acta Physiologica Scandinavica, 71(2),* 140-150.

Bevington, A., Brown, J., Butler, H., Govindji, S., M-Khalid, K., Sheridan, K. & Walls, J. (2002). Impaired system A amino acid transport mimics the catabolic effects of acid in L6 cells. *European journal of clinical investigation, 32(8),* 590-602.

Bevington, A., Brown, J. & Walls, J. (2001). Leucine suppresses acid-induced protein wasting in L6 rat muscle cells. *European journal of clinical investigation, 31(6),* 497-503.

Bevington, A., Brown, J., Pratt, A., Messer, J. & Walls, J. (1998). Impaired glycolysis and protein catabolism induced by acid in L6 rat muscle cells. *European journal of clinical investigation, 28(11),* 908-917.

Biolo, G., Declan Fleming, R.Y. & Wolfe, R.R. (1995). Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. *The Journal of clinical investigation, 95(2),* 811-819.

Bishop, D., Edge, J., Mendez-Villanueva, A., Thomas, C. & Schneiker, K. (2009). High-intensity exercise decreases muscle buffer capacity via a decrease in protein buffering in human skeletal muscle. *Pflugers Archiv*, *458(5),* 929-936.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. & Yancopoulos, G.D. (2001a). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nature cell biology, 3(11),* 1014-1019.

Bodine, S.C., Latres, E., Baumhueter, S., Lai, V.K., Nunez, L., Clarke, B.A., Poueymirou, W.T., Panaro, F.J., Na, E., Dharmarajan, K., Pan, Z.Q., Valenzuela, D.M., DeChiara, T.M., Stitt, T.N., Yancopoulos, G.D. & Glass, D.J. (2001b). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science, 4(5547),* 1704-1708.

Boelens, P.G., Houdijk, A.P., de Thouars, H.N., Teerlink, T., van Engeland, M.I., Haarman, H.J. & van Leeuwen, P.A. (2003). Plasma taurine concentrations increase after enteral glutamine supplementation in trauma patients and stressed rats. *The American Journal of Clinical Nutrition, 77(1),* 250-256.

Borg, G.A. (1982). Psychophysical bases of perceived exertion. *Medicine and science in sports and exercise, 14(5),* 377-381.

Boyce, M.L., Robergs, R.A., Avasthi, P.S., Roldan, C., Foster, A., Montner, P., Stark, D. & Nelson, C. (1997). Exercise training by individuals with predialysis renal failure: cardiorespiratory endurance, hypertension, and renal function. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation, 30(2),* 180-192.

Bradley, J.R., Anderson, J.R., Evans, D.B. & Cowley, A.J. (1990). Impaired nutritive skeletal muscle blood flow in patients with chronic renal failure. *Clinical science, 79(3),* 239-245.

Breitkreutz, R., Holm, S., Pittack, N., Beichert, M., Babylon, A., Yodoi, J. & Droge, W. (2000). Massive loss of sulfur in HIV infection. *AIDS Research and Human Retroviruses, 16(3),* 203-209.

Bronas, U.G. (2009). Exercise training and reduction of cardiovascular disease risk factors in patients with chronic kidney disease. *Advances in chronic kidney disease, 16(6),* 449-458.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W. & Kaelin, W.G.,Jr (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes & development, 18(23),* 2893-2904.

Carney, R.M., McKevitt, P.M., Goldberg, A.P., Hagberg, J., Delmez, J.A. & Harter, H.R. (1983). Psychological effects of exercise training in hemodialysis patients. *Nephron, 33(3),* 179-181.

Carragher, N.O. (2006). Calpain inhibition: a therapeutic strategy targeting multiple disease states. *Current pharmaceutical design, 12(5),* 615-638.

Cartee, G.D. & Funai, K. (2009). Exercise and insulin: Convergence or divergence at AS160 and TBC1D1? *Exercise and sport sciences reviews, 37(4),* 188-195.

Castaneda, C., Gordon, P.L., Uhlin, K.L., Levey, A.S., Kehayias, J.J., Dwyer, J.T., Fielding, R.A., Roubenoff, R. & Singh, M.F. (2001). Resistance training to counteract the catabolism of a low-protein diet in patients with chronic renal insufficiency. A randomized, controlled trial. *Annals of Internal Medicine, 135(11),* 965-976.

Cecchin, F., Ittoop, O., Sinha, M.K. & Caro, J.F. (1988). Insulin resistance in uremia: insulin receptor kinase activity in liver and muscle from chronic uremic rats. *The American Journal of Physiology, 254(4 Pt 1),* E394-401.

Chaudhry, F.A., Schmitz, D., Reimer, R.J., Larsson, P., Gray, A.T., Nicoll, R., Kavanaugh, M. & Edwards, R.H. (2002). Glutamine uptake by neurons: interaction of protons with system a transporters. *The Journal of neuroscience, 2(1),* 62-72.

Cheetham, M.E., Boobis, L.H., Brooks, S. & Williams, C. (1986). Human muscle metabolism during sprint running. *Journal of applied physiology, 61(1),* 54-60.

Chen, Y., Sood, S., Biada, J., Roth, R. & Rabkin, R. (2008). Increased workload fully activates the blunted IRS-1/PI3-kinase/Akt signaling pathway in atrophied uremic muscle. *Kidney international, 73(7),* 848-855.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry, 162(1),* 156-159.

Cibulka, R. & Racek, J. (2007). Metabolic disorders in patients with chronic kidney failure. *Physiological Research / Academia Scientiarum Bohemoslovaca, 56(6),* 697-705.

Coffey, V.G., Zhong, Z., Shield, A., Canny, B.J., Chibalin, A.V., Zierath, J.R. & Hawley, J.A. (2006). Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB, 20(1),* 190-192.

Cohen, P. (2006). The twentieth century struggle to decipher insulin signalling. *Nature reviews. Molecular cell biology, 7(11),* 867-873.

Coloso, R.M. & Stipanuk, M.H. (1989). Metabolism of cyst(e)ine in rat enterocytes. *The Journal of nutrition, 119(12),* 1914-1924.

Condon, J.R. & Asatoor, A.M. (1971). Amino acid metabolism in uraemic patients. *Clinica chimica acta,, 32(3),* 333-337.

Constantin, D., Constantin-Teodosiu, D., Layfield, R., Tsintzas, K., Bennett, A.J. & Greenhaff, P.L. (2007). PPARdelta agonism induces a change in fuel metabolism and activation of an atrophy programme, but does not impair mitochondrial function in rat skeletal muscle. *The Journal of physiology, 583(1),* 381-390.

Coppack, S.W. (2001). Pro-inflammatory cytokines and adipose tissue. *The Proceedings of the Nutrition Society, 60(3),* 349-356.

Corradetti, M.N. & Guan, K.L. (2006). Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene, 25(48),* 6347-6360.

Coyle, E.F., Coggan, A.R., Hemmert, M.K. & Ivy, J.L. (1986). Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *Journal of applied physiology, 61(1),* 165-172.

Crossland, H., Constantin-Teodosiu, D., Greenhaff, P.L. & Gardiner, S.M. (2010). Low-dose dexamethasone prevents endotoxaemia-induced muscle protein loss and impairment of carbohydrate oxidation in rat skeletal muscle. *The Journal of physiology, 588(8),* 1333-1347.

Crossland, H., Constantin-Teodosiu, D., Gardiner, S.M., Constantin, D. & Greenhaff, P.L. (2008). A potential role for Akt/FOXO signalling in both protein loss and the impairment of muscle carbohydrate oxidation during sepsis in rodent skeletal muscle. *The Journal of physiology, 586(Pt 22),* 5589-5600.

D'Amico, G. & Bazzi, C. (2003). Pathophysiology of proteinuria. *Kidney international, 63(3),* 809-825.

Debigare, R. & Price, S.R. (2003). Proteolysis, the ubiquitin-proteasome system, and renal diseases. *American journal of physiology .Renal physiology, 285(1),* F1-8.

de Boer, M.D., Selby, A., Atherton, P., Smith, K., Seynnes, O.R., Maganaris, C.N., Maffulli, N., Movin, T., Narici, M.V. & Rennie, M.J. (2007). The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. *The Journal of physiology,* 585(1), 241-251.

DeFronzo, R.A., Alvestrand, A., Smith, D., Hendler, R., Hendler, E. & Wahren, J. (1981). Insulin resistance in uremia. *The Journal of clinical investigation, 67(2),* 563-568.

DeYoung, M.P., Horak, P., Sofer, A., Sgroi, D. & Ellisen, L.W. (2008). Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes & development, 22(2),* 239-251.

Diem, K. & Lentner, C. (1970). *Documenta Geigy: Scientific Tables.* Seventh edition. Switzerland: Ciba-Geigy Limited.

Diesel, W., Emms, M., Knight, B.K., Noakes, T.D., Swanepoel, C.R., van Zyl Smit, R., Kaschula, R.O. & Sinclair-Smith, C.C. (1993). Morphologic features of the myopathy associated with chronic renal failure. *American Journal of Kidney Diseases, 22(5),* 677-684.

Diesel, W., Noakes, T.D., Swanepoel, C. & Lambert, M. (1990). Isokinetic muscle strength predicts maximum exercise tolerance in renal patients on chronic hemodialysis. *American Journal of Kidney Diseases, 16(2),* 109-114.

Dilger, R.N. & Baker, D.H. (2008). Excess dietary L-cysteine causes lethal metabolic acidosis in chicks. *The Journal of nutrition, 138(9),* 1628-1633.

Dominy, J.E.,Jr, Hwang, J. & Stipanuk, M.H. (2007). Overexpression of cysteine dioxygenase reduces intracellular cysteine and glutathione pools in HepG2/C3A cells. *American journal of physiology. Endocrinology and metabolism, 293(1),* E62-9.

Dreyer, H.C., Drummond, M.J., Pennings, B., Fujita, S., Glynn, E.L., Chinkes, D.L., Dhanani, S., Volpi, E. & Rasmussen, B.B. (2008). Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *American journal of physiology. Endocrinology and metabolism, 294(2),* E392-400.

Dreyer, H.C., Fujita, S., Cadenas, J.G., Chinkes, D.L., Volpi, E. & Rasmussen, B.B. (2006). Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *The Journal of physiology, 576(Pt 2),* 613-624.

