REGULATION OF METABOLIC ACIDOSIS-INDUCED SKELETAL MUSCLE WASTING

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by

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This work is dedicated to all those many people who have contributed so much along the way. However, I make special mention of several groups, without whom this work would not have been possible.

Those who prepared me for this task: Marion, Paul, Mark and Clayton.

Those who gave me inspiration: Dr Ross Bailey, Professor John Walls and my patients and friends with renal disease.

My co-workers on these projects: Alan, Jez, Frease, Izabella, Nicki and Gemma from Leicester; and Dr Russ Price and Professor Bill Mitch from Atlanta.

My supervisors: Professor John Walls who gave me opportunity and direction, and Dr Alan Bevington whose un-dinting support guided this work to completion.

My dear wife Catherine and my children William, Joe and Madeline who sacrificed much family time and provided such great support and inspiration throughout.
Abstract

REGULATION OF METABOLIC ACIDOSIS-INDUCED SKELETAL MUSCLE WASTING

Warren P. Pickering, Department of Infection, Immunity and Inflammation, University of Leicester

Dialysis enables thousands of patients with end stage renal failure to extend both quantity and quality of life. However this group continue to suffer from increased morbidity and mortality compared to those with intact renal function. Malnutrition is widely recognized to be strongly associated with poor outcome for dialysis patients.

This work aims to investigate metabolic acidosis induced skeletal muscle protein wasting, and the inter-relationships between: metabolic acidosis, glucocorticoids (GC), and the ubiquitin proteasome system in this process.

Experiments were undertaken using three key themes and models:

1. The role of glucocorticoids and apoptosis in the process of protein degradation (PD) in L6G8C5 myoblasts in a cell culture system of metabolic acidosis utilizing dexamethasone and the glucocorticoid antagonist RU38486. Stimulation of PD in these cells by acid and GC does not appear to be an artefact of apoptosis or dedifferentiation, but differentiation state does determine whether PD responds spontaneously to acid or (as in vivo) only does so in the presence of GC.

2. The ability of RU38486 to pharmacologically antagonise the suggested permissive effect of glucocorticoid in an in vivo model of acidosis-induced muscle wasting. RU38486 did not prevent the acidosis-induced muscle wasting in this model despite demonstration of significant GC receptor blockade.

3. The role of the ATP-dependent ubiquitin proteasome system in the malnutrition of patients treated by peritoneal dialysis. When serum bicarbonate increased in these patients weight and body mass index increased significantly as did plasma BCAA. Muscle levels of ubiquitin mRNA decreased significantly; serum tumour necrosis factor-α also decreased. These results indicate that even a small correction of serum bicarbonate improves nutritional status, and provide evidence for down-regulation of BCAA degradation and muscle proteolysis via the ubiquitin proteasome system. Whether acidosis and inflammatory cytokines (such as TNF-α) interact to impair nutrition remains to be determined.
Publications arising from this work

Published Papers


Free Communications

**Pickering WP.,** Price SR, Mitch WE, and Walls J. The effects of correction of metabolic acidosis upon the ubiquitin pathway in CAPD patients. 9th International Congress on Nutrition and Metabolism in Renal Disease, Vienna, August 1998.


Poster Presentations

**Pickering WP.,** Bevington A, and Walls J. Dexamethasone decreases apoptosis in L6 myoblasts. 9th International Congress on Nutrition and Metabolism in Renal Disease, Vienna, August 1998.

Pickering WP, Price SR, Bircher G, Mitch WE, and Walls J.
Correction of metabolic acidosis down regulates skeletal muscle ubiquitin mRNA in uraemia.

The glucocorticoid antagonist RU38486 does not prevent muscle wasting in acidotic rats.
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Dr. Russ Price’s Clinical Chemistry Department at Emory University, Atlanta, GA, USA for HLPC assays of 3 methyl-histidine and branched-chain amino acids; and especially Marina Marinovic who performed the Northern analyses for Ubiquitin mRNA from the clinical study (Chapter 5).

Dr. Russ Price for the kind donation of the cDNA probe for ubiquitin used in the in vivo study, in Chapter 4.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)(_2)D(_3)</td>
<td>1,25-Dihydroxyvitamin D(_3)</td>
</tr>
<tr>
<td>3-MH</td>
<td>3-Methylhistidine</td>
</tr>
<tr>
<td>A</td>
<td>Acid-loaded</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched chain amino acid</td>
</tr>
<tr>
<td>BCKAD</td>
<td>Branched chain keto acid dehydrogenase</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>C</td>
<td>Non-acid-loaded control</td>
</tr>
<tr>
<td>C3I</td>
<td>Caspase-3 inhibitor</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous ambulatory peritoneal dialysis</td>
</tr>
<tr>
<td>CCPD</td>
<td>Continuous circulatory peritoneal dialysis</td>
</tr>
<tr>
<td>CPK</td>
<td>Creatine phosphokinase</td>
</tr>
<tr>
<td>CRF</td>
<td>Chronic renal failure</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>EAA</td>
<td>Essential amino acid</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorption assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>HA</td>
<td>High alkali</td>
</tr>
<tr>
<td>HB</td>
<td>Heps buffered salt solution</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-(2-hydroxyethylpiperazine)-N(\prime)-ethanesulphonic acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffered salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor I</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGF-binding protein</td>
</tr>
<tr>
<td>KH</td>
<td>Krebs Henseleit balanced salt solution</td>
</tr>
<tr>
<td>LA</td>
<td>Low alkali</td>
</tr>
<tr>
<td>MAC</td>
<td>Mid-arm circumference</td>
</tr>
<tr>
<td>MEM</td>
<td>Eagle's minimum essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Protein degradation</td>
</tr>
<tr>
<td>PS</td>
<td>Protein synthesis</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RA</td>
<td>Acid-loaded and treated with RU38486</td>
</tr>
<tr>
<td>RC</td>
<td>Non-acid-loaded control treated with RU38486</td>
</tr>
<tr>
<td>Rs</td>
<td>Spearman rank correlation coefficient</td>
</tr>
<tr>
<td>S</td>
<td>Svedburg ultracentrifugation sedimentation coefficient</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard sodium citrate buffer solution</td>
</tr>
</tbody>
</table>
## Abbreviations (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSPE</td>
<td>Saline sodium phosphate EDTA buffer solution</td>
</tr>
<tr>
<td>TA</td>
<td>Triamcinolone</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TSF</td>
<td>Triceps skinfold thickness</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidy transferase end labelling</td>
</tr>
<tr>
<td>Wn</td>
<td>Week number n</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction: the clinical effects of metabolic acidosis in uraemia
1.1 Metabolic acidosis

Metabolic acidosis is characterised by a decrease in pH, a fall in plasma bicarbonate concentration and a compensatory decline in CO2 tension. It is an inevitable consequence of uraemia [1] and a significant contributor to a number of pathophysiological states occurring in uraemia [2, 3].

In normal subjects plasma pH is tightly regulated within a range between 7.35 and 7.45 corresponding to a hydrogen ion concentration between $4.47 \times 10^{-8}$ and $3.55 \times 10^{-8}$ mmol/L [4]. The kidneys regulate the bicarbonate concentration at approximately 24 mmol/L and the lungs maintain the PaCO2 at 40mmHg. This necessitates excretion by the kidneys of the fixed acid production of approximately 60mmol H\(^+\) per day derived from a standard western diet.\(^1\) Approximately 40mmol are excreted in the form of ammonia and 20 mmol as titratable acid [6].

\(^{1}\)It has been suggested that a typical western diet is especially acid producing compared to a traditional hunter-gather diet and that this might be responsible for many of the adverse effects seen with aging in western populations. [5]
1.2 Mechanisms causing metabolic acidosis in uraemia

Table 1.1 Mechanisms causing metabolic acidosis in uraemia

<table>
<thead>
<tr>
<th>Mechanism</th>
</tr>
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<tbody>
<tr>
<td>Decreased ammonia generation</td>
</tr>
<tr>
<td>Decreased ammonia excretion</td>
</tr>
<tr>
<td>Increased bicarbonate excretion</td>
</tr>
<tr>
<td>Decreased excretion of titratable acids</td>
</tr>
</tbody>
</table>

**Ammonia Generation.**

Ammonia was traditionally considered to be a passive urinary buffer [7], but it is now appreciated that ammonia metabolism is a major mechanism responsible for the maintenance of acid-base balance. Proximal tubular cells can deaminate glutamine and glutamic acid producing alpha-ketoglutarate and ammonia. Ammonia (NH₃) is secreted into the proximal lumen, reabsorbed in the loop of Henle and then ultimately excreted into the urine as NH₄⁺ in distal segments, possibly via substitution for hydrogen (H⁺) in the apical Na⁺/H⁺ antiporter or H⁺/K⁺ exchanger [8, 9].

Ammoniagenesis may be impaired by declining nephron mass [5,10,11], as well as changes in circulating volume and electrolytes. Increase in potassium, as may be seen in advanced renal impairment, decreases ammoniagenesis [12] although this may be countered in part by the declining serum calcium of chronic renal failure which tends to increase ammoniagenesis [13].
Ammonia Excretion

With normal renal function ammonia excretion can be increased significantly in acute and chronic metabolic acidosis (up to tenfold in the latter). However, as the pKa of the ammonia buffer system is 9.3, over 99% of available ammonia is present as NH₄⁺ when luminal fluid pH is below 7.3. As a result ammonia is not a significant buffer of H⁺ ions in luminal fluid, but its metabolic production appears responsible for significant bicarbonate (HCO₃⁻) regeneration. By this mechanism, ammonium excretion accounts for approximately two thirds of the daily net acid excretion [14] and HCO₃⁻ regeneration is dependent upon functioning renal mass.

Bicarbonate Excretion

Filtered bicarbonate is normally reclaimed by the proximal tubule and sufficient HCO₃⁻ also regenerated to replace that lost internally in titrating metabolic acid and that lost externally through the gastro-intestinal tract. However, these processes are influenced by a number of factors which may alter the T_max. Extracellular volume expansion and increase in parathyroid hormone, both features of advanced renal failure, decrease the T_max for HCO₃⁻ as does decreased PaCO₂ in metabolic acidosis [15]. Conversely proximal re-absorption may be increased by chronic hypokalaemia [16, 17].

Titratable Acids

Titratable acids refers to those buffer systems which can reversibly bind to significant numbers of protons allowing their excretion into the urine without changing luminal pH [18]. These include inorganic phosphate, creatinine, uric acid
and citrate. The rate of formation and excretion of these titratable acids is affected by the relative tubular transport of the acid and conjugate-base forms of the buffer and the amount of buffer available for excretion. There is evidence that both phosphate [19,20] and citrate [21, 22] may have a significant role in the regulation of acid-base homeostasis. Stimulation of the renin-angiotensin-aldosterone axis by volume contraction enhances distal acid secretion [23], which may be relevant to the use of angiotensin converting enzyme inhibitors in renal failure. Blockade of the renin angiotensin system with ace–inhibitors or angiotensin II receptor antagonists might impair acid excretion by blocking the distal exchange of sodium for H⁺ or K⁺. In advanced renal failure this might exacerbate metabolic acidosis and hyperkalaemia.

1.3 Effects of metabolic acidosis

Table 1.2 Pathophysiological conditions resulting from metabolic acidosis of uraemia

<table>
<thead>
<tr>
<th>Acute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altered respiratory pattern</td>
</tr>
<tr>
<td>Shift of oxyhaemoglobin dissociation curve</td>
</tr>
<tr>
<td>Altered cardiovascular function</td>
</tr>
<tr>
<td>Cognitive change</td>
</tr>
<tr>
<td>Hyperkalaemia</td>
</tr>
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<table>
<thead>
<tr>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin resistance/glucose intolerance</td>
</tr>
</tbody>
</table>
Renal osteodystrophy
Protein catabolism/malnutrition
Growth failure in children

Acute effects

Respiratory function

Mild acidosis leads to a clinically observable increase in ventilatory effort. Severe acidosis (pH<7.20) produces deep rapid respirations known as Kussmaul’s respiration. The failure to lower PaCO$_2$ (expressed in mmHg) by 1.0 to 1.5 times the fall in plasma [HCO$_3^-$] measured in mmol/L implies the co-existence of respiratory acidosis [24]. The hypocapnia which results from respiratory compensation may, however, limit the renal component of correction of metabolic acidosis by shifting the production of CO$_2$ to the left [25].

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+
\]

ie the ventilatory loss of CO$_2$ decreases the amount of bicarbonate available to buffer H$^+$ although it has consumed H$^+$ in the process.

Oxygen-haemoglobin dissociation curve.

Acidosis may have opposite effects on the oxyhaemoglobin dissociation curve depending on the duration of the disturbance [26,27]. At the sub-physiological pH of acute acidosis the curve may be shifted to the right diminishing the affinity of haemoglobin for oxygen, thereby beneficially increasing oxygen delivery to the tissues [26]. However, the development of chronic acidosis may produce decreased concentrations of 2,3-diphosphoglycerate by a direct effect on red cell metabolism
[28], shifting the curve to the left and impairing tissue oxygenation. This effect may be exaggerated by the use of oral phosphate binders in uraemia.

**Cardiovascular function**

In mild acidosis this may be essentially normal, but severe acidosis (pH<7.10) may induce hypotension as myocardial contractility is depressed [29], and peripheral resistance falls [30]. Furthermore, it may contribute to the development of pulmonary oedema [31] and eventually ventricular standstill [32]. The mechanisms involved include alteration of myocardial Beta receptor density and function, as well as impaired responses to cytosolic calcium concentration, and decreased actin myosin crossbridge cycling due to alterations in inorganic phosphate metabolism and energy production. In practice metabolic acidosis may cause hypotension which is resistant to pressor agents, improving only when the acidosis is corrected [23].

**Central nervous system.**

Metabolic acidosis has been associated with the development of altered mentation, confusion and convulsions [33], but the mechanisms are poorly understood.

**Hyperkalaemia**

Under normal circumstances serum potassium closely reflects total body K+ stores. During severe acidosis H+ ions enter cells and this results in the efflux of K+ to maintain electroneutrality. This effect is more often observed in metabolic rather than respiratory acidosis [34], and may be at least in part due to the underlying pathology for example lactic acid production during significant tissue necrosis. It
has previously been estimated that each decrease of 0.1 in pH is accompanied by an increase in potassium of 0.5mEq/litre, but the true increase in potassium may be within the range of 0.2-1.7mEq/litre [35]. A common clinical observation in diabetic ketoacidosis is the co-existence of high plasma K⁺ despite depletion of total body potassium due to urinary losses, although this is less likely to be a problem in chronic renal insufficiency.

In chronic renal failure hyperkalaemia is often not seen until the glomerular filtration rate falls below 25% of normal [36, 37]. Despite the progressive decline in nephron number, potassium balance is maintained by increased excretion per nephron [38], and adaptation to increase K⁺ excretion by the colon [39], effects which are at least partly due to increased aldosterone secretion.

**Chronic effects**

**Insulin resistance/glucose intolerance**

Metabolic acidosis has been observed to reduce gluconeogenesis [40], but whether it truly induces insulin resistance is controversial [41]. Improvement in insulin resistance has been observed following both peritoneal dialysis [42] and haemodialysis [41]. The exact mechanism of improvement is uncertain and indeed DeFronzo demonstrated significant improvements in tissue insulin sensitivity following haemodialysis using both euglycaemic and hyperglycaemic clamps, but none of the patients studied had significant acidosis [41]. No significant difference in carbohydrate tolerance was observed after correction of acidosis in chronic uraemia using a one hour glucose infusion with measurement of glucose and insulin concentrations both before and post transfusion [43]. However, the combined L-[1-
\(^{13}\)C\] leucine-euglycaemic clamp technique revealed that in patients with chronic renal failure treatment with sodium bicarbonate significantly increases insulin mediated glucose uptake, but acidosis does not prevent insulin's ability to reduce proteolysis [44].

