The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin-proteasome pathway.

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Chronic renal failure (CRF) is associated with negative nitrogen balance and loss of lean body mass. To identify specific proteolytic pathways activated by CRF, protein degradation was measured in incubated epitrochlearis muscles from CRF and sham-operated, pair-fed rats. CRF stimulated muscle proteolysis, and inhibition of lysosomal and calcium-activated proteases did not eliminate this increase. When ATP production was blocked, proteolysis in CRF muscles fell to the same level as that in control muscles. Increased proteolysis was also prevented by feeding CRF rats sodium bicarbonate, suggesting that activation depends on acidification. Evidence that the ATP-dependent ubiquitin-proteasome pathway is stimulated by the acidemia of CRF includes the following findings: (a) An inhibitor of the proteasome eliminated the increase in muscle proteolysis; and (b) there was an increase in mRNAs encoding ubiquitin (324%) and proteasome subunits C3 (137%) and C9 (251%) in muscle. This response involved gene activation since transcription of mRNAs for ubiquitin and the C3 subunit were selectively increased in muscle of CRF rats. We conclude that CRF stimulates muscle proteolysis by activating the ATP-ubiquitin-proteasome-dependent pathway. The mechanism depends on acidification and increased expression of genes encoding components of the system. These responses could contribute to the loss of muscle mass associated with CRF.

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The Acidosis of Chronic Renal Failure Activates Muscle Proteolysis in Rats by Augmenting Transcription of Genes Encoding Proteins of the ATP-dependent Ubiquitin–Proteasome Pathway

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Abstract

Chronic renal failure (CRF) is associated with negative nitrogen balance and loss of lean body mass. To identify specific proteolytic pathways activated by CRF, protein degradation was measured in incubated epitrochlearis muscles from CRF and sham-operated, pair-fed rats. CRF stimulated muscle proteolysis, and inhibition of lysosomal and calcium-activated proteases did not eliminate this increase. When ATP production was blocked, proteolysis in CRF muscles fell to the same level as that in control muscles. Increased proteolysis was also prevented by feeding CRF rats sodium bicarbonate, suggesting that activation depends on acidification. Evidence that the ATP-dependent ubiquitin–proteasome pathway is stimulated by the acidemia of CRF includes the following findings: (a) An inhibitor of the proteasome eliminated the increase in muscle proteolysis; and (b) there was an increase in mRNAs encoding ubiquitin (324%) and proteasome subunits C3 (137%) and C9 (251%) in muscle. This response involved gene activation since transcription of mRNAs for ubiquitin and the C3 subunit were selectively increased in muscle of CRF rats. We conclude that CRF stimulates muscle proteolysis by activating the ATP–ubiquitin–proteasome–dependent pathway. The mechanism depends on acidification and increased expression of genes encoding components of the system. These responses could contribute to the loss of muscle mass associated with CRF. (J. Clin. Invest. 1996. 97:1447–1453.) Key words: gene transcription • proteasome • ubiquitin • chronic renal failure • protein degradation

Introduction

Increased protein catabolism and negative nitrogen balance are characteristic of chronic renal failure (CRF). Early studies by Coles documented that CRF patients had low serum proteins and loss of lean body mass (1). Anthropometric measurements reveal that hemodialysis patients have a reduced muscle mass (2, 3), and Williams et al. reported that patients with CRF complicated by metabolic acidosis have increased excretion of 3-methylhistidine (4). They concluded that this represented excessive muscle protein catabolism, resulting in negative nitrogen balance. Despite documentation of the problem in patients, cellular processes causing protein catabolism in CRF remain poorly understood. In particular, the proteolytic pathways activated by CRF and the signals resulting in activation of protein degradation have not been identified.

In muscle, there are at least four pathways that can degrade proteins: lysosomal, calcium-activated proteases, ATP-dependent, and ATP-independent proteolytic pathways (5). Endocytosed proteins are degraded by lysosomal proteases, whereas calcium-activated proteases seem to be important in certain types of muscular dystrophy and in the response to muscle damage (6–8). The ATP-dependent pathways include the ubiquitin–proteasome system, which degrades abnormal and short-lived proteins (9–12). This pathway involves a soluble, multienzyme system that requires ATP (9). Proteins to be degraded are covalently linked to multiple ubiquitins by a ubiquitin–conjugase complex, and degradation occurs in the 26S proteasome complex (9, 10, 12). Little is known about the ATP-independent proteolytic systems (5, 13).

When normal rats were fed NH₄Cl to induce metabolic acidosis, we found that muscle protein degradation was increased. When muscles from these rats were depleted of ATP, the increase in protein degradation was abolished (14, 15). We also found increased levels of mRNAs encoding both ubiquitin and subunits of the proteasome, suggesting that the increase in muscle protein degradation involved the ubiquitin–proteasome pathway. These results obtained in studies of rats fed NH₄Cl may be relevant to the catabolism associated with CRF because we have also found that muscle protein degradation is increased in rats with experimental CRF and that adding sodium bicarbonate to their diet eliminated the excess protein breakdown (16). In that study, we did not identify the proteolytic pathway activated by CRF.