Du, J., Hu, Z. & Mitch, W.E. (2005). Molecular mechanisms activating muscle protein degradation in chronic kidney disease and other catabolic conditions. *European journal of clinical investigation, 35(3),* 157-163.

Du, J., Wang, X., Miereles, C., Bailey, J.L., Debigare, R., Zheng, B., Price, S.R. & Mitch, W.E. (2004). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *The Journal of clinical investigation, 113(1),* 115-123.

Durozard, D., Pimmel, P., Baretto, S., Caillette, A., Labeeuw, M., Baverel, G. & Zech, P. (1993). 31P NMR spectroscopy investigation of muscle metabolism in hemodialysis patients. *Kidney international, 43(4),* 885-892.

Eley, H.L., Russell, S.T. & Tisdale, M.J. (2007). Effect of branched-chain amino acids on muscle atrophy in cancer cachexia. *The Biochemical journal, 407(1),* 113-120.

Elia, M., Carter, A., Bacon, S., Winearls, C.G. & Smith, R. (1981). Clinical usefulness of urinary 3-methylhistidine excretion in indicating muscle protein breakdown. *British medical journal, 282(6261),* 351-354.

Ensunsa, J.L., Hirschberger, L.L. & Stipanuk, M.H. (1993). Catabolism of cysteine, cystine, cysteinesulfinate, and OTC by isolated perfused rat hindquarter. *The American Journal of Physiology, 264(5 Pt 1),* E782-9.

Evans, K., Nasim, Z., Brown, J., Clapp, E., Amin, A., Yang, B., Herbert, T.P. & Bevington, A. (2008). Inhibition of SNAT2 by metabolic acidosis enhances proteolysis in skeletal muscle. *Journal of the American Society of Nephrology, 19(11),* 2119-2129.

Evans, K., Nasim, Z., Brown, J., Butler, H., Kauser, S., Varoqui, H., Erickson, J.D., Herbert, T.P. & Bevington, A. (2007). Acidosis-sensing glutamine pump SNAT2 determines amino acid levels and mammalian target of rapamycin signalling to protein synthesis in L6 muscle cells. *Journal of the American Society of Nephrology, 18(5),* 1426-1436.

Farrington, K., Hodsman, A., Casula, A., Ansell, D. & Feehally, J. (2009). UK Renal Registry 11th Annual Report (December 2008): Chapter 4 ESRD prevalent rates in 2007 in the UK: national and centre-specific analyses. *Nephron, Clinical practice, 111 Suppl 1*, c43-68.

Fayard, E., Tintignac, L.A., Baudry, A. & Hemmings, B.A. (2005). Protein kinase B/Akt at a glance. *Journal of cell science, 118(Pt 24),* 5675-5678.

Flugel-Link, R.M., Jones, M.R. & Kopple, J.D. (1983). Red cell and plasma amino acid concentrations in renal failure. *Journal of parenteral and enteral nutrition, 7(5),* 450-456.

Foster, R.R., Saleem, M.A., Mathieson, P.W., Bates, D.O. & Harper, S.J. (2005). Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes. *American journal of physiology.Renal physiology, 288(1),* F48-57.

Franch, H.A., Raissi, S., Wang, X., Zheng, B., Bailey, J.L. & Price, S.R. (2004). Acidosis impairs insulin receptor substrate-1-associated phosphoinositide 3-kinase signaling in muscle cells: consequences on proteolysis. *American journal of physiology. Renal physiology, 287(4),* F700-6.

Frost, R.A., Huber, D., Pruznak, A. & Lang, C.H. (2009). Regulation of REDD1 by insulin-like growth factor-I in skeletal muscle and myotubes. *Journal of cellular biochemistry, 108(5),* 1192-1202.

Fujita, S., Dreyer, H.C., Drummond, M.J., Glynn, E.L., Cadenas, J.G., Yoshizawa, F., Volpi, E. & Rasmussen, B.B. (2007). Nutrient signalling in the regulation of human muscle protein synthesis. *The Journal of physiology, 582(Pt 2),* 813-823.

Garcia, R.A. & Stipanuk, M.H. (1992). The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. *The Journal of nutrition, 122(8),* 1693-1701.

Garibotto, G., Russo, R., Sofia, A., Sala, M.R., Robaudo, C., Moscatelli, P., Deferrari, G. & Tizianello, A. (1994). Skeletal muscle protein synthesis and degradation in patients with chronic renal failure. *Kidney international, 45(5),* 1432-1439.

Glover, E.I., Phillips, S.M., Oates, B.R., Tang, J.E., Tarnopolsky, M.A., Selby, A., Smith, K. & Rennie, M.J. (2008). Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion. *The Journal of physiology, 586( 24),* 6049-6061.

Going, S.B., Massett, M.P., Hall, M.C., Bare, L.A., Root, P.A., Williams, D.P. & Lohman, T.G. (1993). Detection of small changes in body composition by dual-energy x-ray absorptiometry. *The American Journal of Clinical Nutrition, 57(6),* 845-850.

Goldberg, A.L., Stein, R. & Adams, J. (1995). New insights into proteasome function: from archaebacteria to drug development. *Chemistry & biology, 2(8),* 503-508.

Goldberg, A.P., Geltman, E.M., Gavin, J.R.,3rd, Carney, R.M., Hagberg, J.M., Delmez, J.A., Naumovich, A., Oldfield, M.H. & Harter, H.R. (1986). Exercise training reduces coronary risk and effectively rehabilitates hemodialysis patients. *Nephron, 42(4),* 311-316.

Goldberg, A.P., Geltman, E.M., Hagberg, J.M., Gavin, J.R.,3rd, Delmez, J.A., Carney, R.M., Naumowicz, A., Oldfield, M.H. & Harter, H.R. (1983). Therapeutic benefits of exercise training for hemodialysis patients. *Kidney international .Supplement, 16,* S303-9.

Goldberg, A.L., Tischler, M., DeMartino, G. & Griffin, G. (1980). Hormonal regulation of protein degradation and synthesis in skeletal muscle. *Federation proceedings, 39(1),* 31-36

Goldberg, A.L., Etlinger, J.D., Goldspink, D.F. & Jablecki, C. (1975). Mechanism of work-induced hypertrophy of skeletal muscle. *Medicine and science in sports, 7(3),* 185-198.

Goldspink, G. (2006). Impairment of IGF-I gene splicing and MGF expression associated with muscle wasting. *The international journal of biochemistry & cell biology, 38*[(3)], 481-489.

Goll, D.E., Neti, G., Mares, S.W. & Thompson, V.F. (2008). Myofibrillar protein turnover: the proteasome and the calpains. *Journal of animal science, 86(14 Suppl),* E19-35.

Goodman, M.N. (1994). Interleukin-6 induces skeletal muscle protein breakdown in rats. *Proceedings of the Society for Experimental Biology and Medicine, 205(2),* 182-185.

Goodman, M.N. (1991). Tumor necrosis factor induces skeletal muscle protein breakdown in rats. *The American Journal of Physiology, 260(5 Pt 1),* E727-30.

Goto, K., Okuyama, R., Sugiyama, H., Honda, M., Kobayashi, T., Uehara, K., Akema, T., Sugiura, T., Yamada, S., Ohira, Y. & Yoshioka, T. (2003). Effects of heat stress and mechanical stretch on protein expression in cultured skeletal muscle cells. *Pflugers Archive, 447(2),* 247-253.

Granner, D.K. (1996). Hormones of the Adrenal Cortex. In *Harper's Biochemistry.* (edited by R.K. Murray, D.K. Granner, P.A. Mayes and V.W. and Rodwell), twenty-fourth edition. pp. 547. USA: Appleton and Lange.

Greenhaff, P.L., Karagounis, L.G., Peirce, N., Simpson, E.J., Hazell, M., Layfield, R., Wackerhage, H., Smith, K., Atherton, P., Selby, A. & Rennie, M.J. (2008). Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *American journal of physiology.Endocrinology and metabolism, 295(3),* E595-604.

Greenhaff, P.L., Gleeson, M. & Maughan, R.J. (1987). The effects of dietary manipulation on blood acid-base status and the performance of high intensity exercise. *European journal of applied physiology and occupational physiology, 56(3),* 331-337.

Griffiths, R.D. (1996). Muscle mass, survival, and the elderly ICU patient. *Nutrition, 12(6),* 456-458.

Groppo, R. & Richter, J.D. (2009). Translational control from head to tail. *Current opinion in cell biology, 21(3),* 444-451.

Gul, A., Gilbert, S. & Levey, A. (2007). Assessment of Renal Function. In *Comprehensive Clinical Nephrology.* (edited by J. Feehally, J. Floege and R. and Johnson), Third Edition. pp. 27. China: Mosby Elsevier.

Gulyassy, P.F., Aviram, A. & Peters, J.H. (1970). Evaluation of amino acid and protein requirements in chronic uremia. *Archives of Internal Medicine, 126(5),* 855-859.

Gulyassy, P.F., Peters, J.H., Lin, S.C. & Ryan, P.M. (1968). Hemodialysis and plasma amino acid composition in chronic renal failure. *The American Journal of Clinical Nutrition, 21(6),* 565-573.

Hack, V., Gross, A., Kinscherf, R., Bockstette, M., Fiers, W., Berke, G. & Droge, W. (1996). Abnormal glutathione and sulfate levels after interleukin 6 treatment and in tumor-induced cachexia. *FASEB, 10(10),* 1219-1226.

Hagberg, J.M., Goldberg, A.P., Ehsani, A.A., Heath, G.W., Delmez, J.A. & Harter, H.R. (1983). Exercise training improves hypertension in hemodialysis patients. *American Journal of Nephrology, 3(4),* 209-212.

Haines, D.J., Swan, C.H., Green, J.R. & Woodley, J.F. (1989). Experimental uraemia with associated plasma amino acid abnormalities but without retarded food intake and weight gain. *Nephron, 53(3),* 233-237.

Hannaford, M.C., Goldstein, M.B., Josse, R.G. & Halperin, M.L. (1982). Role of acidosis in the protein wasting of fasting in the rat and the rabbit. *Canadian journal of physiology and pharmacology, 60(3),* 331-334.

Haraldsson, B., Nystrom, J. & Deen, W.M. (2008). Properties of the glomerular barrier and mechanisms of proteinuria. *Physiological Reviews, 88(2),* 451-487.

Haraldsson, B. & Sorensson, J. (2004). Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. *News in physiological sciences, 19,* 7-10.