**Renal osteodystrophy**

Metabolic acidosis is a significant causative factor in renal bone disease. The skeleton contains both organic and mineral phases. The latter contains over 99% of body calcium and 80% of body bicarbonate [45, 46]. The organic matrix is largely composed of Type-1 collagen as well as other proteins including osteonectin and osteopontin. There are three cell types: the matrix synthesising osteoblast, the osteocyte which has ceased matrix production and which becomes embedded in the mineral phase, and the bone resorbing osteoclast. Acting together this system allows for a mechanically stable, but chemically dynamic skeleton which can undergo turnover of up to 15% per year.

Bone chemistry is dependent upon calcium and phosphate levels and regulated by parathyroid hormone (PTH) and 1,25 dihydroxy vitamin D\(_3\) (1,25(OH)\(_2\) D\(_3\)). PTH mobilises bone calcium by increasing osteoclastic resorption and it can also increase 1,25(OH)\(_2\) D\(_3\) production. This in turn can stimulate either bone resorption or formation depending on the ionic micro-environment, and suppress the secretion of PTH. Finally 1,25(OH)\(_2\) D\(_3\) is a powerful stimulus for calcium and phosphate absorption by the intestine.
Renal failure interferes with this balance by causing retention of phosphate and hydrogen ions and through decreased hydroxylation of the 1-position of 25-OHD₃. The 1α hydroxylase enzyme is located in the mitochondria of proximal tubular cells and decreases in quantity with declining nephron mass. [47] These changes contribute to the complex pathology of renal bone disease which conceptually may be divided into high-turnover (osteitis fibrosa) or low-turnover (osteomalacia and adynamic) bone disease. In reality there is a considerable degree of overlap between these two states. As GFR falls there is a progressive increase in the serum PTH level due to loss of suppression by 1,25(OH)₂ D₃, which allows for increased urinary phosphate excretion and thereby maintenance of a stable serum phosphate. However, the metabolic cost of this is an elevated PTH which stimulates osteoblastic and, indirectly via coupling factors, osteoclastic activity [48].

Infusion of mineral acid into nephrectomised animals causes a rapid increase in serum calcium [49] which implies mineral dissolution of bone. The effect of pH has been demonstrated by a neonatal mouse calvariae model incubated for three hours with medium with three different concentrations of bicarbonate. While calcium was released into the acidic medium, no change was seen in the calcium concentration of the neutral medium, and the alkaline medium induced influx of calcium into bone [50, 51]. Similar experiments were conducted with media containing agents which stimulate or suppress osteoclastic bone resorption which produced a constant release of bone calcium indicating that bone dissolution and deposition can occur purely due to physicochemical changes [51]. This was further supported by a cell-free model using synthetic carbonated apatite disks in which decreased pH again induced calcium release [52]. It appears to be bicarbonate and not apatite that is in
equilibrium with the culture medium and becomes solubilised during acidosis. At a constant pH, whether physiologically neutral or acidic, the lower the bicarbonate concentration the greater the calcium efflux [53]. Adding PTH to these models inhibits the inward movement of H+ [54], and effects bone resorption by inducing the secretion of acid by osteoclasts into the microenvironment between these cells and bone mineral [55,56,57].

In chronic metabolic acidosis there appears to be increased cell mediated bone resorption as well as the direct physicochemical effect described above. This is supported by the observation that osteoclast inhibitors such as colchicine or calcitonin reduce the increase in serum calcium induced by acid [49], consistent with evidence that acidosis increases osteoclastic and decreases osteoblastic, (including collagen synthesis) activity in vitro [58]. Furthermore, in partially nephrectomised rats uraemic acidosis caused further bone resorption beyond that seen with mineral acids such as hydrochloric acid (HCl) alone [59].

Interestingly, respiratory acidosis appears to cause deposition of calcium on bone surfaces in addition to calcium efflux [60]. During metabolic acidosis, the low bicarbonate favours bone dissolution, but during respiratory acidosis the increased PaCO₂ favours the deposition of carbonated apatite [61]. These observations suggest a strong case for the correction of metabolic acidosis in patients with renal disease. A number of groups have reported beneficial effects on renal osteodystrophy and in particular Lefebvre et al showed that, in the first 18 months on haemodialysis, patients in whom acidosis had been corrected did not show the increase in secondary hyperparathyroidism seen in the control group [62]. This
study suggested not only that optimal correction of acidosis reduces hyperparathyroidism in patients with high bone-turnover, but that it also stimulates bone turnover in adynamic states. Bushinsky, therefore recommends that prior to endstage, acidosis should be corrected with restoration of serum bicarbonate to the normal range, and that in haemodialysis patients the pre-dialysis bicarbonate should also be normalised [48]. The optimal serum bicarbonate is, however, not well defined as too much calcium (in the form of dialysate or phosphate binders) has been associated with over-suppression of PTH and adynamic osteodystrophy [63] and there are new concerns about accelerated calcification of (coronary) arteries with aggressive use of calcium-containing phosphate binders [64].

1.4 Protein metabolism and malnutrition

1.4.1 Role of malnutrition in ESRF

Acidosis degrades muscle as well as bone. The restoration of growth seen in renal tubular acidosis [65] implies improvement in protein as well as bone chemistry. Such malnutrition is widely accepted as a leading cause of morbidity and mortality in renal failure. Lowrie and Lew examined numerous parameters in 12 000 patients undergoing haemodialysis in the United States and identified low serum albumin (a surrogate marker of malnutrition) as the strongest predictor of poor prognosis in this patient group [66]. The causes of malnutrition are multifactorial, (see Table 1.3 but the evidence for metabolic acidosis as a major uraemic toxin in this process is now compelling.
Table 1.3 Causes of malnutrition in renal failure

Uraemia
Metabolic acidosis
Poor dietary intake due to anorexia and or social circumstance/poverty
Hormone resistance
Side effects of medications and or dialysis
Catabolic cytokines
Intercurrent (inflammatory) illness
Catabolic effects of dialysis process

However, this remains a complex and imperfectly understood phenomenon. In the early 1700s Peter the Great suffered from chronic renal failure, but his court physicians who carefully recorded his body weight would not have recognized that his body habitus remained relatively constant because salt and water retention hid the loss of lean muscle mass [67]. Since the time of Bright, however, several themes have emerged as clinicians and scientists have strived to piece together the components and relationships involved in this deleterious process.

Table 1.4 Central themes in skeletal muscle wasting of renal failure

Clinical observations and studies
Adaptive responses to reduced dietary protein intake
Role of essential amino acids
Metabolic acidosis as a uraemic toxin

The ubiquitin proteasome pathway

Role of glucocorticoids

Clinical studies in dialysis patients examining the role of correction of metabolic acidosis

1.4.2 Clinical observations and studies

Richard Bright in 1831 suggested that alkali therapy might benefit those with renal disease [68], but it was exactly a hundred years later that Lyon et al published their observations that blood urea levels were higher in patients receiving acid rather than alkali diets [69], although they wrongly concluded that this was not a consequence of altered protein degradation. In 1963, Giordano showed that restriction in dietary protein improved the uraemic symptoms of CRF patients [70]. By the early 1970s interest in this area was accelerating. Coles carefully documented that patients became malnourished in concert with a loss of lean muscle mass (like Peter the Great often hidden by oedema) [71]. Blom van Assendelf and Dorhoulut-Mees randomised patients with mild metabolic acidosis to receive either sodium chloride or sodium bicarbonate. A decrease in blood urea nitrogen (BUN) was seen in both groups (thought to be due to sodium induced volume expansion or increased urinary nitrogen excretion), but the decrease in urea generation rate was greater in the sodium bicarbonate group [72]. McSherry and Morris noted the improvement in growth rates of children with renal tubular acidosis by correction of the acidosis alone [65]. Papadoyannakis et al reported that in CRF patients supplementation with calcium carbonate increased plasma bicarbonate levels and improved their
nitrogen balance. [73]. Williams et al reported that metabolic acidosis in CRF patients reduced their adaptation to a low protein diet [74]. Bergström showed that malnutrition is common in patients with CRF and demonstrated a strong positive correlation between plasma bicarbonate concentration and free valine concentrations in muscle biopsies from uraemic patients treated by haemodialysis (HD) [75]. Reaich et al have made several important observations in this field. Metabolic acidosis induced in adults with intact renal function by feeding ammonium chloride also stimulated protein degradation [76]. Supplementing CRF patients with sodium bicarbonate for 2 weeks increased the mean serum bicarbonate from 15 to 21 mmol/L and the rate of protein degradation decreased by 28% [76]. Furthermore, using the euglycaemic clamp technique they demonstrated that acidosis-induced proteolysis was not abrogated by insulin [44].

In dialysis patients there is evidence that increasing the dose of bicarbonate results in improvement in body weight. In 21 haemodialysis patients Seyffart et al examined the effect of increasing the dialysate bicarbonate concentration from 32 mmol/L to 36 mmol/L and showed that the rate of weight gain increased or the rate of weight loss decreased in this group [77]. Stein et al conducted a prospective randomised study comparing nutritional indices in 200 consecutive patients starting on CAPD who were randomised to receive low alkali (lactate 35mmol/L) or high alkali (lactate 40mmol/L) dialysate. Those receiving high alkali dialysate had a significantly greater increase in total body weight and midarm muscle circumference at one year. Although not reaching significance they also had greater increase in triceps skin fold thickness, and fewer and shorter hospitalisations [78].
1.4.3 Adaptive responses to reduced dietary protein intake

If uraemia induced skeletal muscle protein wasting by inhibiting the normal adaptive responses to reduced dietary protein intake, then in CRF patients anorexia or social deprivation might be expected to result in significant net changes in protein wasting. As explained below, this is not so. The daily turnover of protein in normal humans is 4g per kg/body weight or approximately 280g [79]. As skeletal muscle is the largest body store of protein, even quite small increases in protein degradation might be expected rapidly to cause muscle protein malnutrition if there were no compensatory increase in protein synthesis or amino acid metabolism. It has been demonstrated, however, that the compensatory mechanisms that protect against changes in protein degradation rate are intact unless some other catabolic stimulus intervenes.

1.4.4 Role of essential branched-chain amino acids

The key to unlocking this adaptive response is provided by investigations of the role of the essential branched-chain amino acids leucine, isoleucine and valine. The beauty of studying these is threefold; quantitatively they constitute 18% of muscle protein, the bulk of their oxidation takes place in muscle, liver and kidney, but as muscle constitutes 40% of body mass it is the major site, and they appear to have an important regulatory role. The BCAA can act as markers of malnutrition in CRF and their rate of catabolism is thought to be regulated by the activity of the rate limiting enzyme branched-chain ketoacid dehydrogenase (BCKAD). When the activity of this enzyme is increased, BCAA degradation rises and reflects a parallel
increase in accompanying protein degradation [80]. Consequentially, BCKAD activity can act as a marker of nutritional status in health and certain disease states [81]. The turnover kinetics of the essential amino acids (EAA) especially L-[\textsuperscript{13}C\textsubscript{1}] leucine provides important insights [81]. This technique provides estimates of protein synthesis, protein degradation and irreversible oxidation of EAA \textit{in vivo}. Reducing dietary protein to the minimum daily requirement of 0.6g/kg body weight/day results in reduction of the irreversible oxidation of leucine suggesting more efficient utilization of dietary protein to provide EAA for protein synthesis [82]. Restricting dietary protein below 0.6g/kg body weight/day did not decrease amino acid oxidation further, but whole body protein degradation decreased. Now, if uraemia per se were to impair these adaptive responses negative nitrogen balance would follow because: 1) if EAA degradation was not depressed following reduction in dietary protein intake, EAA elimination would exceed dietary supply and nitrogen balance would be more negative and 2) if protein degradation was not suppressed under these conditions, it would exceed protein synthesis and cause negative nitrogen balance.

Two important studies reveal that renal failure per se does not impair these adaptive responses. Goodship et al [83] examined the effect of feeding 0.6 or 1.0g protein/kgBW/day to CRF patients without metabolic acidosis versus control subjects and found that their dietary responses to protein restriction did not differ. Masud et al [84] extended this using a more severe protein restriction 0.3g/kg body weight/day supplemented with EAA or ketoacids. Again the adaptive responses to protein restriction were intact, but these patients were not acidotic: as discussed
below acidosis may significantly disturb the normal regulation of protein degradation, protein synthesis and EAA metabolism that preserves lean tissue mass.

1.4.5 Metabolic acidosis as an uraemic toxin

Several key contributions to our understanding of this process have been made by Mitch’s group initially in Harvard and more recently Atlanta. Hara et al demonstrated in rats that it is acidosis not azotaemia that stimulates BCAA catabolism [85], May et al reported abnormal regulation of BCAA metabolism in rat muscle in acidosis [86], and England et al that rat muscle BCKAD activity and mRNAs increase with extracellular acidaemia [87].

1.4.6 Proteolytic pathways in skeletal muscle

In a normal 70kg adult approximately 280g of protein is synthesised and degraded each day. In health this is a finely balanced and tightly regulated process [79]. The loss of lean body mass characteristic of many disease states is primarily due to accelerated proteolysis of skeletal muscle, although at least in acute metabolic acidosis, impaired protein synthesis has also been reported [88]. The protein degradation provides a supply of amino acids for gluconeogenesis, but some (especially the branched-chain amino acids: leucine, isoleucine and valine) are consumed as a source of energy by muscle itself.

In some pathological states, including acidosis, there is disproportionate breakdown of skeletal muscle rather than visceral protein. This involves particularly degradation of the myofibrillar proteins actin and myosin. These contain a unique amino acid 3-methyl histidine produced by post-translational modification which is
not re-incorporated into protein following proteolysis. Measurement of 3-MH, therefore, allows monitoring of muscle catabolism [79].

Four main pathways for proteolysis have been identified in skeletal muscle:

1. Lysosomal acidic proteases

This pathway degrades endocytosed proteins and most membrane components and in disease states can increase breakdown of some cytosolic components. It is involved in major histo-compatibility complex II (MHC II) antigen presentation. Its activity can be decreased by insulin and amino acids and blocked by weak bases.

2. Calcium activated proteases

This pathway involves enzymes (calpains I and II) which are activated by increases in intracellular calcium, and appear important in muscular dystrophy or damage. This pathway has recently been implicated in uraemic cardiomyopathy [89].

3. A poorly characterized system that does not require lysosomes, calcium or ATP

4. The ATP-dependent ubiquitin proteasome pathway. (see below)
As above Reaich et al demonstrated in normal adults that induction of metabolic acidosis by ammonium chloride stimulates intracellular protein degradation and oxidation of branched-chain amino acids. [76]. The induction of acidosis in non-uraemic rats by addition of 20mmol HCl to their diet resulted in impaired weight gain and increased excretion of non-urea nitrogen when compared to pair-fed controls. Urinary 3-methylhistidine/creatinine ratios were measured as a marker of skeletal muscle proteolysis and this ratio was significantly elevated in the acidotic group [90]. Furthermore, this proteolysis can be reversed simply by the addition of alkali in the form of sodium bicarbonate [86]. The exact mechanism by which this suppression of increased proteolysis occurs is not yet defined, but leucine turnover studies indicate that acidosis increases both protein synthesis and degradation, but the latter more so [76]. Acidosis is associated with increased activity of the branch-chain keto acid dehydrogenase, a key step in metabolism of these amino acids. Consequently low serum levels of valine, leucine, and isoleucine are observed in acidotic rats and these improve with correction of acidosis [87].

1.4.7 The ATP-dependent ubiquitin proteasome pathway

(see Figure 1.1 The ubiquitin proteasome pathway)

Ubiquitin is a small protein containing 76 amino acids. It is found in all eukaryotic cells and is one of the most highly conserved of all proteins with an amino acid sequence identical from insects to man [91].

The proteasome is also highly conserved in eukaryotes and simpler forms are even found in archaebacteria. It is the major neutral proteolytic activity in mammalian cells and constitutes up to 1% of cell protein. The 20S (700-kDa) proteasome is a
Figure 1.1 The ubiquitin proteasome pathway
barrel-shaped structure of 4 stacked rings of proteins each containing 7 subunits around a central cavity. It contains multiple peptidase activities and functions through a novel proteolytic mechanism involving a threonine active site. The 26S (2000k-Da) complex, which degrades ubiquitinated proteins contains in addition a 19S regulatory complex composed of multiple ATPases and components necessary for binding protein substrates.

This is an important nonlysosomal proteolytic pathway responsible for the highly selective degradation of most intracellular proteins under basal conditions and also for proteolysis of some intracellular proteins under conditions of stress. This pathway is now recognized to catalyse the rapid degradation of many rate limiting enzymes, transcriptional regulators (e.g. IκB), and critical regulatory proteins. It is also involved in major histo-compatibility complex I (MHC I) antigen presentation, and has a primary role in the slower degradation of the majority of intracellular proteins, and the acceleration of proteolysis in many pathological states. [92]

Proteolysis via the ubiquitin pathway involves two distinct steps: signaling of the protein for degradation by sequential attachment of multiple ubiquitin molecules, and proteolysis itself by the proteasome with the release of peptide fragments and ubiquitin which is then available to be recycled. Conjugation of ubiquitin to proteins is generally a three-step process. First, the C-terminal Gly of ubiquitin is activated by ATP to a high energy thiol ester, catalysed by the ubiquitin activating enzyme E1. Next, E2 (ubiquitin carrier protein or ubiquitin conjugating enzyme) transfers ubiquitin from E1 to the substrate protein destined for degradation bound to a ubiquitin-protein ligase, E3. Here an isopeptide bond is formed between the
activated C-terminal Gly of ubiquitin and an \(\epsilon\)-NH\(_2\) group of a Lys residue of the substrate. As E3 has substrate binding specificity this probably plays an important role in selectivity. (In some instances E2 transfers ubiquitin directly to the protein substrate so E2 may also determine selectivity. In successive reactions, a polyubiquitin chain is formed by progressive transfer of ubiquitin moieties to Lys-48 of the previous ubiquitin molecule. [93]

Poly-ubiquitinated protein substrates then enter the 20S core proteasome through the outer ring, but only following unfolding of the substrate which is believed to be catalysed by the 19S subunit. Within the 20S core, proteins are sequentially cut into small peptides until completely degraded. The peptides are then released and rapidly hydrolysed to amino acids by cytosolic exopeptidases, or transported to the cell surface by MHC I molecules [93].

The next major advance came in 1994 with the demonstration by Mitch’s group that experimental acidosis-induced skeletal muscle proteolysis in rats occurs via a pathway involving ATP, ubiquitin and the intracellular proteasome complex. The ubiquitin proteasome pathway has been shown to be active in a number of pathologic states: fasting, denervation atrophy, sepsis, burns and cancer cachexia. Messenger RNA levels for ubiquitin and 20S subunits increase despite a reduction in total RNA content of muscles [79]. Mitch et al demonstrated such alterations in rats with acidosis from renal insufficiency [94] and further showed that correction of the acidosis prevents not only the increase in proteolysis, but also the increase in mRNAs for ubiquitin and the proteasome subunits [94].
1.4.8 Role of glucocorticoids in metabolic acidosis-induced protein degradation

The initial signal for up-regulation of the ubiquitin proteasome pathway has not yet been defined, but some studies have suggested a regulatory role for glucocorticoids.

In cell culture using BC₃H₁ myocytes, acidification of the medium did not significantly increase protein degradation, but acid and dexamethasone together increased protein degradation, and this process could be inhibited by the glucocorticoid antagonist RU38486 [95].

May et al. noted that urinary corticosterone levels in CRF rats increased in proportion to metabolic acidosis [96]. If acidosis was corrected, corticosterone levels were still high (sham-operated pair-fed controls), but only acidic animals had increased protein degradation. Garibotto [97] reported an inverse correlation between serum cortisol and bicarbonate levels and demonstrated in forearm muscles of CRF patients, that protein degradation was inversely related to serum bicarbonate, but directly related to serum cortisol. A more detailed experiment by May et al. [3] demonstrated an apparent requirement for glucocorticoids (GC) as well as acidosis to stimulate this process. Metabolic acidosis was induced in rats as previously, but acidic rats which had undergone adrenalectomy did not show acid-induced muscle protein degradation. However, when glucocorticoid was restored by administering dexamethasone the acid-induced protein degradation returned, suggesting a requirement for GC as a permissive factor.
1.4.9 Aims of the work covered in this thesis

Although the clinical entities of malnutrition in renal failure, and metabolic acidosis-induced skeletal muscle protein degradation are now well recognized; the biochemical processes by which these conditions occur remain uncertain. The current work aims to address this difficulty through examination of the inter-relationships between metabolic acidosis, protein degradation, glucocorticoids and uraemia. This is approached via three themes:

1. Examination of the validity of the L6G8C5 cell culture model of acidosis-induced protein degradation; studying the potential confounding factors of apoptosis and de-differentiation.

2. Investigation *in vitro* and *in vivo* of RU38486 (mefipristone) as a potential therapeutic agent via its role as a glucocorticoid antagonist.

3. Investigation of the role of the ubiquitin proteasome pathway in the metabolic acidosis-induced proteolytic malnutrition of end-stage renal failure patients treated by peritoneal dialysis.
Chapter 2 Methods and Materials
2.1 Studies in vitro – Effects of pH and glucocorticoid on cultured skeletal muscle cells

2.1.1 Choice of cell culture model

Previous studies of the effects of metabolic acidosis and glucocorticoid have used BC₃H₁ cells [98] which have a muscle phenotype, but show some features of smooth muscle e.g. they do not fuse to form myotubes. The L6-G8C5 skeletal muscle cell line from rat was chosen here because it has previously been shown to be a useful in vitro model for acid-induced protein wasting and can differentiate in low serum medium to form myotubes. Unlike other skeletal muscle cell lines [99] L6-G8C5 only slowly acidifies its culture medium, allowing stable pH studies to be performed. Primary cultures of myocytes derived from human skeletal muscle have also been described. However, these must routinely be cultured in the presence of insulin to promote differentiation and possibly because of this anabolic pre-treatment show a slow catabolic response to low pH [100].

2.1.2 Cell culture conditions

L6 rat skeletal muscle cells sub-clone G8C5 (European Collection of Animal Cell Cultures ref. 92121114) were seeded at passage 13-23 at 4.5 x 10⁴ cells/cm² on plastic wells in Growth Medium comprising Dulbecco’s Modified Eagle’s Medium (Life Technologies 11880), with 5mmol/l glucose and 1 mmol/l pyruvate, supplemented with 2mmol/l glutamine, penicillin (10⁵ IU/l), streptomycin (100mg/l), phenol red (10mg/l) and 10% vol/vol foetal bovine serum (FBS). FBS was obtained from Life
Technologies (10106) and contained less than 20 nmoles of cortisol per litre. All sera were heat-inactivated for 30 min at 56°C before use. Cultures were incubated at 37°C under humidified 95% air/5% CO₂.

By Day 4 (i.e. after 3 days), the myoblasts were confluent and aligned (Fig 3.1a) and were switched to Eagle’s Minimum Essential Medium with Earle’s Salts (MEM) (Life Technologies 21090) with 2% vol/vol FBS and antibiotics and glutamine as above. Fresh MEM/2% FBS was added on Day 6. By Day 8 a network of multinucleated myotubes had formed, interspersed with dense patches of small myotubes and unfused cells (Fig 3.1b) and remained stable and viable in the medium with 2% serum for the duration of the study (55 hours). More complete fusion was obtained if FBS was used without heat inactivation, if horse serum was substituted for FBS, if cells were used at lower passage number and seeded at lower density, and if myoblast division was blocked with cytosine arabinoside [101]. However such highly fused myotubes detached and died after about 24 hours. Detachment is preventable, both in L6-G8C5 and in human myotubes by routine addition of glucocorticoid supplements [102], but as this would be an obvious confounding factor these were not employed.
2.1.3 Test media for experiments

For experiments, medium at pH 7.1 to 7.5 comprising MEM as above, but with dialysed FBS (Life Technologies 10110), was added from Day 8 onwards. (The relationship between net protein wasting and extracellular pH has been shown previously to be linear over this pH range [99].) The pH was adjusted by addition of HCl or NaHCO$_3$, with extra NaCl at low pH to maintain a constant Na concentration.

Dexamethasone (DEX) (Sigma D-1756) and RU38486 (Mifepristone from Roussel UCLAF/Exogyn) were added dissolved in dimethylsulphoxide (DMSO). The same concentration of DMSO (0.011% vol/vol final concentration) was added to control cultures.

Further experiments were designed to obtain test media depleted of steroid. MEM was made up without Phenol Red and was supplemented with charcoal-stripped dialysed FBS. Activated charcoal (0.075g of Merck 33032) was wetted with 2ml of water and centrifuged at 900g for 5 minutes at 20°C. The supernatant was discarded and the charcoal pellet was re-suspended in 10 ml of dialysed FBS followed by incubation at 4°C for 30 minutes. The charcoal was removed by centrifugation at 3000g for 10 minutes at 4°C and the supernatant was sterilized by filtration through a 0.8μm filter followed by a 0.2μm filter.
2.1.4 Cell Differentiation

Fusion was routinely monitored by estimating the percentage of the culture covered by large myotubes (Fig 3.1b). For studies relating fusion to the pH sensitivity of protein degradation (PD), all estimates of fusion were made blind i.e. before measurement of PD in the same cultures. For experiments requiring more precise quantification of differentiation, the muscle marker enzyme creatine phosphokinase (CPK) was assayed using a commercially available kit (Sigma 45-1). The assay uses the following coupled enzyme reactions:

\[
\begin{align*}
\text{ADP} + \text{Phosphocreatine} & \rightarrow^{\text{cpk}} \text{ATP} + \text{creatine} \\
\text{ATP} + \text{Glucose} & \rightarrow^{\text{hexokinase}} \text{ADP} + \text{Glucose-6-phosphate} \\
\text{Glucose-6-phosphate} + \text{NADP}^+ & \rightarrow^{\text{G6PDH}} 6\text{-phosphogluconate} + \text{NADPH}
\end{align*}
\]

where G6PDH is glucose-6-phosphate dehydrogenase and NADP$^+$ is nicotinamide adenine dinucleotide phosphate. The reaction is monitored from the rate of production of NADPH from its absorbance at 340nm.

After 24 hours in serum free medium at pH 7.1, myoblasts (Fig 3.1c) expressed 0.87±0.08 mIU (of CPK)/μg DNA (n=4). After 24 hours in medium with 2% serum at pH 7.1, fused cultures (Fig 3.1b) expressed 4.1±0.3 mIU/μg DNA (n=4). Switching to control pH or incubating with 5nM dexamethasone (DEX) for 24 hours had no effect. More detailed data on differentiation are presented in Chapter 3.
2.1.5 Protein degradation rate in intact cells.

Protein degradation was assessed by pre-labelling cellular proteins with radioactive phenylalanine followed by measuring the rate of release of acid-soluble radioactivity into the culture medium [99].

L-[U-\textsuperscript{14}C] phenylalanine (Amersham CFB 70) was added to L6-G8C5 cells in 35mm diameter culture wells during the fusion period (Days 4 to 8 inclusive) at a final concentration of 0.23 mCi/l (8.5MBq/l) to label the cellular proteins. At the end of this labeling period, cultures were incubated in 2 ml of unlabelled MEM + 2% FBS for 2 hours to eliminate radioactivity from rapidly degraded proteins [99]. The medium was then discarded and at this point (designated time zero), 3ml of test medium was added. From this point onwards, test media were supplemented with unlabelled L-phenylalanine (2mmol/l) to minimize re-incorporation of labeled phenylalanine into cellular protein [99]. Aliquots of culture medium (0.3ml) were sampled after 7, 21, 31, 41, and 55 hours. The labeled medium was mixed with an equal volume of 20% vol/vol trichloroacetic acid and chilled at +4°C for at least 30 minutes to precipitate protein. The samples were then centrifuged at 3000g for 10 minutes at +4°C and \textsuperscript{14}C-phenylalanine activity determined in the supernatant by liquid scintillation counting as an index of protein degradation [99]. Protein degradation rates are expressed as the rate of decline of log\textsubscript{10} of the percentage of the total \textsuperscript{14}C (acid-soluble \textsuperscript{14}C released into the medium plus total \textsuperscript{14}C remaining in the cells) recovered in each culture well [99]. Rates were calculated as the linear regression slope through the 6 time points (including time zero) in 55 hour experiments (Fig 4.5.b and Fig 4.9) or as the slopes of lines
interpolated between time points 0 and 7h (Fig 4.10). $^{14}$C-phenylalanine activity was also determined in the protein pellet derived from the medium, as an index of cell detachment and leakage of intact labeled proteins [103]. None of the experimental conditions had any significant effect on this activity nor on the total $^{14}$C recovered.

At the end of the incubations, the cultures were placed on ice and rinsed 3 times with 0.9% weight/vol NaCl to remove extracellular protein and $^{14}$C-phenylalanine before storage at -20°C. Cultures were subsequently thawed at room temperature, scraped in 1.20ml of 1.75mol/l (10% weight/vol) perchloric acid, digested at 70°C for 20 minutes and then chilled at +4°C for at least 30 minutes to maximize protein precipitation. The digests were then centrifuged at 3000g for 10 minutes at +4°C, and $^{14}$C-phenylalanine activity and total DNA [104] were determined in the supernatant. The protein pellet was dissolved in 1.00ml of 0.5M NaOH and $^{14}$C-phenylalanine activity and total protein [105] were determined.

2.1.6 DNA Assay (Colour development by the Burton reaction)

To 100μl aliquots of perchloric acid digest supernatant, 100μl of Diphenylamine (DPA, 4% weight/vol in glacial acetic acid) was added and vortexed. Immediately, 20μl of acetaldehyde (0.2% vol/vol in glacial acetic acid) was added and the samples again vortexed. After incubation for 16-20 hours at room temperature 190μl aliquots were transferred to a 96 well multititre plate and the optical density was read at 595nm and
710nm. For each sample the difference in optical density between the two wavelengths was compared with a calibration constructed using digests of calf thymus DNA standards [104].

2.1.7 Protein assay

Total protein was determined colorimetrically in 50 µl aliquots of NaOH digest (see above) by the method of Lowry et al [105] reading the optical density at 660nm against bovine serum albumin standards.

2.1.8. Quantification of apoptosis

Bisbenzamide Hoechst 33342 method for detection of apoptosis.

Cells (4 x 10^5) were plated in Growth Medium on 9cm diameter Petris containing sterile glass microscope slides. On Day 4, the cultures were rinsed with Hanks Balanced Salt Solution (HBSS) to remove serum, and serum-free test medium (MEM at pH 7.1 or 7.5 with or without 5nmol/l dexamethasone (DEX)) was added. After incubation for 24h, fresh test medium containing 10µg/ml of Hoechst 33342 fluorescent dye (Sigma B2261) was added [106]. After a further 2h incubation in the culture incubator to stain DNA in the intact cells, cultures were rinsed with HBSS, followed by fixing of the cells in 15ml of phosphate-buffered saline (PBS) with 1% w/vol paraformaldehyde for 15 min at room temperature. The slides were then dried in air, covered with Immuno-mount (Shandon 9990402) and examined on a fluorescence microscope with a Nikon UV-2A filter block (Fig3.1a-e) Apoptotic cells (showing cytoplasmic shrinkage and nuclei with condensed fragmented chromatin) and total cells
were counted over 6 randomly selected fields on each slide. Three to four slides were examined under each set of incubation conditions in each of 5 independent experiments, the examiner being blind to the test condition of individual slides.

**TUNEL method**

In one further experiment, apoptotic DNA strand breaks were labelled with fluorescein-tagged nucleotides using a terminal deoxynucleotidyl transferase (TUNEL) labelling kit (Roche Molecular Biochemicals ref. 1 684 795). Apoptotic cells showing fluorescein fluorescence and total cells (detected by phase contrast) were counted in 12 randomly selected fields for each set of incubation conditions [107].