Harris, R., Dunnet, M. & Greenhaff, P. (1998). Carnosine and taurine contents in individual fibres of human vastus lateralis muscle. *Journal of sports sciences, 16,* 639-643.

Harris, R.C., Marlin, D.J., Dunnett, M., Snow, D.H. & Hultman, E. (1990). Muscle buffering capacity and dipeptide content in the thoroughbred horse, greyhound dog and man. *Comparative biochemistry and physiology. A, Comparative physiology, 97(2),* 249-251.

Hasselgren, P.O., Wray, C. & Mammen, J. (2002). Molecular regulation of muscle cachexia: it may be more than the proteasome. *Biochemical and biophysical research communications, 290(1),* 1-10.

Hatanaka, T., Hatanaka, Y. & Setou, M. (2006). Regulation of amino acid transporter ATA2 by ubiquitin ligase Nedd4-2. *The Journal of biological chemistry, 281(47)*, 35922-35930.

Hawley, J.A. (2009). Molecular responses to strength and endurance training: are they incompatible? *Applied physiology, nutrition, and metabolism, 34(3),* 355-361.

Hay, N. & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes & development, 18(16),* 1926-1945.

Headley, S., Germain, M., Mailloux, P., Mulhern, J., Ashworth, B., Burris, J., Brewer, B., Nindl, B.C., Coughlin, M., Welles, R. & Jones, M. (2002). Resistance training improves strength and functional measures in patients with end-stage renal disease. *American Journal of Kidney, 40(2),* 355-364.

Henriksen, E.J., Schneider, M.C. & Ritter, L.S. (1993). Regulation of contraction-stimulated system A amino acid uptake in skeletal muscle: role of vicinal sulfhydryls. *Metabolism: clinical and experimental, 42(4),* 440-445.

Henriksen, E.J., Louters, L.L., Stump, C.S. & Tipton, C.M. (1992). Effects of prior exercise on the action of insulin-like growth factor I in skeletal muscle. *The American Journal of Physiology, 263(2 Pt 1),* E340-4.

Hernandez, J.M., Fedele, M.J. & Farrell, P.A. (2000). Time course evaluation of protein synthesis and glucose uptake after acute resistance exercise in rats. *Journal of applied physiology, 88(3),* 1142-1149.

Hill, C.A., Harris, R.C., Kim, H.J., Harris, B.D., Sale, C., Boobis, L.H., Kim, C.K. & Wise, J.A. (2007). Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino acids*, *32(2),* 225-233.

Holloszy, J.O. & Coyle, E.F. (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *Journal of applied physiology: respiratory, environmental and exercise physiology, 56(4),* 831-838.

Holz, M.K., Ballif, B.A., Gygi, S.P. & Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell, 123(4),* 569-580.

Hood, D.A. (2009). Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Applied physiology, nutrition, and metabolism, 34(3),* 465-472.

Hornberger, T.A., Sukhija, K.B. & Chien, S. (2006). Regulation of mTOR by mechanically induced signaling events in skeletal muscle. *Cell cycle, 5(13),* 1391-1396.

Houdijk, A.P., Rijnsburger, E.R., Jansen, J., Wesdorp, R.I., Weiss, J.K., McCamish, M.A., Teerlink, T., Meuwissen, S.G., Haarman, H.J., Thijs, L.G. & van Leeuwen, P.A. (1998). Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. *Lancet, 352(9130),* 772-776.

Hu, Z., Wang, H., Lee, I.H., Du, J. & Mitch, W.E. (2009). Endogenous glucocorticoids and impaired insulin signaling are both required to stimulate muscle wasting under pathophysiological conditions in mice. *The Journal of clinical investigation, 119(10),* 3059-3069.

Hultman, E. & Sahlin, K. (1980). Acid-base balance during exercise. *Exercise and sport sciences reviews, 8,* 41-128.

Hyde, R., Taylor, P.M. & Hundal, H.S. (2003). Amino acid transporters: roles in amino acid sensing and signalling in animal cells. *The Biochemical journal, 373(Pt 1),* 1-18.

Hyde, R., Peyrollier, K. & Hundal, H.S. (2002). Insulin promotes the cell surface recruitment of the SAT2/ATA2 system A amino acid transporter from an endosomal compartment in skeletal muscle cells. *The Journal of biological chemistry, 277(16),* 13628-13634.

Hyde, R., Christie, G.R., Litherland, G.J., Hajduch, E., Taylor, P.M. & Hundal, H.S. (2001). Subcellular localization and adaptive up-regulation of the System A (SAT2) amino acid transporter in skeletal-muscle cells and adipocytes. *The Biochemical journal, 355(Pt 3),* 563-568.

Imae, M., Fu, Z., Yoshida, A., Noguchi, T. & Kato, H. (2003). Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16. *Journal of Molecular Endocrinology, 30(2),* 253-262.

Janzen, D.M. & Geballe, A.P. (2004). The effect of eukaryotic release factor depletion on translation termination in human cell lines. *Nucleic acids research, 32(15),* 4491-4502.

Jastrzebski, K., Hannan, K.M., Tchoubrieva, E.B., Hannan, R.D. & Pearson, R.B. (2007). Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. *Growth factors, 25(4),* 209-226.

Jemiolo, B. & Trappe, S. (2004). Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise. *Biochemical and biophysical research communications, 320(3),* 1043-1050.

Johansen, K.L. (2007). Exercise in the end-stage renal disease population. *Journal of the American Society of Nephrology, 18(6),* 1845-1854.

Johansen, K.L. (2005). Exercise and chronic kidney disease: current recommendations. *Sports medicine, 35(6),* 485-499.

Johansen, K.L., Doyle, J., Sakkas, G.K. & Kent-Braun, J.A. (2005). Neural and metabolic mechanisms of excessive muscle fatigue in maintenance hemodialysis patients. *American journal of physiology. Regulatory, integrative and comparative physiology, 289(3),* R805-13.

Johansen, K.L., Shubert, T., Doyle, J., Soher, B., Sakkas, G.K. & Kent-Braun, J.A. (2003). Muscle atrophy in patients receiving hemodialysis: effects on muscle strength, muscle quality, and physical function. *Kidney international, 63(1),* 291-297.

Johansen, K.L., Chertow, G.M., Ng, A.V., Mulligan, K., Carey, S., Schoenfeld, P.Y. & Kent-Braun, J.A. (2000). Physical activity levels in patients on hemodialysis and healthy sedentary controls. *Kidney international, 57(6),* 2564-2570.

Jones, N.L., Sutton, J.R., Taylor, R. & Toews, C.J. (1977). Effect of pH on cardiorespiratory and metabolic responses to exercise. *Journal of applied physiology: respiratory, environmental and exercise physiology, 43(6),* 959-964.

Kaizu, Y., Ohkawa, S., Odamaki, M., Ikegaya, N., Hibi, I., Miyaji, K. & Kumagai, H. (2003). Association between inflammatory mediators and muscle mass in long-term hemodialysis patients. *American Journal of Kidney Diseases, 42(2),* 295-302.

Kajiyama, H., Nojima, Y., Mitsuhashi, H., Ueki, K., Tamura, S., Sekihara, T., Wakamatsu, R., Yano, S. & Naruse, T. (2000). Elevated levels of serum sulfite in patients with chronic renal failure. *Journal of the American Society of Nephrology, 11(5),* 923-927.

Kalantar-Zadeh, K. & Balakrishnan, V.S. (2006). The kidney disease wasting: inflammation, oxidative stress, and diet-gene interaction. *Hemodialysis international, 10(4),* 315-325.

Kalhoff, H., Manz, F., Diekmann, L., Kunz, C., Stock, G.J. & Weisser, F. (1993). Decreased growth rate of low-birth-weight infants with prolonged maximum renal acid stimulation. *Acta Paediatrica, 82(6-7),* 522-527.

Kandel, E.S. & Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Experimental cell research, 253(1),* 210-229.

Kapp, L.D. & Lorsch, J.R. (2004). The molecular mechanics of eukaryotic translation. *Annual Review of Biochemistry, 73,* 657-704.

Karim, Z., Szutkowska, M., Vernimmen, C. & Bichara, M. (2005). Renal handling of NH3/NH4+: recent concepts. *Nephron, 101(4),* p77-81.

Karlsson, H.K., Nilsson, P.A., Nilsson, J., Chibalin, A.V., Zierath, J.R. & Blomstrand, E. (2004). Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *American journal of physiology. Endocrinology and metabolism, 287(1),* E1-7.

Karlsson, J., Funderburk, C.F., Essen, B. & Lind, A.R. (1975). Constituents of human muscle in isometric fatigue. *Journal of applied physiology, 38(2),* 208-211.

Kayali, A.G., Goodman, M.N., Lin, J. & Young, V.R. (1990). Insulin- and thyroid hormone-independent adaptation of myofibrillar proteolysis to glucocorticoids. *The American Journal of Physiology, 259(5 Pt 1),* E699-705.

Kelly, D.P. & Scarpulla, R.C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes & development, 18(4),* 357-368.

Kemp, G.J., Crowe, A.V., Anijeet, H.K., Gong, Q.Y., Bimson, W.E., Frostick, S.P., Bone, J.M., Bell, G.M. & Roberts, J.N. (2004). Abnormal mitochondrial function and muscle wasting,

but normal contractile efficiency, in haemodialysed patients studied non-invasively in vivo. *Nephrology, dialysis, transplantation, 19(6),* 1520-1527.

Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C.E., Peltonen, L., Holmberg, C., Olsen, A. & Tryggvason, K. (1998). Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Molecular cell, 1(4),* 575-582.

Kimball, S.R. (2007). The role of nutrition in stimulating muscle protein accretion at the molecular level. *Biochemical Society transactions, 35(Pt 5),* 1298-1301.

Kimball, S.R. & Jefferson, L.S. (2006). Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *The Journal of nutrition, 136(1 Suppl),* 227S-31S.

Kimball, S.R., Shantz, L.M., Horetsky, R.L. & Jefferson, L.S. (1999). Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *The Journal of biological chemistry, 274(17),* 11647-11652.

Kimball, S.R., Vary, T.C. & Jefferson, L.S. (1994). Regulation of protein synthesis by insulin. *Annual Review of Physiology, 56,* 321-348.

King, P.A. (1994). Effects of insulin and exercise on amino acid transport in rat skeletal muscle. *The American Journal of Physiology, 266(2 Pt 1),* C524-30.

Kleger, G.R., Turgay, M., Imoberdorf, R., McNurlan, M.A., Garlick, P.J. & Ballmer, P.E. (2001). Acute metabolic acidosis decreases muscle protein synthesis but not albumin synthesis in humans. *American Journal of Kidney, 38(6),* 1199-1207.

Koch, A. & Kaske, M. (2008). Clinical efficacy of intravenous hypertonic saline solution or hypertonic bicarbonate solution in the treatment of inappetent calves with neonatal diarrhea. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine, 22(1),* 202-211.

Koeppen, B.M. (2009). The kidney and acid-base regulation. *Advances in Physiology Education, 33(4),* 275-281.

Konstantinidou, E., Koukouvou, G., Kouidi, E., Deligiannis, A. & Tourkantonis, A. (2002). Exercise training in patients with end-stage renal disease on hemodialysis: comparison of three rehabilitation programs. *Journal of rehabilitation medicine, 34(1),* 40-45.

Koopman, R., Zorenc, A.H., Gransier, R.J., Cameron-Smith, D. & van Loon, L.J. (2006). Increase in S6K1 phosphorylation in human skeletal muscle following resistance exercise occurs mainly in type II muscle fibers. *American journal of physiology. Endocrinology and metabolism, 290(6),* E1245-52.

Kooman, J.P., Deutz, N.E., Zijlmans, P., van den Wall Bake, A., Gerlag, P.G., van Hooff, J.P. & Leunissen, K.M. (1997). The influence of bicarbonate supplementation on plasma levels of

branched-chain amino acids in haemodialysis patients with metabolic acidosis. *Nephrology, dialysis, transplantation, 12(11),* 2397-2401.

Kopple, J.D. (1978). Abnormal amino acid and protein metabolism in uremia. *Kidney international, 14(4),* 340-348.

Kopple, J.D., Wang, H., Casaburi, R., Fournier, M., Lewis, M.I., Taylor, W. & Storer, T.W. (2007). Exercise in maintenance hemodialysis patients induces transcriptional changes in genes favoring anabolic muscle. *Journal of the American Society of Nephrology, 18(11),* 2975-2986.

Kopple, J.D., Mercurio, K., Blumenkrantz, M.J., Jones, M.R., Tallos, J., Roberts, C., Card, B., Saltzman, R., Casciato, D.A. & Swendseid, M.E. (1981). Daily requirement for pyridoxine supplements in chronic renal failure. *Kidney international, 19(5),* 694-704.

Kosmadakis, G.C., Bevington, A., Smith, A.C., Clapp, E.L., Viana, J.L., Bishop, N.C. & Feehally, J. (2010). Physical Exercise in Patients with Severe Kidney Disease. *Nephron. Clinical practice, 115(1),* c7-c16.

Kossi, M. & and Nahas, M. (2007). Epidemiological and Pathophysiology of Chronic Kidney Disease: Natural History, Risk Factors, and Management. In *Comprehensive Clinical Nephrology.* (edited by J. Feehally, J. Floege and R. and Johnson), Third Edition. pp. 813. China: Mosby Elsevier.

Koufaki, P., Nash, P.F. & Mercer, T.H. (2002). Assessing the efficacy of exercise training in patients with chronic disease. *Medicine and science in sports and exercise, 34(8),* 1234-1241.

Kouidi, E., Grekas, D., Deligiannis, A. & Tourkantonis, A. (2004). Outcomes of long-term exercise training in dialysis patients: comparison of two training programs. *Clinical nephrology, 61 Suppl 1,* S31-8.

Kouidi, E., Albani, M., Natsis, K., Megalopoulos, A., Gigis, P., Guiba-Tziampiri, O., Tourkantonis, A. & Deligiannis, A. (1998). The effects of exercise training on muscle atrophy in haemodialysis patients. *Nephrology, dialysis, transplantation, 13(3),* 685-699.

Kouidi, E., Iacovides, A., Iordanidis, P., Vassiliou, S., Deligiannis, A., Ierodiakonou, C. & Tourkantonis, A. (1997). Exercise renal rehabilitation program: psychosocial effects. *Nephron, 77(2),* 152-158.

Kovacic, V., Roguljic, L. & Kovacic, V. (2003). Metabolic acidosis of chronically hemodialyzed patients. *American Journal of Nephrology, 23(3),* 158-164.

Kraut, J.A. & Kurtz, I. (2005). Metabolic acidosis of CKD: diagnosis, clinical characteristics, and treatment. *American Journal of Kidney Disease, 45(6),* 978-993.

Kriz, W. & Elger, M. (2007). Chapter 1: Renal Anatomy. In *Comprehensive Clinical Nephrology.* (edited by J. Feehally, J. Floege and R. Johnson), Third Edition. pp. 1. China: Mosby Elsevier.

Kucukatay, V., Hacioglu, G., Savcioglu, F., Yargicoglu, P. & Agar, A. (2006). Visual evoked potentials in normal and sulfite oxidase deficient rats exposed to ingested sulfite. *Neurotoxicology, 27(1),* 93-100.

Lecker, S.H., Goldberg, A.L. & Mitch, W.E. (2006). Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology, 17(7),* 1807-1819.

Lecker, S.H., Jagoe, R.T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S.R., Mitch, W.E. & Goldberg, A.L. (2004). Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB, 18(1),* 39-51.

Lecker, S.H., Solomon, V., Mitch, W.E. & Goldberg, A.L. (1999). Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *The Journal of nutrition, 129(1S Suppl),* 227S-237S.

Lefebvre, A., de Vernejoul, M.C., Gueris, J., Goldfarb, B., Graulet, A.M. & Morieux, C. (1989). Optimal correction of acidosis changes progression of dialysis osteodystrophy. *Kidney international, 36(6),* 1112-1118.

Lemann, J.Jr., & Relman, A.S. (1959). The relation of sulfur metabolism to acid-base balance and electrolyte excretion: the effects of DL-methionine in normal man. *The Journal of clinical investigation, 38,* 2215-2223.

Lizcano, J.M. & Alessi, D.R. (2002). The insulin signalling pathway. *Current biology, 12(7),* R236-8.

Lofberg, E., Essen, P., McNurlan, M., Wernerman, J., Garlick, P., Anderstam, B., Bergstrom, J. & Alvestrand, A. (2000). Effect of hemodialysis on protein synthesis. *Clinical nephrology, 54(4),* 284-294.

Lofberg, E., Wernerman, J., Anderstam, B. & Bergstrom, J. (1997). Correction of acidosis in dialysis patients increases branched-chain and total essential amino acid levels in muscle. *Clinical nephrology, 48(4),* 230-237.

Lote, J.L. (1990). *Principles of Renal Physiology.* Second Edition. Bristol: Chapman and Hall.

Lundin, A.P., Stein, R.A., Brown, C.D., LaBelle, P., Kalman, F.S., Delano, B.G., Heneghan, W.F., Lazarus, N.A., Krasnow, N. & Friedman, E.A. (1987). Fatigue, acid-base and electrolyte changes with exhaustive treadmill exercise in hemodialysis patients. *Nephron, 46(1),* 57-62.

Lundin, A.P., Stein, R.A. & and Frank, F. (1981). Cardiovascular status in long-term hemodialysis patients: An exercise and echocardiographic study. *Nephron, 28,* 234-238.

Ma, R.S. & Chan, J.C. (1973). Endogenous sulphuric acid production: a method of measurement by extrapolation. *Clinical biochemistry, 6(2),* 82-87.

Mackenzie, B. & Erickson, J.D. (2004). Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Archiv, 447(5),* 784-795.

MacRae, H.S., Dennis, S.C., Bosch, A.N. & Noakes, T.D. (1992). Effects of training on lactate production and removal during progressive exercise in humans. *Journal of applied physiology, 72(5),* 1649-1656.

Mahoney, D.J., Carey, K., Fu, M.H., Snow, R., Cameron-Smith, D., Parise, G. & Tarnopolsky, M.A. (2004). Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiological genomics, 18(2),* 226-231.

Mak, R.H. & Cheung, W. (2006). Energy homeostasis and cachexia in chronic kidney disease. *Pediatric nephrology, 21(12),* 1807-1814.

Malagoni, A.M., Catizone, L., Mandini, S., Soffritti, S., Manfredini, R., Boari, B., Russo, G., Basaglia, N., Zamboni, P. & Manfredini, F. (2008). Acute and long-term effects of an exercise program for dialysis patients prescribed in hospital and performed at home. *Journal of nephrology, 21(6),* 871-878.

Mallinson, J.E., Constantin-Teodosiu, D., Sidaway, J., Westwood, F.R. & Greenhaff, P.L. (2009). Blunted Akt/FOXO signalling and activation of genes controlling atrophy and fuel use in statin myopathy. *The Journal of physiology, 587(Pt 1),* 219-230.

Maroni, B.J., Haesemeyer, R.W., Kutner, M.H. & Mitch, W.E. (1990). Kinetics of system A amino acid uptake by muscle: effects of insulin and acute uremia. *The American Journal of Physiology, 258(5 Pt 2),* F1304-10.

Marsiglia, J.C., Cingolani, H.E. & Gonzalez, N.C. (1973). Relevance of beta receptor blockade to the negative inotropic effect induced by metabolic acidosis. *Cardiovascular research, 7(3),* 336-343.

Mascher, H., Andersson, H., Nilsson, P.A., Ekblom, B. & Blomstrand, E. (2007). Changes in signalling pathways regulating protein synthesis in human muscle in the recovery period after endurance exercise. *Acta physiologica, 191(1),* 67-75.

Maughan, R.J. (1982). A simple, rapid method for the determination of glucose, lactate, pyruvate, alanine, 3-hydroxybutyrate and acetoacetate on a single 20-mul blood sample. *Clinica chimica acta, 122(2),* 231-240.

Maughan, R., Gleeson, M. & Greenhaff, P.L. (1997a). Physiology and Biochemistry of Skeletal Muscle and Exercise. In *Biochemistry of Exercise and Training.* pp. 1. UK: Oxford University Press.

Maughan, R., Gleeson, M. & Greenhaff, P. (1997b). Metabolic Responses to Prolonged Exercise. In *Biochemistry of Exercise and Training.* pp. 158. UK: Oxford University Press.