**Annexin V**

This is based on the principle that apoptosis results in the translocation of phosphatidyl serine from the cytosolic to the external leaflet of the plasma membrane allowing its detection by modified ELISA using annexin V as a detection system [108]. In the presence of serum, dividing cells with retracted cytoplasm and condensed chromatin made counting apoptotic cells difficult by the Hoechst 33342 method. Annexin V was therefore used, as it has been validated previously as an assay for phosphatidylserine exposure on the surface of apoptotic cells in skeletal muscle cultures [108]. Starting on Day 4, cells on 96-well culture plates (Costar C3595 (low non-specific binding)) were incubated in test media (MEM + 10% FBS at pH 7.1 or 7.5 with or without 5 nmol/l DEX). After 24h, the cells were rinsed with HB buffer comprising: 10 mmol/l
Hepes/NaOH pH 7.4, 150 mmol/l NaCl, 5 mmol/l KCl, 1mmol/l MgCl₂, 1.8 mmol/l CaCl₂. FITC-labelled Annexin V (Bio-Whittaker BMS-306F1) diluted 1:1000 in HB was added and incubated for 8 min at room temperature. The cells were rinsed 3 times with HB and fixed for 10 min at +4°C in 4% w/vol paraformaldehyde in PBS, followed by 3 rinses with PBS. Non-specific binding sites were blocked for 30 min at room temperature with 3% w/vol bovine serum albumin (BSA) followed by incubation for 30 min with alkaline phosphatase-conjugated anti-FITC Fab antibody fragment (Roche Diagnostics 1426338) diluted 1:1000 in 3% BSA. Wells were washed 3 times with 0.02% Tween 20 in PBS followed by incubation with alkaline phosphatase substrate (1mg/ml of Sigma 104-105) in 1 mol/l diethylamine titrated to pH 9.8 with HCl. Absorbance was read at 405 and 660nm after 2h and the difference taken as an index of Annexin V binding [108].

2.1.9 Statistical analysis.

Values are expressed as mean ± SEM, with “n” values denoting the number of independent experiments. Statistical significance of changes was assessed by Analysis of Variance with post hoc testing by Duncan’s Multiple Range Test (or Tamhane’s Test for data sets with widely differing variance). Skewed apoptosis data in Fig 3.2 were analysed by Friedman’s Analysis of Variance with paired analysis of test and control data by the Wilcoxon Signed Ranks Test. Correlation is expressed as the Spearman Rank correlation coefficient. Changes were taken to be significant if P<0.05.
2.2 Studies *in vivo*-Glucocorticoid antagonism in acid-loaded rats

2.2.1 Design of the animal study

Experiments were conducted in an approved facility and in accordance with Home Office approved practice, (the author having attended a Small Animals course) and the appropriate personal and project licenses were obtained.

Twenty adult male rats were randomly allocated to 4 groups designated C (Control), RC (Control receiving RU38486), A (Acidotic) and RA (Acidotic receiving RU38486). The animals were housed in conditions of constant temperature and humidity with a 12 hour light/dark cycle. They were fed a diet containing 20g of protein (as casein) per 100g dry weight (ICN 960259) with free access to water. To make the diet easier to handle, its viscosity was increased with methyl cellulose (Sigma M0512, 4000 centipoises viscosity at 2% w/vol at 20°C) by mixing 24.5g of dry diet with 24.5ml of water and 1g of methyl cellulose. After a run-in period of 15 days, the diet of animals in Groups A and RA was made up in 0.25 mol/l HCl instead of water, a dose calculated to administer 35mmol of HCl per day per Kg body weight as in an earlier study in this laboratory [109]. As a positive control, to confirm the effect of heavier acid-loading on muscle PD reported previously [3, 110], a separate group of 5 rats (with 3 simultaneous non-acidotic controls) was given a dietary acid load of 70mmol of HCl per day per Kg body weight.

Animals in Groups RC and RA received RU38486 by gavage in a single daily dose of 50mg per Kg body weight in suspension in an aqueous vehicle [111] of 0.25% w/vol carboxymethyl-cellulose and 0.2% w/vol polysorbate 80. Animals in Groups C and A
received vehicle alone. Pair-feeding was performed to ensure that food intake in the acidotic animals was matched to that in the corresponding non-acidotic controls. Animals were weighed on days 0, 5, 10 and 15. Urine was collected in the last 24h before sacrifice and stored at -20°C. After 15 days (approximately 24h after the last dose of drug) the animals were sacrificed under light anaesthesia and exsanguinated by aortic cannulation. Blood pH and bicarbonate were immediately determined on a Corning 238 blood gas analyzer. Skeletal muscle was then processed as follows:

2.2.2 Glucocorticoid receptor binding in a soluble protein fraction

The left gastrocnemius muscle (approximately 2g) was weighed, and homogenized using a T8 motorised homogenizer (IKA Labortechnik, Germany) in 5ml of ice-cold buffer [111] comprising 25mmol/l Na₂HPO₄, 1.5mmol/l Na₂EDTA, 10% (vol/vol) glycerol, 2mmol/l dithiothreitol, 10mmol/l Na₂MoO₄, and 1mmol/l phenylmethylsulfonyl fluoride, adjusted to pH 7.2 at room temperature with HCl. The homogenate was stored under liquid nitrogen. Homogenates were thawed on ice and centrifuged for 60 min at 110,000g at +4°C in a Beckman L8-60M preparative ultracentrifuge. The supernatant (soluble protein fraction) was used immediately for determination of total protein [105] and specific glucocorticoid receptor binding with a

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1In a pilot study with another 20 rats, RU38486 was administered via osmotic mini pumps (AlzetCorp CA) in an attempt to reduce any stress arising from repeated gavage. However, on removal from the animals, the pumps were still found to contain large amounts of drug so their dose delivery was deemed unreliable.
single saturating dose (15nmol/l) of \(^3\)H-triamcinolone acetonide (New England Nuclear NET 470) [111]. A similar procedure was used for whole homogenates of L6-G8C5 cells exactly as described by Konagaya et al [112].

2.2.3 Ubiquitin mRNA determination

The right gastrocnemius muscle was immediately removed and frozen in liquid nitrogen. Total RNA was isolated using an acid phenol-guanidinium reagent (Trizol, Life Technologies 15596) and was quantified by measuring absorbance at 260/280nm. RNA (30 µg per sample) was separated by electrophoresis in a 1% agarose 1.9% formaldehyde gel, transferred to a nylon membrane (Hybond, Amersham RPN 203N) and cross-linked to the membrane by ultraviolet irradiation. Membranes were incubated at 42°C for 4h in a pre-hybridization solution containing 5x SSPE (Sigma S2015), 5x Denhardt's solution (1x = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50% deionized formamide, 1% sodium dodecyl sulphate (SDS), and 200 µg/ml salmon sperm DNA. Membranes were then hybridized sequentially with \(^32\)P-dCTP-labelled cDNA probes (Promega "Prime-a-Gene) for human ubiquitin and human cyclophilin in a hybridization solution of the same composition as above at 42°C overnight. After hybridization, membranes were washed twice with 2x SSPE, 0.2% SDS, at room temperature for 10 min, followed by two washes with 0.2x SSPE, 0.2% SDS at 65°C for 30 min each. Autoradiographic signals were quantified in arbitrary units using a Bio-Rad GS700 Imaging Densitometer. Densitometric signals for ubiquitin were normalized by expressing them relative to the corresponding cyclophilin values to correct for variations in the amount of RNA loaded onto the gel.
2.2.4 Ex vivo determination of muscle protein degradation rate

Total protein degradation rate was measured as described [113] from the rate of tyrosine (Tyr) output from extensor digitorum longus (EDL) muscles incubated in vitro in the presence of cycloheximide to block Tyr reincorporation by protein synthesis. Briefly, EDLs were excised, weighed, attached to steel supports to maintain them at resting length [113] and transferred to 3ml of Krebs-Henseleit bicarbonate buffer (K-H) with 5mmol/l glucose and 20mmol/l Hepes. This was incubated at 37°C under 95%O2/5%CO2 at pH 7.4 for 30 min with frequent swirling of the buffer to maximise gas exchange between the muscles and the K-H. The buffer was then discarded and incubated as above for a further 2h in fresh K-H without Hepes, and supplemented with 0.5mmol/l cycloheximide. Tyrosine release into the buffer was determined by ion exchange chromatography [109] and expressed as nmol/g wet weight over the 2h incubation.

2.2.5 Extraction and assay of corticosterone from urine.

The procedure was based on that described previously [3]. Urine was centrifuged for 10 min at 2000g. To 1ml of supernatant, 10nCi (370Bq) of [1,2,6,7-3H] corticosterone (Amersham TRK 406) was added as internal standard in 20μl of DMSO. Corticosterone was extracted by vortexing with 1ml of dichloromethane followed by centrifugation at 3000g for 10 min at +4°C. The yellow aqueous phase was discarded. The residue was dried down under oxygen-free nitrogen, dissolved in 200μl of 0.1mol/l Tris which had been titrated to pH 7.4 with HCl, and applied to a 360 mg C18 reverse-
phase column (Waters Corporation, Sep-Pak ref. WAT051910) which had been wetted with 1ml of acetonitrile at 1ml/min, followed by 2ml of 0.1mol/l Tris, pH 7.4 at 1ml/min. The corticosterone-loaded column was washed with 10ml of H2O at 2-3 ml/min, followed by 2ml of 20% v/v aqueous acetonitrile at 1-2 ml/min. Corticosterone was eluted with 1.5ml of acetonitrile at 0.5ml/min. The first 0.5ml of eluate was discarded as it contained negligible corticosterone. The remainder was dried down as above and dissolved by vortexing for 10 min in 0.5ml of 0.1mol/l Tris, pH 7.4. Total corticosterone was determined in this extract by 125I radio-immunoassay (Amersham, Biotrak ref. RPA 548) and 3H-corticosterone recovery (65 ± 2%, n = 20) was assessed by liquid scintillation counting.

2.2.6 Statistical analysis.

Values are expressed as mean ± SEM. Statistical significance of changes was assessed by unpaired Student’s t test or, for multiple comparisons, by Analysis of Variance and Duncan’s Multiple Range Test. Correlation is expressed as the Spearman Rank Correlation Coefficient Rs. Effects were regarded as significant if P<0.05.
2.3 Studies in vivo. Metabolic acidosis in peritoneal dialysis patients

2.3.1 Design of the patient study

Continuous peritoneal dialysis patients were enrolled in a prospective examination of the relationship between an increase in serum HCO$_3^-$ and nutritional status. Informed written consent in accordance with the procedures of the Leicester General Hospital ethics committee was obtained from eight continuous ambulatory peritoneal dialysis (CAPD)/continuous cycling peritoneal dialysis (CCPD) patients (7 men, 1 woman with a mean age of 61.9 ± 6 years). The duration of peritoneal dialysis before the start of the study averaged 29.8 ± 3 months (minimum 10 months). The patients were well nourished with a body mass index (BMI) of 25.5 ± 1.2 kg/m$^2$, while the serum albumin averaged 36.6 ± 1.1 g/L. No patient had diabetes mellitus, malignancy, vasculitis, a recent episode of peritonitis or was eating a vegetarian diet.

During an initial three-month period, the patients received dialysis with a standard dialysate of 35 mmol/L lactate. Alkali supplements (calcium carbonate and/or sodium bicarbonate) were withheld and patients were observed to ensure there was no complicating illness. Throughout the study, the prescribed dialysis volumes and frequencies were kept constant. At week 0 (w0), anthropometric indices were measured and a three-day food diary was evaluated by a clinical dietician. A blood sample was obtained to measure serum chemistries and transferrin, pre-albumin, insulin, and tumor necrosis factor-α (TNF-α) values plus plasma BCAA levels. In addition, arterial blood was drawn for blood gas and pH analysis and under local anesthesia, a muscle biopsy
from the vastus lateralis muscle was obtained using the conchotome method [114]. Serum cortisol was measured at four timepoints between 0800 and 1600 hours; and on the first five patients 24 hour urinary cortisol and dialysate effluent cortisol were also measured. This was done because of the well documented interactions among glucocorticoids, metabolic acidosis and muscle metabolism [110, 97, 114]. Cortisol values in the dialysate and urine were below the detection level of the assay (<25 nmol/24h) and serum cortisol measurements were within the expected range for normal adults. Since no pattern of changes in serum cortisol values emerged, these measurements were discontinued for subsequent patients.

After their initial evaluations, the patients were randomized to continue with the low alkali (LA) 35 mmol/L lactate dialysate, or to change to a high alkali (HA) 40 mmol/L lactate dialysate plus oral supplements of sodium bicarbonate (up to 600 mg thrice daily) and or calcium carbonate (up to 500 mg twice daily). In the HA group the oral alkali doses were adjusted in an attempt to maintain the serum bicarbonate above 25 mmol/L as described [78]. Serum chemistry values were monitored weekly. After four weeks a second muscle biopsy, anthropometric measurements and other blood values were obtained as above.

2.3.2 Serum and plasma chemistry

Serum chemistry values were measured with an Ektachem multiple analyzer and arterial blood gases with a Corning 238 blood gas analyzer. Insulin was measured by radioimmunoassay, TNF-α by enzyme-linked immunosorbant assay (ELISA;
Quantikine DTA50; R&D Systems, Minneapolis, MN, USA) pre-albumin by immunoprecipitation (IncStar Corporation, Stillwater, MN, USA) and 3-methylhistidine by high pressure liquid chromatography (HPLC) [90]. Plasma concentrations of the branched-chain amino acids were measured by HPLC [116].

2.3.3 Ubiquitin mRNA determination

Ubiquitin mRNA in muscle biopsies was measured by Northern blotting [79, 94]. Briefly, RNA was isolated from the muscle samples using TriReagent (Molecular Research Center, Cincinnati, OH, USA) and separated in a formaldehyde/agarose gel by electrophoresis before transfer to a ZetaProbe GT membrane (Bio-Rad, Richmond, CA, USA). The blots were stained with methylene blue to visualize the 18S and 28S ribosome RNA bands, and then hybridized with a cDNA probe for chicken ubiquitin (this cDNA is highly homologous with mammalian ubiquitin mRNAs, and thus, it hybridizes with human ubiquitin mRNA) and that was labeled with ${}^{32}$P-dCTP by the random primer method. After overnight hybridization at 65°C, the membranes were washed once with 2x standard sodium citrate (SSC)/0.5% sodium dodecyl sulfate (SDS) at 42°C followed by two washes with 0.5 x SSC/0.5% SDS at 65°C. The washed membranes were then exposed to x-ray film at -80°C with intensifier screens. Autoradiographic bands were quantified using the SigmaGel software package (Jandel Software, San Rafael, CA, USA). To account for differences in lane loading, the intensity of each ubiquitin radiographic band was divided by the intensity of the methylene blue-stained 18S ribosomal RNA band (18S and 28S ribosomal RNA levels were not different between treatment groups).
2.3.4 statistical analysis

Results are presented as mean ± SEM. Statistical analyses included analysis of variance (ANOVA) in comparing values between the groups, and the paired $t$ test when comparing changes in values occurring in the individual patients at week 0 and week 4. Differences were considered significant when $P < 0.05$. 
Chapter 3. Are cultured skeletal muscle cells a valid model for the catabolic state in metabolic acidosis?
3.1 Introduction

As explained in chapter 1, catabolic states in chronic diseases such as chronic renal failure (CRF), cancer cachexia, burns and sepsis are major causes of morbidity and mortality [79]. In these states there is considerable loss of lean body mass, especially in skeletal muscle, and in CRF this is largely attributed to increased protein degradation (PD) during metabolic acidosis (section 1.4) [79]. Much has been learned of these disorders from studies in vivo and from ex vivo incubations of freshly isolated skeletal muscle, but isolated muscle preparations remain viable for only a few hours which restricts the studies that can be performed. Organ culture or cell culture methods are therefore needed for the long-term metabolic and molecular basis of these catabolic states to be investigated.

Increased PD in muscle during metabolic acidosis in vivo, appears to require the presence of glucocorticoid (GC) (section 1.4.8) [3, 97, 110, 115]. The basis of this catabolic effect of acid and the permissive effect of GC is not understood. Studies with cultured L6G8C5 rat skeletal muscle cells suggest that acid acts by inhibiting the pH sensitive System A amino acid transporter in the plasma membrane [117], but these cells differ from muscle in vivo in that they respond spontaneously to low pH without the need for GC [117]. Indeed the GC requirement has been difficult to model in vitro. A possible explanation for the discrepancy between muscle in vivo and L6G8C5 cells in vitro is that confounding factors in vitro affect PD.

Two such confounding factors are apoptosis and cell differentiation. The rate of turnover of confluent cultures of skeletal muscle cells (cell division balanced by apoptosis [102]) is much higher than for skeletal muscle cells in vivo, in which the
slow turnover rate has made muscle a favoured target for gene transfer therapy. In principle, therefore, the proteolysis which is an integral part of the process of apoptosis might become a significant fraction of the total PD rate in vitro and, in studies of PD rate in intact cells, accelerated apoptosis might be misinterpreted as a catabolic response. Differentiation of skeletal muscle cells from myoblasts to mature skeletal muscle also involves dramatic changes in total cell protein content with the protein/DNA ratio rising from 10µg protein/µg DNA in myoblasts [99] to 300 in mature skeletal muscle [109]. For this reason, dedifferentiation of cultures might also be misinterpreted as a catabolic effect, and changes in differentiation state might alter the cells' response to catabolic stimuli.

The aim of the study described in this chapter was therefore to examine the effects of the two related catabolic stimuli (low pH and GC) on L6G8C5 cells under culture conditions varying from simple unfused myoblasts to differentiated myotubes, to determine conditions under which catabolic actions of acid and GC can be studied with minimum risk of interference from changes in apoptosis and differentiation.

3.2 Methods

The laboratory and statistical methods used in this chapter are described in sections 2.1.1 to 2.1.9
Figure 3.1. (a) - (d) Phase contrast micrographs of L6-G8C5 cells (X100). (a) Confluent unfused myoblasts on Day 4. (b) The same culture on Day 8 showing large myotubes covering approximately 25% of the area of the culture (c) Myoblasts on Day 5 after 18h of exposure to serum-free MEM, pH 7.1 showing large numbers of shrunken, retracted apoptotic cells. (d) As for (c) but with DEX (5 nmol/l) suppressing apoptosis. (e) Fluorescence micrograph (X400) of the same culture as (c) stained with Hoechst 33342, showing intensely fluorescent condensed fragmented chromatin in retracted apoptotic cells.
3.3 Results

3.3.1 Quantification of apoptosis

3.3.1.1 Unfused cells

In serum-free medium, unfused myoblasts rapidly underwent apoptosis (Figure 3.1c and e) which readily allowed the direction of effects of pH and GC on apoptosis to be determined. No primary necrosis was detected [118]. Apoptosis was strongly inhibited by low dose GC (5 nmol/l DEX) (Figure 3.1d and Figure 3.2). Increasing pH also suppressed apoptosis, but this was very variable and did not reach statistical significance (Figure 3.2). Even when 10% serum was added (which strongly suppresses apoptosis), apoptosis was still detectable (Figure 3.3) and this residual apoptosis was again suppressed by DEX, although more weakly than in serum-free medium (Figure 3.3 vs Figure 3.2). In the presence of serum, pH had no detectable effect on apoptosis (Figure 3.3).

3.3.1.2 Cells fused spontaneously in 2% serum

Apoptosis was also suppressed by 2% serum (Figure 3.1a vs c) and, unlike 10% serum, low serum medium promoted fusion and differentiation of the cells to myotubes (Figure 3.1b). In these spontaneously fused cultures (Figure 3.1b), blockade of apoptosis with the specific Caspase 3 inhibitor, (C3I) Ac-Asp-Met-Gln-Asp-CHO (Calbiochem 235421) at 50μmol/l (a dose that abolishes apoptosis in intact cells [119]) only decreased total PD rate at pH 7.4 by 9% (Figure 3.4a). In contrast, MG132 (an inhibitor of the ubiquitin-proteasome pathway) has been shown previously in this laboratory [120] to cause strong inhibition of PD and almost abolished the increment in PD induced by acid, suggesting that as in skeletal
Figure 3.2. (Main figure) Effect of DEX and pH on the percentage of cells showing apoptotic morphology in L6-G8C5 cultures stained with Hoechst 33342 on Day 5 after 24h of incubation in serum-free MEM at the specified pH. *P < 0.05 relative to corresponding values with DEX. (Inset) As for main figure but apoptotic cells were detected by labelling DNA strand breaks using a TUNEL protocol. Counts are mean ± SEM for 12 randomly selected fields.
Figure 3.3 (Main figure) Effect of DEX and pH on apoptosis assessed using Annexin V in L6-G8C5 cultures on Day 5 after 24h of incubation in MEM + 10% FBS at the specified pH. *P < 0.05 relative to corresponding values with DEX. (Inset) Effect on the Annexin V signal of 24h of incubation in MEM with or without foetal bovine serum.
Figure 3.4. The effect of pH, Caspase 3 inhibitor (C3I), and Dexamethasone (DEX) on protein degradation rate. Open bars denote medium at pH 7.1. Hatched bars denote medium at pH 7.4. All rates were determined by linear regression over a time course 0-45h after the simultaneous addition of the pH-adjusted media and C3I or DEX to the cells. Data in (a) and (b) (n = 4) are from spontaneously fused cultures to which these media were added after 8 days in culture. *P<0.05 vs the corresponding cultures at pH 7.4. ** P<0.05 vs cultures at pH 7.4 without C3I.
muscle *in vivo* [3] the catabolic effect of acid occurs largely through this pathway. This and the small effect of C3I suggests that the relatively small effects of pH and GC on residual apoptotic rate (Figure 3.3) will have little confounding effect on total PD, particularly as not all caspase proteolytic activity in muscle cells is coupled to apoptosis [121].

### 3.3.2 Effect of pH and GC on differentiation

Acute exposure of unfused serum-free cultures (Figure 3.1c) or spontaneously fused cultures with 2% serum (Figure 3.1b) to low pH or DEX for 24 h had no detectable effect on differentiation of the cells either assessed from the degree of fusion or from measurements of CPK activity (Figure 3.5, main figure). More prolonged exposure of fused cultures to a pH of 7.1 (for 4 days, the longest period routinely used for PD and acidosis studies) had no significant effect on CPK activity compared with controls at pH 7.4 (Table 3.1). Only prolonged severe blockade of the action of endogenous GC in the serum for the whole of the growth and fusion period of the cultures, by using the GC receptor antagonist RU38486, had any significant impact on CPK activity (Figure 3.5, inset), reducing CPK in the cultures and impairing myotube formation. Blockade of mineralocorticoid receptors with spironolactone had no additional effect (Figure 3.5, inset).

### 3.3.4 Effect of apoptosis and differentiation state on the response of PD to pH and GC

In spontaneously fused cultures with 2% serum, low pH gave a clear stimulation of PD rate (Figure 3.4b) and this effect was not blocked when apoptosis was inhibited with C3I (Figure 3.4a). Even though stimulation of PD by metabolic acidosis *in vivo*
Figure 3.5. (Main figure) Effect of pH and Dexamethasone (5nmol/l) on creatine phosphokinase activity in L6 cells (n = 4) during 24h incubations in medium (MEM) with or without 2% dialysed serum. Each data point denotes a separate culture well. For fused cultures, incubations commenced after 8 days on the culture plate. For unfused cultures, incubations commenced after 4 days. Open symbols denote cultures with Dexamethasone. Horizontal bars denote means.

(Inset) Effect of glucocorticoid receptor antagonist RU38486 and mineralocorticoid receptor antagonist spironolactone on creatine phosphokinase activity in L6 cells. Cells were cultured for 3 days in growth medium (DMEM with 10% serum), followed by 5 days in fusion medium (MEM with 2% serum), and then 3 days in test medium (MEM with 2% dialysed serum at pH 7.4). A denotes cultures without receptor antagonists, B with 5 pmol/l RU38486 on Days 9-11, C with 5 pmol/l RU38486 throughout, D with 5 pmol/l RU38486 throughout plus 5 pmol/l spironolactone on Days 9-11. *P < 0.05 versus A.
**Table 3.1** Total protein and DNA content and expression of CPK in cells spontaneously fused in low serum medium

<table>
<thead>
<tr>
<th></th>
<th>Spontaneously fused cells (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE (DAY 8)</td>
</tr>
<tr>
<td>Protein</td>
<td>296</td>
</tr>
<tr>
<td>µg/35mm well</td>
<td>± 10</td>
</tr>
<tr>
<td>DNA</td>
<td>37.0</td>
</tr>
<tr>
<td>µg/35mm well</td>
<td>± 0.5</td>
</tr>
<tr>
<td>CPK</td>
<td>0.40*</td>
</tr>
<tr>
<td>MU/ µg protein</td>
<td>± 0.02</td>
</tr>
</tbody>
</table>

Measurements were made at the end of the fusion period (PRE) and after 4 days in pH-adjusted test medium (POST).

*P<0.05 versus spontaneously fused cells PRE

^P<0.05 versus spontaneously fused cells at pH 7.4

*Spontaneously fused cultures* were incubated in growth medium (DMEM + 10% serum) for 3 days, in fusion medium (MEM + 2% serum) for 4 days, then in test medium (MEM at pH 7.1 or 7.4 + 2% dialysed serum, 5ml/35mm well) for 4 days.
appears to require GC [3] stimulation occurred here in the absence of added GC (Figure 3.4a and b). Indeed, as shown in chapter 4 (Section 4.3.2), addition of DEX (<500 nmol/l) caused no sustained enhancement of the rise in PD induced by acid (Figure 3.4b).

For generation of spontaneously fused cultures, L6-G8C5 cells are routinely used in this laboratory at passage 13 to 23. At higher passage number, fusion of the cultures is impaired (Figure 3.6a), and unexpectedly under these conditions the responsiveness of the PD rate to low pH increased. Consequently, a significant inverse correlation was observed between the magnitude of the response of PD to low pH and the degree of fusion (differentiation) of the cells (Figure 3.6b). Subsequent experiments in this laboratory using highly fused myotubes obtained by incubation with insulin-like growth factor-I, retinoic acid and creatine have confirmed that the responsiveness of PD to acid is lost in such cultures [120].

3.4 Discussion
The effects described in these studies and earlier work [102] show that, in addition to their well-described effects on protein turnover in skeletal muscle, GC [102] and to a lesser extent pH, exert effects on apoptosis and differentiation of cultured skeletal muscle cells. These observations do not preclude the use of these cells for studies of catabolic states. Culture conditions have been demonstrated under which apoptosis and differentiation are not major confounding factors in measurements of PD. In the presence of serum, pH has no significant effect on apoptosis (Figure 3.3) or differentiation (Figure 3.5; Table 3.1) [120], and GC suppresses rather than increases apoptosis (Figure 3.2 and 3.3) and promotes rather than prevents
Figure 3.6. (a) Impairment of differentiation (fusion to form myotubes) in L6-G8C5 cells with increasing passage number. The approximate percentage of the area of the confluent culture covered with large myotubes was estimated after 8 days in culture. Each data point denotes a separate batch of cells. Rs = -0.65, P < 0.001.

(b) Blunting of the acid-induced increase in protein degradation by increasing cell fusion to form myotubes. The initial protein degradation rate (t = 0-7h after addition of the pH-adjusted media) was measured after 8 days on the culture plate in the same cultures shown in (a). Protein degradation was measured in MEM + 2% dialysed serum at pH 7.1 or 7.4 and the percentage increase was determined. Rs = -0.51, P < 0.001.
differentiation (Figure 3.5); [102] in contrast with the stimulation of apoptosis observed in other cell types [122, 123] The stimulation of total PD by acid and GC cannot therefore, be attributed to PD secondary to increased apoptosis or dedifferentiation. Viewed in the context of earlier work [99, 117], the effects of acid and GC on PD in these cells seem, therefore, to be a reasonable model for the analogous effects in vivo. At least for acid, these occur through the same PD pathway, (i.e. the ubiquitin-proteasome pathway [124] which is activated in uraemic metabolic acidosis in vivo [3].

It should be emphasised, however, that these findings in vitro do not entirely rule out a role for muscle differentiation or apoptosis in the catabolic state or in the changes in muscle fibre composition during chronic renal failure in vivo. Apoptotic and differentiation effects do occur in skeletal muscle in vivo. In addition to the caspase activation in cancer cachexia [121], and apoptosis during muscle wasting following burns [125], impaired differentiation like that observed on prolonged GC receptor blockade (Figure 3.5) has been observed in the muscles of adrenalectomized rats in vivo [126] and is reversed by GC supplements.

3.5 Choice of culture conditions for subsequent experiments

As the experiments described in this chapter (and other work from this laboratory [120] have demonstrated that L6-G8C5 cells spontaneously fused in medium containing 2% serum show an acid-induced increase in PD, with negligible artefacts arising from increased apoptosis or dedifferentiation, these culture conditions were
chosen for the subsequent experiments on the glucocorticoid-dependence of acid-induced PD which are described in Chapter 4.

More highly differentiated (highly fused) L6-G8C5 myotubes are readily obtained with differentiation promoting agents including IGF-I [101, 120]. However, an unexpected finding was that the responsiveness of PD in these cells to low pH was lost as the extent of fusion (differentiation) to myotubes increased (Figure 3.6 and [120]). Such highly fused cultures are not routinely used, therefore, in this laboratory and this apparent suppressive effect of IGF-I on acid-induced PD, and its relevance to the GC dependence of acid-induced PD, is discussed in more detail in Chapter 6.
Chapter 4. The glucocorticoid antagonist RU38486 fails to block acid-induced skeletal muscle wasting in vivo or in vitro.
4.1 Introduction

Few treatment options are available to counter the muscle wasting malnutrition effects of metabolic acidosis other than alkali supplements such as NaHCO$_3$ [78, 127]. Traditionally nephrologists have been cautious about this approach due to concerns about sodium loading, volume overload and hypertension. However, it has been observed in severely acidic rats (arterial pH 7.15) that glucocorticoid secretion increases during metabolic acidosis [3], that the accompanying catabolic state is abolished by adrenalectomy [3, 110] and that the catabolic effect of acid is restored by administration of glucocorticoid in the form of pharmacologic doses of dexamethasone [3, 110]. It is possible, therefore, that the glucocorticoid rather than the acid itself is the main determinant of protein catabolism during metabolic acidosis.

The glucocorticoid receptor antagonist 11β-(4-dimethylaminophenyl)-17β-hydroxy,-17a-(prop-1-nyl)-estra-4,9-dien-3-one (RU38486) has been shown to block muscle wasting induced by externally administered glucocorticoid in rats [111]. Consequently, experiments were undertaken to determine whether the glucocorticoid-dependent nature of this process could be inhibited in vivo using RU38486 in rats rendered acidic by administration of a dietary acid load.

The failure of this in vivo experiment to show the expected anabolic effect of RU38486 then prompted us to re-examine the role of glucocorticoid in acid-induced muscle wasting, using the L6 skeletal muscle cell model that was characterised in Chapter 3. The transience of the effects of glucocorticoid observed in this in vitro model are then discussed in the light of recent findings from this laboratory on the
interaction between glucocorticoid and insulin-like growth factor I (IGF-I) and its possible role in vivo.

4.2 Methods

The laboratory and statistical methods and animal procedures used in this chapter are described in chapter 2 sections 2.2.1 to 2.2.6.

4.3 Results

4.3.1 Effects of glucocorticoid antagonist during metabolic acidosis in vivo

4.3.1.1 Effect on pH and serum bicarbonate

Control and acidotic rats were treated with oral glucocorticoid antagonist RU38486 or vehicle for a period of 15 days. For acid-treated animals receiving vehicle, sufficient dietary acid was administered to induce metabolic acidosis with a moderate reduction in plasma HCO\textsubscript{3}\textsuperscript{-} concentration and no significant decline in arterial pH (Table 4.1), the situation usually encountered in uraemic metabolic acidosis [78, 128]. In the rats receiving RU38486, despite ingestion of the same food and acid intake (Table 4.1), both a significant fall in arterial pH and decline in plasma HCO\textsubscript{3}\textsuperscript{-} concentration were observed in acid-loaded animals relative to the controls receiving drug alone (Table 4.1).