May, R.C., Bailey, J.L., Mitch, W.E., Masud, T. & England, B.K. (1996). Glucocorticoids and acidosis stimulate protein and amino acid catabolism in vivo. *Kidney international, 49(3),* 679-683.

May, R.C., Kelly, R.A. & Mitch, W.E. (1987). Mechanisms for defects in muscle protein metabolism in rats with chronic uremia. Influence of metabolic acidosis. *The Journal of clinical investigation, 79(4),* 1099-1103.

May, R.C., Kelly, R.A. & Mitch, W.E. (1986). Metabolic acidosis stimulates protein degradation in rat muscle by a glucocorticoid-dependent mechanism. *The Journal of clinical investigation, 77(2),* 614-621.

McDonald, W.J., Golper, T.A., Mass, R.D., Kendall, J.W., Porter, G.A., Girard, D.E. & Fischer, M.D. (1979). Adrenocorticotropin-cortisol axis abnormalities in hemodialysis patients. *The Journal of clinical endocrinology and metabolism, 48(1),* 92-95.

McDowell, H.E., Eyers, P.A. & Hundal, H.S. (1998). Regulation of System A amino acid transport in L6 rat skeletal muscle cells by insulin, chemical and hyperthermic stress. *FEBS letters, 441(1),* 15-19.

McGivan, J.D. & Pastor-Anglada, M. (1994). Regulatory and molecular aspects of mammalian amino acid transport. *The Biochemical journal, 299 ( Pt 2)(Pt 2),* 321-334.

McMahon, L.P., McKenna, M.J., Sangkabutra, T., Mason, K., Sostaric, S., Skinner, S.L., Burge, C., Murphy, B. & Crankshaw, D. (1999). Physical performance and associated electrolyte changes after haemoglobin normalization: a comparative study in haemodialysis patients. *Nephrology, dialysis, transplantation, 14(5),* 1182-1187.

McSherry, E. & Morris, R.C.,Jr (1978). Attainment and maintenance of normal stature with alkali therapy in infants and children with classic renal tubular acidosis. *The Journal of clinical investigation, 61(2),* 509-527.

Merrick, W.C. (1992). Mechanism and regulation of eukaryotic protein synthesis. *Microbiological reviews, 56(2),* 291-315.

Mesquita, R.B., Fernandes, S.M. & Rangel, A.O. (2002). Turbidimetric determination of chloride in different types of water using a single sequential injection analysis system. *Journal of environmental monitoring, 4(3),* 458-461.

Métayer, S., Seiliez, I., Collin, A., Duchene, S., Mercier, Y., Geraert, P.A. & Tesseraud, S. (2008). Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. *The Journal of nutritional biochemistry, 19(4),* 207-215.

Miller, B.W., Cress, C.L., Johnson, M.E., Nichols, D.H. & Schnitzler, M.A. (2002). Exercise during hemodialysis decreases the use of antihypertensive medications. *American Journal of Kidney Diseases, 39(4),* 828-833.

Mitch, W.E. (2006). Metabolic and clinical consequences of metabolic acidosis. *Journal of nephrology, 19 Suppl 9*, S70-5.

Mitch, W.E. (2002). Malnutrition: a frequent misdiagnosis for hemodialysis patients. *The Journal of clinical investigation, 110(4),* 437-439.

Mitch, W.E. (1998). Robert H Herman Memorial Award in Clinical Nutrition Lecture, 1997. Mechanisms causing loss of lean body mass in kidney disease. *The American Journal of Clinical Nutrition, 67(3),* 359-366.

Mitch, W.E. & Goldberg, A.L. (1996). Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. *The New England journal of medicine, 335(25),* 1897-1905.

Mitch, W.E., Medina, R., Grieber, S., May, R.C., England, B.K., Price, S.R., Bailey, J.L. & Goldberg, A.L. (1994). Metabolic acidosis stimulates muscle protein degradation by activating the adenosine triphosphate-dependent pathway involving ubiquitin and proteasomes. *The Journal of clinical investigation,* 93(5), 2127-2133.

Mitchell, S.F. & Lorsch, J.R. (2008). Should I stay or should I go? Eukaryotic translation initiation factors 1 and 1A control start codon recognition. *The Journal of biological chemistry, 283(41),* 27345-27349.

Mittendorfer, B., Volpi, E. & Wolfe, R.R. (2001). Whole body and skeletal muscle glutamine metabolism in healthy subjects. *American journal of physiology.Endocrinology and metabolism, 280(2),* E323-33.

Molsted, S., Eidemak, I., Sorensen, H.T. & Kristensen, J.H. (2004). Five months of physical exercise in hemodialysis patients: effects on aerobic capacity, physical function and self-rated health. *Nephron.Clinical practice, 96(3),* c76-81.

Moriscot, A.S., Baptista, I.L., Bogomolovas, J., Witt, C., Hirner, S., Granzier, H. & Labeit, S. (2010). MuRF1 is a muscle fiber-type II associated factor and together with MuRF2 regulates type-II fiber trophicity and maintenance. *Journal of structural biology, 170(2),* 344-353.

Movilli, E., Viola, B.F., Camerini, C., Mazzola, G. & Cancarini, G.C. (2009). Correction of metabolic acidosis on serum albumin and protein catabolism in hemodialysis patients. *Journal of renal nutrition, 19(2),* 172-177.

Murton, A.J., Constantin, D. & Greenhaff, P.L. (2008). The involvement of the ubiquitin proteasome system in human skeletal muscle remodelling and atrophy. *Biochimica et biophysica acta, 1782*(*12), 730-743.

Nader, G.A. (2006). Concurrent strength and endurance training: from molecules to man. *Medicine and science in sports and exercise, 38(11),* 1965-1970.

Nader, G.A. & Esser, K.A. (2001). Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *Journal of applied physiology, 90(5),* 1936-1942.

Nakamura, H., Kajikawa, R. & Ubuka, T. (2002). A study on the estimation of sulfur-containing amino acid metabolism by the determination of urinary sulfate and taurine. *Amino acids, 23(4),* 427-431.

Nakanishi, T., Otaki, Y., Hasuike, Y., Nanami, M., Itahana, R., Miyagawa, K., Nishikage, H., Izumi, M. & Takamitsu, Y. (2002). Association of hyperhomocysteinemia with plasma

sulfate and urine sulfate excretion in patients with progressive renal disease. *American Journal of Kidney Diseases, 40(5),* 909-915.

Narins, R.G. & Cohen, J.J. (1987). Bicarbonate therapy for organic acidosis: the case for its continued use. *Annals of Internal Medicine, 106(4),* 615-618.

NHS (2008). New NICE guideline on identification of Chronic Kidney Disease will help save lives.

Nishida, A., Kubo, K. & Nihei, H. (1991). Impaired muscle energy metabolism in uremia as monitored by 31P-NMR. *Nippon Jinzo Gakkai shi, 33(1),* 65-73. Abstract Only.

Nissen, S. (1997). Measurement of muscle proteolysis and the impact on muscle wasting. The *Proceedings of the Nutrition Society, 56(2),* 793-799.

Noble, C.G. & Song, H. (2008). Structural studies of elongation and release factors. *Cellular and molecular life sciences : CMLS, 65(9),* 1335-1346.

Nolan, G.E., Smith, J.B., Chavre, V.J. & Jubiz, W. (1981). Spurious overestimation of plasma cortisol in patients with chronic renal failure. *The Journal of clinical endocrinology and metabolism, 52(6),* 1242-1245.

Nussbaum, J. & Garcia, R.K. (2009). Restorative physical and occupational therapy: a critical need for patients with chronic kidney and end-stage renal disease. *Advances in chronic kidney disease, 16(6),* 529-535.

O'Hare, A.M., Tawney, K., Bacchetti, P. & Johansen, K.L. (2003). Decreased survival among sedentary patients undergoing dialysis: results from the dialysis morbidity and mortality study wave 2. *American Journal of Kidney Diseases, 41(2),* 447-454.

Painter, P. (2005). Physical functioning in end-stage renal disease patients: update 2005. *Hemodialysis international, 9(3),* 218-235.

Painter, P., Moore, G., Carlson, L., Paul, S., Myll, J., Phillips, W. & Haskell, W. (2002). Effects of exercise training plus normalization of hematocrit on exercise capacity and health-related quality of life. *American Journal of Kidney Diseases, 39(2),* 257-265.

Painter, P., Carlson, L., Carey, S., Paul, S.M. & Myll, J. (2000). Physical functioning and health-related quality-of-life changes with exercise training in hemodialysis patients. *American Journal of Kidney Diseases, 35(3),* 482-492.

Painter, P. & Moore, G.E. (1994). The impact of recombinant human erythropoietin on exercise capacity in hemodialysis patients. *Advances in Renal Replacement Therapy, 1(1),* 55-65.

Painter, P.L., Nelson-Worel, J.N., Hill, M.M., Thornbery, D.R., Shelp, W.R., Harrington, A.R. & Weinstein, A.B. (1986). Effects of exercise training during hemodialysis. *Nephron, 43(2),* 87-92.

Palii, S.S., Thiaville, M.M., Pan, Y.X., Zhong, C. & Kilberg, M.S. (2006). Characterization of the amino acid response element within the human sodium-coupled neutral amino acid transporter 2 (SNAT2) System A transporter gene. *The Biochemical journal, 395(3),* 517-527.

Papadoyannakis, N.J., Stefanidis, C.J. & McGeown, M. (1984). The effect of the correction of metabolic acidosis on nitrogen and potassium balance of patients with chronic renal failure. *The American Journal of Clinical Nutrition, 40(3), 623-627.*

Parkhouse, W.S., McKenzie, D.C., Hochachka, P.W. & Ovalle, W.K. (1985). Buffering capacity of deproteinized human vastus lateralis muscle. *Journal of applied physiology, 58(1),* 14-17.

Parkhouse, W.S. & McKenzie, D.C. (1984). Possible contribution of skeletal muscle buffers to enhanced anaerobic performance: a brief review. *Medicine and science in sports and exercise, 16(4),* 328-338.

Pavenstadt, H., Kriz, W. & Kretzler, M. (2003). Cell biology of the glomerular podocyte. *Physiological Reviews, 83(1),* 253-307.

Pecoits-Filho, R., Lindholm, B. & Stenvinkel, P. (2002). The malnutrition, inflammation, and atherosclerosis (MIA) syndrome -- the heart of the matter. *Nephrology, dialysis, transplantation, 17 Suppl 11*, 28-31.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C. & Thomas, G. (2004). S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Molecular and cellular biology, 24(8),* 3112-3124.

Pereira, B.J., Shapiro, L., King, A.J., Falagas, M.E., Strom, J.A. & Dinarello, C.A. (1994). Plasma levels of IL-1 beta, TNF alpha and their specific inhibitors in undialyzed chronic renal failure, CAPD and hemodialysis patients. *Kidney international, 45(3),* 890-896.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research, 29(9),* e45.

Phillips, S.M., Tipton, K.D., Aarsland, A., Wolf, S.E. & Wolfe, R.R. (1997). Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *The American Journal of Physiology, 273(1 Pt 1),* E99-107.

Pickering, W.P., Baker, F.E., Brown, J., Butler, H.L., Govindji, S., Parsons, J.M., Pawluczyk, I.Z., Walls, J. & Bevington, A. (2003). Glucocorticoid antagonist RU38486 fails to block acid-induced muscle wasting in vivo or in vitro. *Nephrology, dialysis, transplantation, 18(8),* 1475-1484.

Price, S.R., England, B.K., Bailey, J.L., Van Vreede, K. & Mitch, W.E. (1994). Acidosis and glucocorticoids concomitantly increase ubiquitin and proteasome subunit mRNAs in rat muscle. *The American Journal of Physiology, 267(4 Pt 1),* C955-60.

Proud, C.G. (2007). Amino acids and mTOR signalling in anabolic function. *Biochemical Society transactions, 35(Pt 5),* 1187-1190.

Proud, C.G. (2006). Regulation of protein synthesis by insulin. *Biochemical Society transactions, 34(Pt 2),* 213-216.

Proud, C.G., Wang, X., Patel, J.V., Campbell, L.E., Kleijn, M., Li, W. & Browne, G.J. (2001). Interplay between insulin and nutrients in the regulation of translation factors. *Biochemical Society transactions, 29(Pt 4),* 541-547.

Qureshi, G.A., Qureshi, A.R. & Bergstrom, J. (1989). Quantitation of free amino acids in plasma and muscle samples in healthy subjects and uremic patients by high-performance liquid chromatography and fluorescence detection. *Journal of pharmaceutical and biomedical analysis, 7(3),* 377-384.

Rajan, V.R. & Mitch, W.E. (2008). Muscle wasting in chronic kidney disease: the role of the ubiquitin proteasome system and its clinical impact. *Pediatric nephrology, 23(4),* 527-535.

Rauch, C. & Loughna, P.T. (2008). Stretch-induced activation of ERK in myocytes is p38 and calcineurin-dependent. *Cell biochemistry and function, 26(8),* 866-869.

Reaich, D., Graham, K.A., Channon, S.M., Hetherington, C., Scrimgeour, C.M., Wilkinson, R. & Goodship, T.H. (1995). Insulin-mediated changes in PD and glucose uptake after correction of acidosis in humans with CRF. *The American Journal of Physiology, 268(1 Pt 1),* E121-6.

Reaich, D., Channon, S.M., Scrimgeour, C.M., Daley, S.E., Wilkinson, R. & Goodship, T.H. (1993). Correction of acidosis in humans with CRF decreases protein degradation and amino acid oxidation. *The American Journal of Physiology, 265(2 Pt 1),* E230-5.

Reaich, D., Channon, S.M., Scrimgeour, C.M. & Goodship, T.H. (1992). Ammonium chloride-induced acidosis increases protein breakdown and amino acid oxidation in humans. *The American Journal of Physiology, 263(4 Pt 1),* E735-9.

Rennie, M.J. (1999). An introduction to the use of tracers in nutrition and metabolism. The *Proceedings of the Nutrition Society, 58(4)*, 935-944.

Rennie, M.J., Bohe, J., Smith, K., Wackerhage, H. & Greenhaff, P. (2006). Branched-chain amino acids as fuels and anabolic signals in human muscle. *The Journal of nutrition, 136(1 Suppl)*, 264S-8S.

Rhen, T. & Cidlowski, J.A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. The New England journal of medicine, *353(16),* 1711-1723.

Robertson, S.A. (1989). Simple acid-base disorders. *The Veterinary clinics of North America. Small animal practice, 19(2),* 289-306. Abstract Only.

Robins, A.J., Milewczyk, B.K., Booth, E.M. & Mallick, N.P. (1972). Plasma amino acid abnormalities in chronic renal failure. *Clinica chimica acta, 42(1),* 215-217.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. & Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell, 78(5),* 761-771.

Rosman, P.M., Farag, A., Peckham, R., Benn, R., Tito, J., Bacci, V. & Wallace, E.Z. (1982). Pituitary-adrenocortical function in chronic renal failure: blunted suppression and early escape of plasma cortisol levels after intravenous dexamethasone. *The Journal of clinical endocrinology and metabolism, 54(3),* 528-533.

Rosner, M., Hanneder, M., Siegel, N., Valli, A., Fuchs, C. & Hengstschlager, M. (2008). The mTOR pathway and its role in human genetic diseases. *Mutation research, 659(3),* 284-292.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P. & Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes & development, 19(18),* 2199-2211.

Saad, M.J., Folli, F., Kahn, J.A. & Kahn, C.R. (1993). Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *The Journal of clinical investigation, 92(4),* 2065-2072.

Sadiq, F., Hazlerigg, D.G. & Lomax, M.A. (2007). Amino acids and insulin act additively to regulate components of the ubiquitin-proteasome pathway in C2C12 myotubes. *BMC molecular biology, 8,* 23.

Sakamoto, K., Arnolds, D.E., Ekberg, I., Thorell, A. & Goodyear, L.J. (2004). Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle. *Biochemical and biophysical research communications, 319(2),* 419-425.

Sakkas, G.K., Sargeant, A.J., Mercer, T.H., Ball, D., Koufaki, P., Karatzaferi, C. & Naish, P.F. (2003). Changes in muscle morphology in dialysis patients after 6 months of aerobic exercise training. *Nephrology, dialysis, transplantation, 18(9),* 1854-1861.

Salamone, L.M., Fuerst, T., Visser, M., Kern, M., Lang, T., Dockrell, M., Cauley, J.A., Nevitt, M., Tylavsky, F. & Lohman, T.G. (2000). Measurement of fat mass using DEXA: a validation study in elderly adults. *Journal of applied physiology, 89(1),* 345-352.

Sale, C., Saunders, B. & Harris, R.C. (2010). Effect of beta-alanine supplementation on muscle carnosine concentrations and exercise performance. *Amino acids, 39(2),* 321-333.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S.H. & Goldberg, A.L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell, 117(3),* 399-412.

Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W. & Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry, 278(17),* 14599-14602.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. & Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science, 307(5712),* 1098-1101.

Schaffer, S.W., Azuma, J. & Mozaffari, M. (2009). Role of antioxidant activity of taurine in diabetes. *Canadian journal of physiology and pharmacology, 87(2),* 91-99.

Schakman, O., Gilson, H. & Thissen, J.P. (2008). Mechanisms of glucocorticoid-induced myopathy. *The Journal of endocrinology, 197(1),* 1-10.

Scheid, M.P., Marignani, P.A. & Woodgett, J.R. (2002). Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Molecular and cellular biology, 22(17),* 6247-6260.

Scheltinga, M.R., Young, L.S., Benfell, K., Bye, R.L., Ziegler, T.R., Santos, A.A., Antin, J.H., Schloerb, P.R. & Wilmore, D.W. (1991). Glutamine-enriched intravenous feedings attenuate extracellular fluid expansion after a standard stress. *Annals of Surgery, 214(4),* 385-93; discussion 393-5.

Scott, S.V. & Klionsky, D.J. (1998). Delivery of proteins and organelles to the vacuole from the cytoplasm. *Current opinion in cell biology, 10(4),* 523-529.

Sebastian, A., Frassetto, L.A., Sellmeyer, D.E., Merriam, R.L. & Morris, R.C.,Jr (2002). Estimation of the net acid load of the diet of ancestral preagricultural Homo sapiens and their hominid ancestors. *The American Journal of Clinical Nutrition, 76(6),* 1308-1316.

Seino, S., Seino, M., Nishi, S. & Bell, G.I. (1989). Structure of the human insulin receptor gene and characterization of its promoter. *Proceedings of the National Academy of Sciences of the United States of America, 86(1),* 114-118.

Shah, O.J., Iniguez-Lluhi, J.A., Romanelli, A., Kimball, S.R. & Jefferson, L.S. (2002). The activated glucocorticoid receptor modulates presumptive autoregulation of ribosomal protein S6 protein kinase, p70 S6K. *The Journal of biological chemistry, 277(4),* 2525-2533.

Shah, O.J., Kimball, S.R. & Jefferson, L.S. (2000). Glucocorticoids abate p70(S6k) and eIF4E function in L6 skeletal myoblasts. *American journal of physiology. Endocrinology and metabolism, 279(1),* E74-82.

Shepherd, P.R., Withers, D.J. & Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *The Biochemical journal, 333 ( Pt 3)(Pt 3),* 471-490.

Sherman, W.M., Peden, M.C. & Wright, D.A. (1991). Carbohydrate feedings 1 h before exercise improves cycling performance. *The American Journal of Clinical Nutrition, 54(5),* 866-870.

Sherman, W.M., Costill, D.L., Fink, W.J., Hagerman, F.C., Armstrong, L.E. & Murray, T.F. (1983). Effect of a 42.2-km footrace and subsequent rest or exercise on muscle glycogen and enzymes. *Journal of applied physiology: respiratory, environmental and exercise physiology, 55(4),* 1219-1224.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G. & Kozma, S.C. (1998). Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *The EMBO journal, 17(22),* 6649-6659.

Shirley, D., Capasso, G. & Unwin, R. (2007). Chapter 2: Renal Physiology. In *Comprehensive Clinical Nephrology.* (edited by J. Feehally, J. Floege and R. Johnson), Third edition. pp. 13. China: Mosby Elsevier.

Silverthorn, D. (2001a). Chapter 12: The Muscles. In *Human Physiology - an Integrated Approach.* , Second Edition edn. pp. 345. New Jersey: Prentice Hall.

Silverthorn, D. (2001b). Chapter 18: The Kidneys. In *Human Physiology - an Integrated Approach.*, Second edition edn. pp. 542. New Jersey: Prentice Hall.