4.3.1.2 Effect on body weight gain, and induction of catabolism

Body weight gain over the 15 day study was significantly blunted in the acidotic animals compared with the controls (Table 4.1 and Figure 4.1 and 4.2) and a catabolic state was detected in the skeletal muscle (gastrocnemius) of the acidotic group with an increase in mRNA for the catabolic marker ubiquitin (Table 4.1 and Figure 4.3) and a net decline in muscle protein as plasma [HCO\textsubscript{3}] declined (Figure
Table 4.1 Influence of 15 days of acid-loading (35 mmol/Kg body weight/day) and of RU38486 (50mg/Kg body weight/day) in rats. (n = 5 for all groups).

<table>
<thead>
<tr>
<th></th>
<th>Group C Control</th>
<th>Group RC RU38486</th>
<th>Group A Acidotic</th>
<th>Group RA Acidotic + RU38486</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pH (Day 15)</td>
<td>7.33 ± 0.02</td>
<td>7.36 ± 0.01</td>
<td>7.26 ± 0.03</td>
<td>7.24&lt;sup&gt;d&lt;/sup&gt; ± 0.04</td>
</tr>
<tr>
<td>Arterial HCO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt; (mmol/l)</td>
<td>23.4 ± 0.4</td>
<td>22.9 ± 0.8</td>
<td>19.7&lt;sup&gt;e&lt;/sup&gt; ± 1.2</td>
<td>18.6&lt;sup&gt;d&lt;/sup&gt; ± 1.5</td>
</tr>
<tr>
<td>Body weight (Day 15) (g)</td>
<td>328 ± 4</td>
<td>330 ± 4</td>
<td>306&lt;sup&gt;e&lt;/sup&gt; ± 6</td>
<td>296&lt;sup&gt;d&lt;/sup&gt; ± 10</td>
</tr>
<tr>
<td>Body weight gain (Days 0-15) (g)</td>
<td>42 ± 4</td>
<td>44 ± 3</td>
<td>26&lt;sup&gt;e&lt;/sup&gt; ± 3</td>
<td>16&lt;sup&gt;d,f&lt;/sup&gt; ± 3</td>
</tr>
<tr>
<td>Food intake per rat per day (g)</td>
<td>44.5 ± 1.1</td>
<td>43.2 ± 1.3</td>
<td>45.4 ± 0.8</td>
<td>41.1 ± 1.6</td>
</tr>
<tr>
<td>Free glucocorticoid receptors&lt;sup&gt;a&lt;/sup&gt; (fmol/mg protein)</td>
<td>18.7 ± 3.0</td>
<td>3.7&lt;sup&gt;e&lt;/sup&gt; ± 0.8</td>
<td>16.0 ± 1.9</td>
<td>3.5&lt;sup&gt;f&lt;/sup&gt; ± 0.9</td>
</tr>
<tr>
<td>Ubiquitin mRNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.34 ± 0.17</td>
<td>1.60 ± 0.39</td>
<td>3.35&lt;sup&gt;e&lt;/sup&gt; ± 0.40</td>
<td>3.64&lt;sup&gt;d&lt;/sup&gt; ± 0.39</td>
</tr>
<tr>
<td>Protein degradation&lt;sup&gt;c&lt;/sup&gt; (nmol Tyr/g wet weight/2h)</td>
<td>390 ± 48</td>
<td>657&lt;sup&gt;e&lt;/sup&gt; ± 31</td>
<td>344 ± 17</td>
<td>295 ± 39</td>
</tr>
<tr>
<td>Urine Output (Day 15) (ml/24h)</td>
<td>20 ± 1</td>
<td>21 ± 1</td>
<td>32&lt;sup&gt;e&lt;/sup&gt; ± 2</td>
<td>29&lt;sup&gt;d&lt;/sup&gt; ± 3</td>
</tr>
<tr>
<td>Urine Corticosterone Output (Day 15) (µg/Kg body weight/24h)</td>
<td>3.7 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>5.2&lt;sup&gt;e&lt;/sup&gt; ± 0.2</td>
<td>5.1&lt;sup&gt;d&lt;/sup&gt; ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific binding of <sup>3</sup>H-triamcinolone acetonide in supernatants of gastrocnemius homogenates.

<sup>b</sup> Expressed as ratio to cyclophilin mRNA in gastrocnemius.

<sup>c</sup> Rate of release of tyrosine from EDLs in the presence of 0.5mmol/l cycloheximide

<sup>d</sup> P < 0.05 versus Group RC.

<sup>e</sup> P < 0.05 versus Group C.

<sup>f</sup> P < 0.05 versus Group A.
4.4c). However, with this degree of acid-loading, stimulation of protein degradation rate (tyrosine output from the animals' EDLs) was too small to detect (figure 4.5a).

In a separate group of rats the anticipated increase in protein degradation with decreasing plasma \([\text{HCO}_3^-]\) was demonstrable (Figure 4.5a) but, as in L6-G8C5 cells in vitro (Figure 4.5b), a much wider range of \(\text{HCO}_3^-\) concentration (13 – 27mM, arterial pH range ~ 7.1 to 7.45) had to be imposed for the effect to be observed.

4.3.1.3 Glucocorticoid receptor blockade

Oral RU38486 strongly inhibited specific binding of \(^3\text{H}-\text{TA}\) to glucocorticoid receptors in both acidic and control rats in a soluble protein fraction derived from gastrocnemius (Table 4.1 and Figure 4.6), suggesting that about 80% of the receptors had been blocked by the drug. The \(^3\text{H}-\text{TA}\) binding data were corrected for the decline in total soluble protein content that occurred in these muscles with declining plasma \([\text{HCO}_3^-]\) (Figure 4.4c).

In spite of this receptor blockade, RU38486 did not significantly blunt the catabolic effect of acidosis on body weight (Table 4.1 and Figure 4.1 and 4.2), wet weight of individual muscles (Figure 4.4a), or ubiquitin mRNA (Table 4.1) and unexpectedly Tyr output from the EDLs of non-acidotic animals was increased by the drug (Table 4.1). Furthermore, the 15 day body weight gain in the acidic group receiving RU38486 was less than that in the acidotic group receiving vehicle (Table 4.1, Figure 4.1, Figure 4.2), suggesting that the drug was worsening the catabolic state. In contrast acidosis exerted a hypertrophic effect on the rats' kidneys (Figure 4.4b) as
Figure 4.1 Mean body weight gain throughout time course of experiment, by group.
Statistical analysis as per Table 4.1
Figure 4.2. Effect of acid and or RU38486 on total body weight gain by group mean ± SEM. Statistical analysis as shown in table 4.1.
Figure 4.3 Expression of Ubiquitin in Rat Gastrocnemius During Metabolic Acidosis *in vivo*

**Acidic diet 15 days**

<table>
<thead>
<tr>
<th>Ubiquitin</th>
<th>Cyclophilin</th>
<th>Arterial $\text{HCO}_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 kB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2 ± 1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 ± 0.04</td>
</tr>
</tbody>
</table>

Ubiquitin / Cyclophilin Ratio

$*$P < 0.05
Figure 4.4 Effect of 15 days of acid loading (35 mmol/Kg body weight/day) and RU38486 (50mg/Kg body weight/day) on tissue weight and muscle protein (5 rats in each group). (a) Muscle mean wet weights: gastrocnemius (open circle), heart (filled circle), soleus (open square), EDL (filled square). Acidosis significantly decreased weight (P<0.05) in all except soleus. (b) Kidney wet weights. Acidosis (A) significantly increased weight relative to control (C) (*P<0.05). (c) Influence of plasma [HCO₃⁻] on soluble protein content of gastrocnemius in rats receiving vehicle only (correlation coefficient Rₛ = -0.80, P = 0.006).
Figure 4.5 (a) Relationship between protein degradation rate in rat skeletal muscle cells and severity of metabolic acidosis. (a) Tyrosine output from rat extensor digitorum longus muscles incubated for 2h ex vivo in Krebs-Henseleit, pH 7.4 at 37°C with 0.5mmol/l cycloheximide after 15 days with (n=5) or without (n=3) oral HCl supplements of 70mmol/Kg body weight/day. (Correlation coefficient $R_s = -0.76$, $P=0.03$). Vertical dashed lines indicate the mean plasma $\text{HCO}_3^-$ concentration for the acidotic and control groups of rats in Table 4.1 and Fig 4.1. (b) Rate of release of $^{14}$C-Phe from pre-labelled cell protein in cultures of L6-G8C5 cells incubated for 45h in MEM + 2% v/v dialysed serum at the specified $\text{HCO}_3^-$ concentration starting on day 8 (see Section 2.1.5). (Correlation coefficient $R_s = -0.946$, $P<0.01$).
Figure 4.6 Effect of glucocorticoid receptor blockade by RU38486 expressed as free receptors per mg protein (mean ± SEM). Statistical analysis is shown in table 4.1.
Figure 4.7 Urinary corticosterone excretion day +15, (group mean ± SEM).
Statistical analysis is shown in table 4.1.
reported previously [129] and, unlike the effects on muscle (Figure 4.4a), this effect was no longer detectable in the presence of RU38486 (Figure 4.4b).

In the acidotic rats, urinary corticosterone excretion was increased (Table 4.1 Figure 4.7) as reported previously [3], and treatment with RU38486 had no effect on this. The failure of RU38486 to block muscle wasting in the acidotic animals cannot, therefore, be attributed to a compensatory increase in corticosterone secretion in response to receptor blockade by the drug.

4.3.2 Effects of glucocorticoid and acid in vitro

4.3.2.1 Sensitivity of L6-G8C5 cells to glucocorticoid

The presence of glucocorticoid receptors in L6-G8C5 myoblasts and in fused cultures was demonstrated by measuring specific binding of $^3$H-triamcinolone acetonide ($^3$H-TA) (Figure 4.8). In homogenates from both types of culture, binding of this ligand was displaced by Dexamethasone (DEX) and by RU38486 over a dose range of 5-500 nmol/l (Figure 4.8) implying that both agents essentially saturate the glucocorticoid receptors at 500 nmol/l irrespective of the degree of cell differentiation.

4.3.2.2 Effect of low dose glucocorticoid on L6-G8C5 cells

No catabolic effect of 5nmol/l DEX was detected when the rate of PD was determined over a 55h time course (Figure 4.9), indeed the rate was slightly decreased by DEX both at pH 7.1 and 7.5. As reported previously [99] lowering the pH of the culture medium to 7.1 was sufficient to increase PD even in the absence
Figure 4.8 Displacement by competing ligands of specific binding of $^3$H-triamcinolone acetonide (15 nmol/l) to glucocorticoid receptors in homogenates of L6-G8C5 cells. ( ■ ) DEX and ( □ ) RU38486 in myoblasts on day 4. ( ▲ ) DEX and ( △ ) RU38486 in fused cultures on day 8.
Figure 4.9 Effect of DEX (5 nmol/l) and RU38486 (50 nmol/l) on protein degradation (PD) rate of L6-G8C5 cells (n = 4). PD rate was determined over 55h commencing on day 8. Open bars denote cultures at pH 7.1. Hatched bars denote cultures at pH 7.5. *P < 0.03 versus corresponding values at pH 7.5. *P < 0.03 versus Control values at the same pH.
Figure 4.10 a) Dose-response curves (n = 3) of the effect of DEX on PD rate measured over the first 7h. Solid line denotes pH 7.1; dashed line denotes pH 7.5. *P < 0.05 versus corresponding values without DEX. b) Effect of DEX (5 nmol/l) (□) on the time course of the acid-induced rise in PD in L6-G8C5 cells, commencing on day 8. Each data point represents the mean PD rate (n = 10) measured over the preceding time interval. * P < 0.01 versus the simultaneous value without DEX (■). c) Effect of RU38486 (5 µmol/l) on the acid-induced rise in PD rate in L6-G8C5 cells (n = 4). PD rate was determined over the first 7h, commencing on Day 8. **P < 0.04 versus Control.
Figure 4.11 Time Course of Effect of 500nM Dex on PD in L6 Cells, pH 7.4

PD \( \log_{10} \% / \text{h} \times 10^3 \) (\( n = 4 \))

Dex

Cont

Time (h)

*P < 0.05 v Cont
Table 4.2 The effect of glucocorticoid and of RU38486 on initial (t = 0-7h) protein degradation rate in L6-G8C5 cells.

Protein degradation rate (log_{10} %/h x 10^3, t = 0-7h, n = 4)

<table>
<thead>
<tr>
<th>pH of the medium</th>
<th>Control 500nmol/l</th>
<th>DEX 500nmol/l</th>
<th>DEX (500nmol/l) + RU38486 (5umol/l)</th>
<th>RU38486 5umol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>9.3 ± 0.1</td>
<td>10.22^a ± 0.07</td>
<td>9.35^b ± 0.06</td>
<td>9.15 ± 0.04</td>
</tr>
<tr>
<td>7.5</td>
<td>8.5 ± 0.1</td>
<td>9.2^a ± 0.1</td>
<td>8.8 ± 0.2</td>
<td>8.72 ± 0.4</td>
</tr>
</tbody>
</table>

^aP<0.05 versus Control value at the same pH.

^bP<0.05 versus value at the same pH with DEX alone.
4.3.2.3 Transient effects on protein degradation rate

Even though DEX did not stimulate the rate of PD averaged over a 55h experiment, it was noted that the initial rate of PD (measured from t = 0 - 7h) (Figure 4.10a) and the accompanying acid-induced rise in PD (Figure 4.10b) were both transiently increased by DEX. In further measurements at t = 2, 4 and 7h (Figure 4.11) the catabolic effect of DEX was detectable from 4h onwards.

At pH 7.1 the stimulation of PD by DEX at t = 0-7h was blocked by RU38486 (Table 4.2), suggesting that classical glucocorticoid receptors were involved. Even in the absence of added glucocorticoid, 5 μM RU38486 halved the acid-induced rise in PD at t = 0-7h (Figure 4.10c), suggesting that the trace of glucocorticoid in the 2% serum in the culture medium was initially enhancing the effect of acid. However, RU38486 failed to blunt the effect of acid in longer incubations (Figure 4.9).

Similar negative results have been obtained in experiments in which glucocorticoid action was blocked by the alternative approach of stripping steroid-like molecules from the medium using charcoal-treated serum and incubation without Phenol Red [124]). It should be emphasised that this persistent stimulation of PD at pH 7.1 in the absence of added glucocorticoid is not an artifact of using an alkalaemic control pH (7.5). The same result is obtained when a control pH of 7.4 is used [124].
4.4 Discussion

4.4.1 The requirement for glucocorticoid: direct or indirect?

The previously reported complete abolition by adrenalectomy of acid-induced PD in muscle *in vivo* and its restoration by glucocorticoid [3, 110] implies a key role for glucocorticoid in the catabolic action of acid. In contrast, the failure of adrenalectomy in the original studies to prevent acid-induced weight loss in rats (Table 1 in [110]), the failure of a high dose of RU38486 to block the wasting effects of acidosis *in vivo* (Table 4.1 and Figure 4.4), the relatively small increase in glucocorticoid secretion observed during moderate metabolic acidosis (Table 4.1 and Figure 4.7), and the weak response of acid-induced PD to glucocorticoid alone seen in L6 cells (Figure 4.9 and 4.10) all suggest that glucocorticoid is not the sole determinant of protein wasting during metabolic acidosis. The observation of direct effects of extracellular pH on PD in L6 and BC3H1 cells *in vitro* ([98, 99], Figures 4.5b, 4.9, 4.10) suggests that low pH itself is important, possibly by inhibiting the pH-sensitive System A amino acid transporter [117]. Indeed, the strong suppression of this response to pH *in vitro* by IGF-I [124] and its reversal by glucocorticoid [124], suggest that glucocorticoid's effect could be indirect, occurring through an endogenous anabolic factor such as IGF-I.

4.4.2 Does glucocorticoid antagonise a transient anabolic factor?

A possible explanation for the transient (0-7h) enhancement of acid-induced PD by DEX (Fig 10b) and the transient blunting of it by RU38486 (Fig 10c in the present study) is that glucocorticoid acts by antagonising the effect of an anabolic factor which suppresses PD only in the first 7h after fresh medium is added to the cultures. Serum factors known to be antagonised by glucocorticoid are insulin and the
insulin-like growth factors (IGFs). IGF-I is a promising candidate as it is gradually inactivated when added to muscle cell cultures by IGF-binding proteins (IGFBPs) secreted by the cells [130].

In L6-G8C5 cultures, more recent studies from this laboratory [124] have shown that large IGF-I supplements (10-100 nmol/l) abolish the acid-induced increase in PD, and a more physiological IGF-I supplement (1 nmol/l) transiently prevents acid-induced PD during the first 7h but not in longer incubations [124]. The re-emergence of acid-induced PD after 7h with 1 nmol/l IGF-I is almost prevented if fresh medium with 1 nmol/l IGF-I is subsequently added at intervals to replenish free IGF-I and remove putative inhibitors such as IGFBPs. As predicted, this suppression by IGF-I is reversed when 5 nmol/l DEX is added with the IGF-I [124].

4.4.3 Why are glucocorticoid effects in L6-G8C5 cells smaller than in adrenalectomised rats?

Even though enhancement of acid-induced PD by DEX has been clearly demonstrated in L6 cells (Figure 4.10b), the response is not as dramatic as in adrenalectomised rats in vivo [3, 110]. The "anti-IGF-I" actions of glucocorticoid in vivo are well documented and involve at least four mechanisms, some of which are absent in L6 cultures. Firstly circulating IGF-I is decreased, in part because of decreased synthesis in liver [131, 132] and other tissues through blockade of IGF-I induction by growth hormone [133]. Secondly endogenous IGF-I expression in muscle may also be decreased [132]. Thirdly the free concentration of IGF-I may decrease because of increased output of the glucocorticoid-inducible IGF-binding protein IGFBP1 from liver [134]. Fourthly IGF-I action on skeletal muscle cells is
blunted by impairment of intracellular signalling through p70 S6 kinase [135]. Of these four mechanisms, only the last has been demonstrated in L6 cells [135] and the first and third are not applicable in isolated muscle cell cultures in vitro.

4.4.4 Is the glucocorticoid receptor a suitable target for therapy?

In spite of blockade of 80% of the glucocorticoid receptors in gastrocnemius, RU38486 failed to prevent the wasting disorder in metabolic acidosis (Table 4.1 and Figure 4.4a). This failure is unlikely to arise from incomplete receptor blockade, because the same dose of RU38486 did prevent the muscle wasting induced in non-acidotic rats by a high dose of exogenous glucocorticoid [111]. This dose of RU38486 also apparently blunted acid-induced renal hypertrophy in the present study (Figure 4.4b). It is possible that an anabolic effect of RU38486 in muscle might have been observed if the rats in the present study had received a larger acid load (as in Figure 4.5a) sufficient to allow direct measurement of increased muscle protein degradation. However, this also seems an inadequate explanation because the severity of acidosis achieved in the present study was close to that in chronic uraemia [78, 128] and led to clear weight loss (Table 4.1), muscle wasting (Table 4.1 and Figure 4.4a) and increased ubiquitin mRNA (Figure 4.3) which all failed to improve with RU38486.

Unexpectedly RU38486 seemed to worsen the wasting disorder in two ways (Table 1). Firstly in non-acidotic rats, muscle Tyr output increased. The reason is unknown but may relate to the reproducible longer-term suppression of PD observed with low dose glucocorticoid in vitro (Figure 4.9) and to the myopathy [136], myalgia, rhabdomyolysis and loss of the muscle differentiation marker creatine
phosphokinase.[126] reported on prolonged corticosteroid deprivation in vivo, effects which are at least partly attributable to glucocorticoid [126]. Secondly, the already impaired 15 day weight gain in acidotic rats was worsened by the drug (Table 4.1 and Figure 4.2). The same effect of glucocorticoid withdrawal is observed in adrenalectomised rats (Table 1 in [1110]). As food intake was not significantly affected (Table 4.1), this did not arise from greater acid intake in the RU38486 animals (Table 4.1). It appears, however, that RU38486 did reach functionally significant concentrations in the rats’ kidneys, with an apparent blunting of acid-induced renal hypertrophy (Figure 4.4b). There is abundant evidence that in kidney glucocorticoid stimulates ammoniagenesis [137] and renal acid excretion [138] and that RU38486 blunts urinary NH4+ excretion in acid loaded rats [139]. Such a renal action of the drug may explain the statistically significant fall in arterial pH in the acid-loaded RU38486 group (Table 1). A related problem is encountered in adrenalectomy experiments [140] and necessitates giving sodium supplements [3, 110, 140] to prevent acidosis [140]. Clearly in renal patients, in whom acid excretion is already seriously impaired, such acid retention would not be a desirable feature of glucocorticoid antagonism.

A final reason for caution over the idea that glucocorticoid is the key determinant of acid-induced protein wasting is that, to date, the enhancing effect of glucocorticoid has only been shown at an arterial pH of ~7.15 in vivo [3, 110] and an extracellular pH of 7.1 in vitro ([95 Isozaki, 124). It may therefore be unwise to extrapolate from these results to the more moderate acidosis observed in chronic uraemia. The failure of RU38486 to block wasting in moderate acidosis in Table 1 and Figure 4.4a
conceivably arises through some difference between the glucocorticoid requirement in moderate and severe acidosis.

In conclusion, glucocorticoid receptor blockade in moderate acidosis *in vivo* fails to blunt acid-induced wasting, and glucocorticoid alone (even in severe acidaemia [124]) exerts only a weak effect on acid-induced PD. In contrast IGF-I is a potent determinant of acid-induced PD *in vitro* and is antagonised by glucocorticoid [124]. Glucocorticoid action in metabolic acidosis may, therefore, be indirect, and in the absence of adrenalectomy exerts an effect too weak to be a useful therapeutic target.
Chapter 5 The role of the ATP-dependent ubiquitin proteasome system on muscle wasting and malnutrition due to metabolic acidosis in peritoneal dialysis patients.
5.1 Introduction

5.1.1 Metabolic acidosis and muscle wasting: clinical observations and effect of bicarbonate.

Lowrie and Low identified malnutrition as the leading cause of morbidity and mortality in patients on end-stage renal replacement therapy [66]. The causes are multifactorial and include: poor dietary intake, hormone resistance, and the presence of other co-morbidities. However, there is also compelling evidence for a pivotal role for metabolic acidosis-induced skeletal muscle proteolysis. The clinical connection between renal failure, metabolic acidosis and malnutrition was first documented by Lyon et al who in 1931 observed that blood urea levels were higher in CRF patients ingesting acid rather than alkali generating diets [69].

Impaired growth rates in non-uraemic children with renal tubular acidosis have been restored to super-normal levels with correction of metabolic acidosis alone, all finally achieving normal stature [65]. In haemodialysis patients, increasing the dialysate bicarbonate concentration from 32 mmol/L to 36 mmol/L increased the rate of weight gain or decreased the rate of weight loss compared to those maintained on lower bicarbonate dialysate [77]. Peritoneal dialysis patients randomised to receive high alkali (lactate 40mmol/L) dialysate had statistically significant increase in total body weight and mid-arm muscle circumference at one year, as well as fewer and briefer hospital admissions compared to those treated with low alkali (lactate 35 mmol/L) dialysate [78].

The exact mechanism by which correction of metabolic acidosis prevents proteolysis in dialysis patients has not previously been elucidated. Experimental
induction of acidosis in pair-fed non-uraemic rats resulted in a significant reduction in total body weight gain coupled with increased excretion of non-urea nitrogen and increased urinary 3-methylhistidine/creatinine (this ratio being a marker of skeletal muscle proteolysis) [90]. This protein degradation can be inhibited simply by the addition of alkali in the form of sodium bicarbonate [90]. Leucine turnover studies in human volunteers, suggested that induction of metabolic acidosis increased protein degradation more than protein synthesis [76]. Correction of acidosis in CAPD patients decreased whole body protein degradation [141]. Animal studies have demonstrated that metabolic acidosis increases skeletal muscle oxidation of branched-chain amino acids (valine, leucine, and isoleucine), the only essential amino acids to be degraded to great extent by extra-hepatic tissues [85].

5.1.2 The ubiquitin proteasome pathway and skeletal muscle

As described in Section 1.4.6 four proteolytic pathways have been identified in skeletal muscle. Mitch and colleagues have demonstrated in non-uraemic rats that metabolic acidosis induces proteolysis through up-regulation of an ATP-dependent pathway involving ubiquitin and proteasomes [94]. Of the transcriptional events involved in this up-regulation, the most striking is the increase in expression of ubiquitin itself ([94] and Figure 4.3), and for this reason ubiquitin expression was chosen as the marker of ubiquitin proteasome pathway activation in the study described below. Ubiquitin is a highly conserved intracellular protein which is sequentially attached to proteins identified for degradation. This process requires catalyst and carrier proteins (E₁, E₂, and E₃) and energy (ATP). Ubiquitinisation induces conformational change in proteins enabling their entry into the 26S proteasome which is a large cytoplasmic “device” which “cuts” proteins into
peptides [91,92,93]. This process has been demonstrated to be up-regulated in several pathological states including burns [79], cancer cachexia [142] and sepsis [143] models.

The current study was designed to determine whether acidosis associated skeletal muscle proteolysis in dialysis patients occurs via this same mechanism and whether it can be corrected by the addition of alkali.

5.2 Methods
The study design, methods and materials are explained in chapter 2 sections 2.3.1 to 2.3.4.

5.3 Results
5.3.1 Acid/base status
During the four weeks of study, each patient had an improvement in their serum bicarbonate concentration: those treated with the 35 mmol/L lactate improved by +2.2 ± 0.6 mmol/L (p=0.02) and those treated with 40 mmol/L lactate by +5.7 ± 0.7 (p=0.014). The final serum bicarbonate concentration in the two groups did not differ significantly. Six of the eight patients had a serum HCO₃⁻ of >25 mmol/L irrespective of their dialysate lactate concentration and the other two patients had an increase in serum HCO₃⁻ of 1 and 4 mmol/L. The hypothesis was that an increase in serum HCO₃⁻ would improve indices of nutritional status and decrease the ubiquitin mRNA in muscle. Because all patients had an increase in serum HCO₃⁻ to similar final values we combined results from all subjects in consequent analyses. This is consistent with the finding by Stein and colleagues that patients treated with
35 mmol/L lactate also had a mean serum HCO$_3^-$ of 26 mmol/L (although by the end of the one year study it was 23 mmol/L) [78]. In the present study arterial blood pH also increased slightly, but the change was not statistically significant (Table 5.1). There were no significant changes in serum creatinine or urea concentrations (Table 5.2).

5.3.2 Nutritional status (Table 5.2)

After 4 weeks, there was a statistically significant increase in body weight and body mass index (BMI). There was no change in midarm muscle circumference nor in triceps skinfold thickness. Analysis of three day food diaries indicated that energy intake after four weeks was slightly, but not statistically lower at the time of the second biopsy. The amount of dietary protein was unchanged.

5.3.3 Factors related to nutritional status

The average serum albumin and prealbumin concentrations did not change significantly. Likewise, no significant changes were seen in serum insulin or transferrin. However, the serum level of tumour necrosis factor alpha (TNF-α) decreased significantly (Table 5.2, Figure 5.1), which was comparable with that previously reported in CAPD [144] and other chronic kidney disease [145] patients.

5.3.4 Evidence that the increase in serum bicarbonate down regulates catabolic pathways

The increase in serum bicarbonate was associated with evidence for down regulation of the catabolic pathways that degrade protein and branched-chain amino acids (BCAA). In muscle, ubiquitin mRNA (corrected for lane loading using the respective 18S rRNA level) decreased significantly over the four weeks (W0 = 1.49 ± 0.07 arbitrary units, W4 = 1.23 ± 0.08 units (p=0.016) (Figure 5.2 and 5.3).
Secondly, the plasma BCAA levels increased with correction of metabolic acidosis (Figure 5.4). The increase in valine and isoleucine values were statistically significant (p=0.04), whilst the increase in leucine approached statistical significance (p=0.07).
Table 5.1 serum bicarbonate and plasma pH values of CAPD patients

<table>
<thead>
<tr>
<th></th>
<th>W0 HCO₃⁻ mmol/L</th>
<th>W4 Change</th>
<th>W0 pH</th>
<th>W4 change</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 mmol/L lactate</td>
<td>22.0±1.1</td>
<td>+2.2±0.6</td>
<td>7.41±0.02</td>
<td>0</td>
</tr>
<tr>
<td>40 mmol/L lactate</td>
<td>24.0±2.5</td>
<td>+5.7±0.7</td>
<td>7.36±0.03</td>
<td>+0.09±0</td>
</tr>
<tr>
<td>Combined</td>
<td>22.8±1.1</td>
<td>+3.5±0.7</td>
<td>7.39±0.02</td>
<td>+0.03±0.02</td>
</tr>
</tbody>
</table>

Data are mean ± SE, W0 and W4 are weeks zero and four respectively

Table 5.2 Laboratory values of CAPD patients before and after treatment

<table>
<thead>
<tr>
<th></th>
<th>W0 value</th>
<th>W4 change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine µmol/L</td>
<td>981±63</td>
<td>-18±40</td>
<td>NS</td>
</tr>
<tr>
<td>Urea mmol/L</td>
<td>17.2±2.3</td>
<td>+1.0±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight kg</td>
<td>74.2±3.3</td>
<td>+0.6±0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>Body mass index kg/m²</td>
<td>25.5±1.2</td>
<td>+0.2±0.1</td>
<td>0.035</td>
</tr>
<tr>
<td>Midarm circumference cm</td>
<td>28.9±1.0</td>
<td>+0.1±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Tricep skinfold thickness mm</td>
<td>12.1±1.2</td>
<td>+0.7±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Dietary protein g/day</td>
<td>64.8±4.2</td>
<td>+0.7±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Energy intake kcal/day</td>
<td>1951±115</td>
<td>-101±74</td>
<td>NS</td>
</tr>
<tr>
<td>Serum albumin g/L</td>
<td>36.6±1.1</td>
<td>-1.5±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Serum prealbumin mg/L</td>
<td>380.5±32.4</td>
<td>+28±32.8</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α pg/L</td>
<td>15.4±0.5</td>
<td>-1.4±0.4</td>
<td>0.008</td>
</tr>
<tr>
<td>Serum transferrin g/L</td>
<td>1.81±0.10</td>
<td>+0.03±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Serum insulin mU/L</td>
<td>29.4±7.0</td>
<td>+1.6±5.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations are: NS, not significant, W, week
Figure 5.1 Serum TNF-α values at week 0 and week 4 all patients combined.
Figure 5.2 Representative autoradiogram of Ubiquitin mRNA from vastus lateralis of 2 peritoneal dialysis patients.
Figure 5.3 Ubiquitin mRNA (arbitrary units) v bicarbonate paired values for individual patients at week 0 (closed diamonds) and week 4 (open diamonds).
Figure 5.4 Increase in serum levels of branched chain amino acids with correction of metabolic acidosis. $P = 0.04$ for valine and isoleucine, $P = 0.07$ for leucine.
5.4 Discussion

Metabolic acidosis is associated with negative nitrogen balance and degradation of BCAA and protein in patients with kidney disease [74, 73, 146, 141, 147]. Animal experiments have demonstrated that the mechanisms involved in these deleterious processes involve stimulation of the activities of branched-chain ketoacid dehydrogenase (BCKAD) and the ubiquitin proteasome proteolytic system [85, 86, 87, 148]. It seems reasonable to contemplate the same processes being active in patients with renal failure. The results of the present study support this conclusion and, furthermore, suggest that these metabolic abnormalities can be improved simply by increasing serum bicarbonate even at relatively modest levels of hypobicarbonataemia as frequently encountered in clinical practice.