Silverthorn, D. (2001c). Chapter 19: Integrated Physiology II: Fluid and Electrolyte Balance. In *Human Physiology - an Integrated Approach.* Second Edition edn. pp. 569. New Jersey: Prentice Hall.

Smith, E.C. (1938). The buffering of muscle in rigor; protein, phosphate and carnosine. *The Journal of physiology, 92(3),* 336-343.

Soderlund, K., Greenhaff, P.L. & Hultman, E. (1992). Energy metabolism in type I and type II human muscle fibres during short term electrical stimulation at different frequencies. *Acta Physiologica Scandinavica, 144(1),* 15-22.

Solomon, V. & Goldberg, A.L. (1996). Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *The Journal of biological chemistry, 271(43),* 26690-26697.

Stein, A., Moorhouse, J., Iles-Smith, H., Baker, F., Johnstone, J., James, G., Troughton, J., Bircher, G. & Walls, J. (1997). Role of an improvement in acid-base status and nutrition in CAPD patients. *Kidney international, 52(4),* 1089-1095.

Stipanuk, M.H., Dominy, J.E.,Jr, Lee, J.I. & Coloso, R.M. (2006). Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *The Journal of nutrition, 136(6 Suppl),* 1652S-1659S.

Stipanuk, M.H. (2004). Role of the liver in regulation of body cysteine and taurine levels: a brief review. *Neurochemical research, 29(1),* 105-110.

Storer, T.W., Casaburi, R., Sawelson, S. & Kopple, J.D. (2005). Endurance exercise training during haemodialysis improves strength, power, fatigability and physical performance in maintenance haemodialysis patients. *Nephrology, dialysis, transplantation, 20(7),* 1429-1437.

Suliman, M.E., Barany, P., Divino Filho, J.C., Qureshi, A.R., Stenvinkel, P., Heimburger, O., Anderstam, B., Lindholm, B. & Bergstrom, J. (2002). Influence of nutritional status on plasma and erythrocyte sulphur amino acids, sulph-hydryls, and inorganic sulphate in end-stage renal disease. *Nephrology, dialysis, transplantation, 17(6),* 1050-1056.

Suliman, M.E., Filho, J.C., Barany, P., Anderstam, B., Lindholm, B. & Bergstrom, J. (2001). Effects of methionine loading on plasma and erythrocyte sulphur amino acids and sulph-

hydryls before and after co-factor supplementation in haemodialysis patients. *Nephrology, dialysis, transplantation, 16(1),* 102-110.

Suliman, M.E., Divino Filho, J.C., Barany, P., Anderstam, B., Lindholm, B. & Bergstrom, J. (1999). Effects of high-dose folic acid and pyridoxine on plasma and erythrocyte sulfur amino acids in hemodialysis patients. *Journal of the American Society of Nephrology, 10(6),* 1287-1296.

Suliman, M.E., Anderstam, B. & Bergstrom, J. (1996). Evidence of taurine depletion and accumulation of cysteinesulfinic acid in chronic dialysis patients. *Kidney international,* 50(5), 1713-1717.

Suzuki, Y., Ito, O., Mukai, N., and Takamatsu, K. (2002). High levels of skeletal muscle carnosine contributes to the latter half of exercise performance during maximal cycle ergometer sprinting. *Japanese Journal of Physiology, 52, 119-205.*

Sykiotis, G.P. & Papavassiliou, A.G. (2001). Serine phosphorylation of insulin receptor substrate-1: a novel target for the reversal of insulin resistance. *Molecular endocrinology, 15(11),* 1864-1869.

Szeto, C.C., Wong, T.Y., Chow, K.M., Leung, C.B. & Li, P.K. (2003). Oral sodium bicarbonate for the treatment of metabolic acidosis in peritoneal dialysis patients: a randomized placebo-control trial. *Journal of the American Society of Nephrology, 14(8),* 2119-2126.

Tan, B.H. & Fearon, K.C. (2008). Cachexia: prevalence and impact in medicine. *Current opinion in clinical nutrition and metabolic care, 11(4),* 400-407.

Tawney, K.W., Tawney, P.J. & Kovach, J. (2003). Disablement and rehabilitation in end-stage renal disease. *Seminars in dialysis, 16(6),* 447-452.

The Association of Public Health Observatories (2009). Chronic Kidney Disease Prevalence Estimates. http://www.apho.org.uk/resource/item.aspx?RID=63798

Thong, F.S., Dugani, C.B. & Klip, A. (2005). Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology, 20*, 271-284.

Tortora, G. & Grabowski, S. (1996a). Chapter 10: Muscle Tissue. In *Principles of Anatomy and Physiology.* Eighth edition. pp. 238. New York: Harper Collins College Publishers.

Tortora, G.:.G., S. (1996b). Chapter 26: The Urinary System. In *Principles of Anatomy and Physiology.* (edited by Anonymous ), Eighth edition edn. pp. 847. New York: Harper Collins College Publishers.

Tortora, G. & Grabowski, S. (1996c). Chapter 27: Fluid, Electrolyte and Acid-Base Homeostasis. In *Principles of Anatomy and Physiology.* Eighth edition. pp. 890. New York: Harper Collins College Publishers.

Trilok, G. & Draper, H.H. (1989). Sources of protein-induced endogenous acid production and excretion by human adults. *Calcified tissue international, 44(5),* 335-338.

Tsintzas, O.K., Williams, C., Boobis, L. & Greenhaff, P. (1996). Carbohydrate ingestion and single muscle fiber glycogen metabolism during prolonged running in men. *Journal of applied physiology, 81(2),* 801-809.

Uchiyama, T., Kulkarni, A.A., Davies, D.L. & Lee, V.H. (2003). Biophysical evidence for His57 as a proton-binding site in the mammalian intestinal transporter hPepT1. *Pharmaceutical research, 20(12),* 1911-1916.

Um, S.H., D'Alessio, D. & Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell metabolism, 3(6),* 393-402.

Vandenburgh, H.H. (1987). Motion into mass: how does tension stimulate muscle growth? *Medicine and science in sports and exercise,* 19(5 Suppl), S142-9.

Vincent, A.S., Lim, B.G., Tan, J., Whiteman, M., Cheung, N.S., Halliwell, B. & Wong, K.P. (2004). Sulfite-mediated oxidative stress in kidney cells. *Kidney international, 65(2),* 393-402.

Wackerhage, H. (2006). Introduction to Molecular Exercise Physiology. In *Genetics and Molecular Biology of Muscle Adaptation.* (edited by N. Spurway and D. MacLaren), pp. 121-163. Italy: Churchill Livingstone Elsevier.

Waddell, D.S., Baehr, L.M., van den Brandt, J., Johnsen, S.A., Reichardt, H.M., Furlow, J.D. & Bodine, S.C. (2008). The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *American journal of physiology.Endocrinology and metabolism, 295(4),* E785-97.

Wagenmakers, A.J. (1999). Tracers to investigate protein and amino acid metabolism in human subjects. *The Proceedings of the Nutrition Society, 58(4)*, 987-1000.

Wang, X.H., Du, J., Klein, J.D., Bailey, J.L. & Mitch, W.E. (2009). Exercise ameliorates chronic kidney disease-induced defects in muscle protein metabolism and progenitor cell function. *Kidney international, 76(7),* 751-759.

Wang, H., Kubica, N., Ellisen, L.W., Jefferson, L.S. & Kimball, S.R. (2006). Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. *The Journal of biological chemistry, 281(51),* 39128-39134.

Welle, S. (1999). Basic Mechanisms of Protein Turnover. In *Human Protein Metabolism.* pp. 11-28. New York: Springer-Verlag.

Westerblad, H., Allen, D.G. & Lannergren, J. (2002). Muscle fatigue: lactic acid or inorganic phosphate the major cause? *News in physiological, 17*, 17-21.

Westerblad, H., Bruton, J.D. & Lannergren, J. (1997). The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. *The Journal of physiology, 500 ( Pt 1)*(Pt 1), 193-204.

Weston, A.R., Myburgh, K.H., Lindsay, F.H., Dennis, S.C., Noakes, T.D. & Hawley, J.A. (1997). Skeletal muscle buffering capacity and endurance performance after high-intensity interval training by well-trained cyclists. *European journal of applied physiology and occupational physiology, 75(1),* 7-13.

Widegren, U., Jiang, X.J., Krook, A., Chibalin, A.V., Bjornholm, M., Tally, M., Roth, R.A., Henriksson, J., Wallberg-henriksson, H. & Zierath, J.R. (1998). Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB, 12(13),* 1379-1389.

Wilkinson, S.B., Phillips, S.M., Atherton, P.J., Patel, R., Yarasheski, K.E., Tarnopolsky, M.A. & Rennie, M.J. (2008). Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. The *Journal of physiology, 586(15)*, 3701-3717.

Wilson, J.R., McCully, K.K., Mancini, D.M., Boden, B. & Chance, B. (1988). Relationship of muscular fatigue to pH and diprotonated Pi in humans: a 31P-NMR study. *Journal of applied physiology, 64(6),* 2333-2339.

Wiseman, R.W., Beck, T.W. & Chase, P.B. (1996). Effect of intracellular pH on force development depends on temperature in intact skeletal muscle from mouse. *The American Journal of Physiology, 271(3 Pt 1),* C878-86.

Witard, O.C., Tieland, M., Beelen, M., Tipton, K.D., van Loon, L.J. & Koopman, R. (2009). Resistance exercise increases postprandial muscle protein synthesis in humans. *Medicine and science in sports and exercise, 41(1),* 144-154.

Workeneh, B.T. & Mitch, W.E. (2010). Review of muscle wasting associated with chronic kidney disease. *The American Journal of Clinical Nutrition, 91(4)*, 1128S – 1132S.

Workeneh, B.T., Rondon-Berrios, H., Zhang, L., Hu, Z., Ayehu, G., Ferrando, A., Kopple, J.D., Wang, H., Storer, T., Fournier, M., Lee, S.W., Du, J. & Mitch, W.E. (2006). Development of a diagnostic method for detecting increased muscle protein degradation in patients with catabolic conditions. *Journal of the American Society of Nephrology, 17(11),* 3233-3239.

Yang, X., Yang, C., Farberman, A., Rideout, T.C., de Lange, C.F., France, J. & Fan, M.Z. (2008). The mammalian target of rapamycin-signaling pathway in regulating metabolism and growth. *Journal of animal science, 86(14 Suppl),* E36-50.

Yang, D. & Brunengraber, H. (2000). Glutamate, a window on liver intermediary metabolism. *The Journal of nutrition, 130(4S Suppl),* 991S-4S.

Yao, D., Mackenzie, B., Ming, H., Varoqui, H., Zhu, H., Hediger, M.A. & Erickson, J.D. (2000). A novel system A isoform mediating Na+/neutral amino acid cotransport. *The Journal of biological chemistry, 275(30),* 22790-22797.

Young, G.A. & Parsons, F.M. (1970). Plasma amino acid imbalance in patients with chronic renal failure on intermittent dialysis. *Clinica chimica acta; international journal of clinical chemistry, 27(3),* 491-496.

Zabetakis, P.M., Gleim, G.W., Pasternack, F.L., Saraniti, A., Nicholas, J.A. & Michelis, M.F. (1982). Long-duration submaximal exercise conditioning in hemodialysis patients. *Clinical nephrology, 18(1),* 17-22.

Zhang, L., Wang, X.H., Wang, H., Du, J. & Mitch, W.E. (2010). Satellite Cell Dysfunction and Impaired IGF-1 Signaling Cause CKD-Induced Muscle Atrophy. *Journal of the American Society of Nephrology , 213*, 419 – 27.

Zhao, J., Brault, J.J., Schild, A., Cao, P., Sandri, M., Schiaffino, S., Lecker, S.H. & Goldberg, A.L. (2007). FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell metabolism, 6(6),* 472-483.

Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kisselev, L. & Philippe, M. (1995). Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *The EMBO journal, 14(16),* 4065-4072.

Zierath, J.R. & Hawley, J.A. (2004). Skeletal muscle fiber type: influence on contractile and metabolic properties. *PLoS biology, 2(10),* e348.

Zong, X., Stieber, J., Ludwig, A., Hofmann, F. & Biel, M. (2001). A single histidine residue determines the pH sensitivity of the pacemaker channel HCN2. *The Journal of biological chemistry, 276(9),* 6313-6319.

<p style="text-align: center;">Appendix A</p>
<p style="text-align: center;">Solutions and Reagents</p>

Folin Reagent A (1 Litre)
20g Anhydrous $Na_2CO_3$
4g NaOH
0.2g K,Na-tartrate. $4H_2O$
Ultra pure water up to one litre

Folin Reagent B (1 Litre)
5g $CuSO_4.5H_2O$
Ultra pure water up to one litre

UIC3 Protease Inhibitor Cocktail (100ml)
8.55g Sucrose
0.47g Hepes
0.032g Sodium Azide
0.076g EGTA
Adjust to pH 7.4 with 0.5M NaOH
Ultra pure water up to 100ml
20 µl PMSF (25mg)
14.2µl E64 (5mg)
13.7µl Pepstatin A (5mg)
9.5µl Leupeptin (10mg)

SDS-PAGE Muscle Lysis Buffer (10ml)
0.08g NaCl
0.002g KCl
0.002g $MgCl_2$
500µl Tris 1M pH 7.5
20µl Ethylenediaminetetraacetic acid (EDTA) 0.5M pH 8
250µl Ethylene glycol tetraacetic acid (EGTA) 40mM
800µl Glycerol
1ml 10% Triton X-100
100µl β-Glycerophosphate 1M

1.18ml Sodium Orthovanadate 8.5mM

1ml Sodium Fluoride 500mM

10μ β-Mercaptoethanol

5.09ml ultra pure water


SDS-PAGE L6 Lysis Buffer (10ml)

500μl Tris 1M pH 7.5

20μl Ethylenediaminetetraacetic acid (EDTA) 0.5M pH 8

250μl Ethylene glycol tetraacetic acid (EGTA) 40mM

1ml 10% Triton X-100

100μl β-Glycerophosphate 1M

1.18ml Sodium Orthovanadate 8.5mM

1ml Sodium Fluoride 500mM

10μ β-Mercaptoethanol

5.89ml ultra pure water


Laemelli Reducing Sample Buffer

4ml Ultra pure $H_2O$

1ml 0.5M Tric HCL pH 6.8

0.8ml Glycerol

1.6ml 10% w/v Sodium Dodecyl Sulphate (SDS)

0.4ml β-Mercaptoethanol

0.2ml 0.05% w/v Bromophenol Blue


10X Running Buffer (1 Litre)

30.3g Trizma Base

144g Glycine

100ml 20% SDS

Ultra pure water to one litre


1X Running Buffer (1 Litre)

100ml 10X Running buffer

900ml Ultra pure water


10X Transfer Buffer (1 Litre)

30.3g Tris-Base

144g Glycine

Ultra pure water to one litre

1X Transfer Buffer (1 Litre)
100ml 10X Transfer Buffer
200ml Methanol
100ml 10% SDS
Ultra pure water to one litre
Blue

10X TBS
60.55g Trizma base
87.66g NaCl
Ultra pure water to 700ml
pH to 7.6 using 6M HCl
Ultra pure water to one Litre

1X TTBS + 0.1% tween (1 Litre)
100ml 10X TBS
Ultra pure water to one litre
1ml Tween 20

RNA Gel Loading Sample Buffer
50% Glycerol
10mM Tris
1mM EDTA
Bromophenol blue 0.05% w/v to give a readily detectable colour
DNA Sample Buffer
0.25% Bromophenol Blue
15% Ficoll (Type 400) in Water

HBS
140mM BaCl
20mM Hepes Acid
2.5mM $MgSO_4$. $7H_2O$
5mM KCl
1mM $CaCl_2$. $2H_2O$

10mg/l Phenol red

Adjust to pH 7.4 with 0.5M NaOH

Pre-hybridisation Solution

5 ml Formamide

2.5ml 20X SSPE

1ml 50X Denhardt's solution

0.5ml 20% SDS w/v

1ml DEPC Water

200μg/ml Salmon sperm DNA

Warm to 42˚C prior to use.

# Appendix B

## Methods Development

No protocols were set up in the Laboratory for working with skeletal, and for western blotting in particular, a great deal of methods development had to be done to get the assay working effectively. Below is a step by step description of this process.

1) I started by practicing the protocol described by Karlsoon et al. (2004) on rat skeletal muscle to determine the phosphorylation status of P-rpS6, P-PKB, P-P$^{70}$S6K, and P-4E-BP1. Westerns were run with 30μg protein, the amount of protein routinely loaded onto the gels in our Laboratory when running cell culture lysates. These Westerns worked well.

2) Professor Ron Maughan kindly taught Dr Kosmadakis the biopsy technique and in the process provided myself with some human skeletal muscle to continue methods development with. Initially, all Westerns were run for P-rpS6 with the aim of setting up this western first. I repeated the protocol that had obtained good results in rodent muscle, but there was only a very faint signal detectable in healthy human skeletal muscle.

3) The amount of protein loaded onto the gel was increased to 100μg protein and gave a better signal than 30μg. This was then increased to 150μg, which gave no improvement over 100μg.

4) Two patients withdrew very early on in the study, but allowed their baseline samples to be used in methods development. The protocol was repeated with 100μg and then 150μg protein, but no signal was detectable. As a result of this, 150μg protein was routinely loaded onto the gel to maximise the possibility of detecting a signal with further modifications.

5) It was decided to always run a positive control on the same gel as the muscle samples to ensure that the lack of a signal was not due to a technical failure of the Western. L6-G8C5 cells stimulated with insulin served as the positive control and a volume containing 10μg protein was loaded onto the gel. (A reduced amount of protein was used here because the length of time the membrane was exposed to the film meant that anything more than 10μg resulted in a very large overexposed band.)

6) The effect of using a signal enhancer reagent and an antibody extender from Thermo Scientific was tested. Due to cost reasons it was decided to use the signal enhancer reagent, but this was later removed from the protocol as it made little difference and prevented the membranes from being re-probed with other antibodies.

7) I tried varying the length of time the membrane was exposed to the film, finally increasing to 24h exposures, which did give stronger signals than shorter exposures and this exposure time was then routinely used.

8) I switched from using nitrocellulose to PVDF membranes, as these are sometimes suggested to be more sensitive. However, they seemed to make little difference, but again, in order to maximise the possibly of detecting a signal, they were continued to be used.

9) I tried increasing the length of time proteins were transferred from the gel to the membrane using the wet transfer system from 2 to 3 hours and finally up to 4 hours to make sure all the protein was transferred out of the gel successfully. However, coomassie blue staining showed there was difference in the amount of protein that remained in the gel between the three different transfer times, and no difference in the signals detected, so the transfer time remained at 2 hours.

10) I started to compare the lysis buffer recipe I was using (which was the same as that used in cell culture experiments in our Laboratory) with those in the literature. It was essentially the same, except some research groups add salts to help dissolve the protein and glycerol to maintain a uniform concentration of the lysate when frozen. Salts and glycerol were then routinely added to the lysis buffer.

11) I tried using sonication following homogenisation to ensure the biopsy had completely gone into solution. This made no difference and was removed from the protocol.

12) The length of time the muscle homogenate was incubated in the lysis buffer was increased from 30 to 60 and finally to 90 minutes, and instead of incubating it on ice, the tubes were rotated in the cold room. After this modification and the improved lysis buffer recipe, the lysates did appear to be more translucent and to contain less insoluble material.

13) Some research groups use the semi-dry transfer method, and this was tried following the modifications made in point 12. This method did seem to give a better transfer and the wet transfer system was no longer used.

14) I changed the blocking agent from 5% milk powder to 5% Bovine Serum Albumin (BSA), but this resulted in a high background and was no longer used.

15) I then applied to the Loughborough University Ethical Advisory Committee to run a healthy volunteer study to obtain muscle biopsies from healthy individuals, in which phosphorylation signals should be detectable. If I could see a signal, then I could be sure that the lack of a detectable signal in the biopsies from CKD patients was the correct result and it was not simply the result of technical difficulties. A signal was detectable, so it was concluded that there is simply very little phosphorylation of rpS6 in these patients.

This low phosphorylation signal was also found to be the case for P-4E-BP1 and P-P$^{70}$SK6, but phosphorylation of PKB could be seen.

The protocol described in Section 2.5 was used in the Western blotting experiments for phosphorylated proteins described in this thesis.