The hypothesis for this study was that an increase in serum HCO$_3^-$ (as a surrogate for an improved acid-base status) would be the signal to suppress catabolic pathways. Serum HCO$_3^-$ increased in both groups as reported previously [78] using the same protocol. One potential explanation for the increase in serum HCO$_3^-$ in those patients continuing low alkali dialysate is better compliance with the dialysis regimen by virtue of being followed in a clinical study. Indeed, Bernardini, et al reported that as many as 30% of CAPD patients completed less than 90% of prescribed dialysis exchanges, although this fraction decreased in patients on longer term dialysis [149]. We cannot exclude the possibility that a change in the dialysis schedule was responsible for the higher plasma levels of BCAA and lower levels of ubiquitin mRNA in muscle. However, the longer-term study of Stein et al [78] in which a higher serum HCO$_3^-$ was associated with greater weight gain and mid arm muscle circumference, suggests that it is the correction of acidosis rather than
changes in dialysis that accounted for the improvements in nutritional status [78]. Although the study only included one woman (all eligible patients were asked whether they would wish to be included in this study, but female patients were much less likely to agree to having two quadriceps biopsies), her values uniformly responded in the same direction as those of the men.

It would have been preferable to demonstrate an effect of a higher serum HCO$_3^-$ by demonstrating a difference between the effect of the two dialysates. Unfortunately, it was not possible to examine this for several reasons: the two dialysates did not yield significantly different values of serum HCO$_3^-$ or arterial pH after one month. However, the number of patients in each group was small and when the results were subjected to ANOVA, there was no significant difference between the two groups in terms of changes in ubiquitin mRNA, BMI or changes in nutritional status. This was an invasive study to examine patho-physiological mechanisms so recruitment was difficult precluding larger numbers of subjects in each group.

May et al reported that plasma and muscle BCAA levels were lower in non-uraemic acidotic rats compared to pair-fed controls, and that this response was related to increased activity of BCKAD [96]. We did not measure BCKAD activity in the muscle of these CAPD patients because of the small muscle sample, but did find that plasma BCAA levels were higher when the serum HCO$_3^-$ levels were increased. This finding is consistent with suppression of BCKAD activity in muscle, the major site of BCAA degradation in vivo [150].
The inference from the decrease in ubiquitin mRNA in muscle is that it reflects a decrease in proteolytic activity of the ubiquitin proteasome system [79]. While protein degradation in muscle was not measured, two types of evidence support this inference. Firstly, Garibotto et al assessed arteriovenous flux of amino acids in the forearm of acidotic, CRF patients and found higher rates of protein degradation in those with lower serum HCO₃⁻ values [97]. The patients’ weights did increase during the four weeks of the present study and a similar protocol pursued for a year found an increase in both total body weight and muscle mass [78], however there was no measurable increase in muscle mass in the current study. Secondly, most studies of experimentally induced catabolic conditions characterized by skeletal muscle protein degradation via the ubiquitin proteasome pathway have revealed a corresponding increase in ubiquitin mRNA in muscle. In the model of metabolic acidosis in uraemic rats, this increase was prevented simply by feeding NaHCO₃ to the acidotic animals [79, 146].

Other investigators have reported that the ubiquitin mRNA level in muscle of patients with catabolic conditions such as head injury, sepsis, trauma, and gastric cancer is high [142, 143, 151, 152]. In some of these studies, there was evidence for increased protein degradation plus an increase in mRNAs encoding subunits of the proteasome or components of the other proteolytic pathways. The present study is the first to provide evidence that changes in ubiquitin mRNA can be therapeutically regulated in a condition associated with loss of muscle mass. The ubiquitin mRNA level in individual patients with renal failure treated by peritoneal dialysis decreased as their serum bicarbonate increased.
The signal(s) leading to higher levels of ubiquitin mRNA were not identified in those reports of clinical conditions associated with increased ubiquitin mRNA in muscle, but in patients with head injury there was an increase in plasma cortisol [151]. Experimental evidence in rats has suggested a permissive role for glucocorticoids in acidosis induced muscle protein degradation [3, 85, 86, 115] (see Chapter 4.) In the current study, measured serum cortisol levels were within the range for normal adults and levels in CAPD dialysis effluent and 24 H urine samples were < 25nmol/L. Therefore, as in Chapter 4, supportive evidence for the role of glucocorticoids was not identified by this work.

Finally, it has been suggested that ureamia activates inflammatory responses that decrease serum albumin and may stimulate protein loss [153, 154]. Cytokine levels have been observed to be elevated in patients with renal failure and experimentally, TNF-α injection into rats is associated with muscle protein and BCAA catabolism [155, 156, 157, 158, 159]. Although a cause and effect relationship cannot be concluded from the current data the possibility of cytokine regulation of this process is raised.

In summary, the results of this study indicate that increasing the serum bicarbonate level in CAPD patients leads to a down-regulation of proteolysis via the ATP-dependent ubiquitin proteasome pathway in muscle. There was also an increase in plasma BCAA consistent with a decrease in their degradation. The number of patients studied was small, but the agreement between these results and those in animals provide evidence that muscle wasting in dialysis patients occurs via stimulation of these catabolic pathways. Furthermore, they suggest that these
pathways can be manipulated by simple interventions which are readily available to clinical practice; i.e. high alkali dialysate and oral sodium bicarbonate.
Chapter 6 General Discussion
6.1 Introduction

As was shown in Chapter 1, metabolic acidosis is an almost inevitable consequence of unmodified renal failure and induces multiple deleterious systemic effects, including a protein wasting (malnutrition) disorder. This thesis has focussed upon this acid-induced malnutrition because, in the long-term, renal dialysis patients are especially susceptible to the morbidity and mortality which are associated with such wasting illness. The reason for this clinically important association is unknown. The aim of this final chapter is therefore to examine the inter-relationships between the glucocorticoid-dependent processes of apoptosis, cell differentiation, muscle protein wasting, and the ubiquitin proteasome pathway which have emerged from Chapters 3 to 5. This overview will be compared with results from recent studies by other workers in an attempt to discern a mechanism for acid-induced protein wasting. Possible explanations of how this mechanism might lead to long-term morbidity and mortality will then be discussed, followed by consideration of the implications of this work for therapy against acid-induced malnutrition.

6.2 Overview of the mechanism of acid-induced protein wasting and the role of glucocorticoid

6.2.1 Increased gene expression

Previous work from other laboratories (reviewed in Section 1.4.3) has shown that acidosis leads to increased expression of mRNAs for genes which code for proteins of the ubiquitin-proteasome pathway, both in acidotic rats [115] and in cultured muscle cells exposed to low pH [95]. One of the clearest examples is the increased expression of ubiquitin mRNA, and this was confirmed in the animal study in this
thesis (Fig 4.3). These increased mRNA levels may therefore stimulate the synthesis of the proteins of the ubiquitin proteasome pathway and hence drive increased protein catabolism. Glucocorticoid is also known to increase expression of ubiquitin mRNA \cite{160} and Proteasome C3 sub-unit mRNA \cite{161} in L6 cells and might therefore be expected to reinforce the effect of acid. However, in the animal and cell culture studies described in Chapter 4, a direct obligatory role for glucocorticoid in stimulating acid-induced protein wasting was not apparent, nor is there any evidence yet that ubiquitin-proteasome pathway proteins increase in muscle cells in response to acidosis or glucocorticoid.

6.2.2. Comparison of studies in humans and rats

The changes in ubiquitin mRNA observed in acidotic humans in Chapter 5 were smaller than in the study with acidotic rats in Chapter 4. In part this could have arisen because some of the patients were less acidotic than the rats but, as explained in Chapter 4, the acid load applied to the animals was not excessive, and led to a moderate decline in plasma bicarbonate with no significant decline in arterial pH, a situation similar to that seen in CAPD patients. It is still not known whether the mRNA responses reported in metabolic acidosis are a pre-requisite for the protein catabolic state i.e. required to synthesise additional proteins of the Ubiquitin-proteasome system, or whether the mRNA response is a consequence of the catabolic state i.e. increased transcription of e.g. the ubiquitin gene to replace copies of the ubiquitin protein degraded in the catabolic state \cite{162} Future studies on the time course of the ubiquitin mRNA response and the accumulation (or otherwise) of free ubiquitin protein and ubiquitinated conjugates in a reliable cell culture model may be helpful in distinguishing these two possibilities.
6.2.3. Validity of the L6G8C5 cell culture model

In vitro experiments described in Chapter 3 suggest that L6G8C5 cells show a protein catabolic state strongly resembling that in acidotic muscle in vivo, with detectable increases in PD within 7h.

In spite of this, initial results from other workers in this laboratory [A. Bevington, J. Brown and H. Butler, unpublished observations] have detected no increase in ubiquitin mRNA response even after 12h, and the initial rise in PD at 7h still occurs even when all transcriptional responses are blocked with Actinomycin D. The ubiquitin mRNA response may not therefore be required for initiation of acid-induced PD in the first 7h, although an important role in the longer term maintenance of the accelerated rate of PD has not been excluded.

6.2.4. Glucocorticoid, acidosis and resistance to insulin-like factors

In Chapter 4 it was suggested that, rather than a direct glucocorticoid requirement for acid-induced protein catabolism, the role of glucocorticoid in acid-induced protein degradation may be indirect, occurring by blunting the protein anabolic effect of insulin-like factors such as IGF-I. The various splicing variants of IGF-I are regarded as major determinants of muscle mass in humans [163] and in situ generation of IGF-I during exercise may account for the promising improvements in nutritional status and morbidity observed in dialysis patients following therapeutic exercise regimens [163, 164]. Following on from the cell culture studies described in Chapter 4, more recent work in this laboratory [124] has shown that IGF-I strongly suppresses acid-induced PD in L6G8C5 cells, and that glucocorticoid apparently
enhances the effect of acid by blunting this effect of IGF-I. This interpretation has been strongly reinforced by two recent studies which have shown that acidosis, like glucocorticoid, can exert a protein wasting effect in L6 cells by inducing a form of insulin resistance. Franch and co-workers [165] have shown that the suppression of PD in L6 cells by insulin is blunted at low extracellular pH, apparently by inhibition of insulin signalling in the region of phosphoinositide-3-kinase. A possible related mechanism has emerged from recent work on the System A (SNAT2) glutamine transporter in the plasma membrane of L6 cells which is strongly inhibited by low extracellular pH [117] and by glucocorticoid. There is considerable evidence that inhibition of this transporter triggers protein wasting in these cells [117] and it has now been shown in this laboratory that extracellular glutamine starvation in L6 cells rapidly blocks the anabolic effect of insulin signalling to protein synthesis [Evans KF unpublished observations].

6.3 Muscle protein wasting, morbidity and mortality
As discussed in Chapter 1, the association between wasting illness and increased morbidity and mortality is well established in renal patients, but the physiological basis of this association is unknown. Two independent randomised prospective studies have shown improvements in patient morbidity following prolonged correction of uraemic metabolic acidosis [78, 127], suggesting that acidosis has in some way caused the morbidity. If this apparent effect of acidosis arises from wasting illness, this implies that the decline in cell mass (particularly skeletal muscle) that is detected in renal patients by body composition studies [166] leads to morbidity. This may arise because muscle contains the body's largest reservoir of amino acids (in the form of protein) that can be liberated under conditions of
metabolic stress for protein synthesis, as metabolic fuels, and as a carbon source for gluconeogenesis in other tissues. An alternative explanation, however, is that the mechanism that leads to wasting in skeletal muscle also exerts harmful effects in other vital organs. As many dialysis patients ultimately die from cardiovascular disease and infections, the influence of insulin resistance through acidosis, glucocorticoid and impaired glutamine transport in the heart, the vasculature and the immune system may merit future investigation.

6.4 Sodium bicarbonate supplements in a dialysis patient: friend or foe?
The current lack of a detailed understanding of protein wasting in acidosis at the molecular level does not preclude a pragmatic approach to therapy at the clinical level. Alkali therapy is a readily available and cheap option and was first recommended by no less a physician than Richard Bright. Alkali in the form of increased lactate or bicarbonate (peritoneal dialysis), or acetate or bicarbonate (haemodialysis) buffer has since been used widely in attempts to correct metabolic acidosis and ameliorate the malnutrition associated with renal failure. The current clinical study (Chapter 5) builds on previous work, especially that of Stein et al [78] showing long-term anthropometric and morbidity benefits with high alkali dialysate. A possible criticism of that study was that additional sodium, from the oral sodium bicarbonate supplements, may simply have led to salt and water retention and that this may have been interpreted erroneously as gain in muscle mass. Had this been the case however, one would have expected to see an increase in morbidity rather than the observed decrease. Moreover, there is evidence that Na administered as NaHCO₃ is more readily excreted and does not have the same pressor effect as Na administered as NaCl [167]. The results reported in Chapter 5 corroborate the
increase in total body weight seen previously, although no significant change was seen in MAC or TSF, presumably due to the short duration of the study. Furthermore, as discussed in Chapter 5, statistically significant improvements were also seen in the catabolic markers ubiquitin mRNA, plasma BCAA levels and TNF-alpha levels.

Two other studies have examined changes in the ubiquitin proteasome pathway in dialysis or pre-dialysis patients following correction of metabolic acidosis. Roberts et al [168] did not see a reduction in ubiquitin mRNA when they administered oral bicarbonate supplements to pre-dialysis patients, but these patients were not directly comparable with those in Chapter 5, not yet having commenced dialysis, and the bicarbonate levels before and after supplementation were lower than in our work, implying that the metabolic acidosis had not been corrected to the same extent. Bossola et al [169] compared ubiquitin mRNA levels in rectus abdominus biopsies from 8 haemodialysis patients and 6 controls. There were no significant differences in the 1.26kb polyubiquitin mRNA, and the 2.4kb species was unexpectedly lower in the HD patients than in the controls. However, the number of subjects was probably too small for a cross-sectional study of this type, and a normal range has yet to be defined for such measurements.

Finally, the optimum plasma bicarbonate concentration to aim for in applying bicarbonate therapy needs to be considered. This is still unclear. A target of 24mmol/L has been proposed [17]. The work in Chapter 5, and other acidosis correction studies [77,78,127], imply however that further clinical benefit may be derived on elevating plasma bicarbonate further within the normal range, and there
is still debate over whether the accepted normal range may in fact be too low owing to the considerable acid-generating potential of typical Western diets consumed by healthy individuals [5].

6.5 Conclusions and future directions

Much remains to be learned about the mechanism(s) whereby metabolic acidosis induces skeletal muscle protein wasting, but the following important new findings have arisen from the work described in this thesis:

1) The L6G8C5 culture model of acid-induced protein wasting has been further validated, and artifacts arising from changes in apoptosis and differentiation state have been shown to be only minor contributors to the observed wasting response.

2) The culture conditions needed to demonstrate the enhancement by glucocorticoid of acid-induced protein degradation previously reported in vivo have now been defined in vitro. The effect of glucocorticoid seems to be indirect and is not an obligatory requirement for acid to trigger protein wasting. Consequently the acid-induced protein wasting observed both in vitro and in acid-loaded rats shows no significant improvement when glucocorticoid action is blocked with the glucocorticoid receptor antagonist RU38486.

3) Correction of metabolic acidosis in peritoneal dialysis patients was achieved using high alkali dialysate and oral bicarbonate supplements, and this led to the first demonstration in humans of suppression of a marker of the ubiquitin-proteasome pathway (ubiquitin mRNA).
An important priority for future clinical research is the question of whether sodium bicarbonate therapy leads to salt and water retention and hence spurious weight gain and possible cardiovascular side effects. To that end, an investigation of the effects of this type of supplementation in dialysis patients, accompanied by rigorous measurement of body composition [166] should be a high priority for future research.
7 References


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