Increases in both muscle protein degradation and levels of mRNAs encoding components of the ubiquitin–proteasome pathway have been found in association with other catabolic conditions, including sepsis (17), cancer (18–20), burns (21), starvation, and denervation (22). Consequently, insights into the mechanisms leading to increased mRNAs for components of this pathway could have widespread interest.

The present experiments were designed to explore which proteolytic pathway is activated by CRF as well as the mechanism of activation. In studies of rats after subtotal nephrectomy, we used a pair-feeding strategy to eliminate the influence of variations in dietary protein, which can change protein turnover in muscle (22, 23). There was direct evidence that

1. Abbreviations used in this paper: CRF, chronic renal failure; EDL, extensor digitorum longus; GAPDH, glyceraldehyde-3-phosphate; SO, sham operated.

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ATP-dependent, Ubiquitin-Proteasome Proteolysis and Renal Failure
CRF stimulates muscle protein degradation by activating the ubiquitin–proteasome system, including up-regulation of expression of genes encoding components of this system.

**Methods**

Male Sprague-Dawley rats weighing 50–75 g obtained from Charles River Laboratories (Raleigh, NC) were housed in temperature-controlled quarters with a 12-h light–dark cycle. Water and 23% protein pellets (Purina Animal Chow, Inc., Chicago, IL) were provided ad libitum. Rats, anesthetized with 60 mg ketamine and 12 mg xylazine/kg intraperitoneally, underwent a right nephrectomy through a flank incision and were fed ad libitum 8% protein chow prepared by mixing 36% ground pellet chow (Agway 2000, Madison, WI) and 64% dextrin (U.S. Biochem. Corp., Cleveland, OH). 1 wk later, rats were anesthetized and branches of the left renal artery were ligated to produce a 7/8 nephrectomy. After surgery, the rats resumed the 8% protein diet and were given 0.225 g/dl NaHCO<sub>3</sub> to drink. 10 d later, rats were paired by weight to rats that had their abdomen opened and the kidneys manipulated but not damaged (sham-operated [SO] rats). CRF and SO rats were pair-fed a high protein diet for at least 2 wk before the experiment to induce azotemia and acidosis (16). The diet contained 46% protein, consisting of 35% casein (Teklad Premier Labo-
ATP-dependent, Ubiquitin-Proteasome Proteolysis and Renal Failure

Table I. CRF Complicated by Acidemia Increases ATP-dependent Protein Degradation in Muscle

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>CRF</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma HCO₃</td>
<td>22±1</td>
<td>14±2*</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>119±9</td>
<td>174±11*</td>
<td>55±14*</td>
</tr>
<tr>
<td>No lysosome, no calcium</td>
<td>92±6</td>
<td>118±9*</td>
<td>26±11*</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Plasma HCO₃</td>
<td>25±1</td>
<td>20±2*</td>
</tr>
<tr>
<td>No lysosome, no calcium</td>
<td>60±5</td>
<td>84±8*</td>
<td>24±9*</td>
</tr>
<tr>
<td>ATP depletion</td>
<td>54±5</td>
<td>64±2</td>
<td>10±7</td>
</tr>
</tbody>
</table>

Values are mean±SEM of plasma bicarbonate concentration (HCO₃, mM) and protein degradation (nmol tyrosine released/g muscle per h). In Experiment 1, muscles of six pair-fed CRF and SO control rats were fixed at resting length and incubated in KRB with glucose and cycloheximide to measure the basal rate of protein degradation, and the contralateral muscle was incubated in the same media but with insulin, branched-chain amino acids, methylamine, and E64 to block lysosomal function and calcium-activated proteases. In Experiment 2, muscles of five CRF and control rats were studied to compare the rates of protein degradation when lysosomal function and calcium-activated proteases were inhibited and when the muscles were incubated with the same media but 2-deoxyglucose was substituted for glucose and DNP was added to deplete ATP (see Methods). *P < 0.05 compared with values measured in control rats by paired Student’s t test; †P < 0.05 compared with values measured in control rats by ANOVA.

Since the cell content of amino acids varies with the amount of dietary protein (24, 31), this factor may explain the higher values we obtained in epitrochlearis compared with values Tiao et al. found in extensor digitorum longus (EDL) muscles of rats fasted for 16 h (17).

Since our earlier study indicated that it was the acidosis of CRF that activated muscle proteolysis (16), we tested whether

Table II. Intracellular Tyrosine in Muscles Following Incubation under Conditions Designed to Isolate Different Proteolytic Pathways

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Control</th>
<th>CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol tyrosine/g muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal proteolysis</td>
<td>183±12</td>
<td>230±15*</td>
</tr>
<tr>
<td>No lysosome, no calcium</td>
<td>105±12</td>
<td>113±13</td>
</tr>
<tr>
<td>ATP depletion</td>
<td>92±10</td>
<td>81±14</td>
</tr>
<tr>
<td>MG 132</td>
<td>82±9</td>
<td>76±5</td>
</tr>
</tbody>
</table>

Values are mean±SEM of intracellular tyrosine content (nmol tyrosine/g muscle) in muscles of six pairs of CRF and SO pair-fed control rats after incubation in media containing KRB, glucose, and cycloheximide (basal proteolysis) or the same media without calcium but with insulin, branched-chain amino acids, and inhibitors to block lysosomal function and calcium-dependent proteases (see Methods). Muscles from six other pairs of CRF and control rats were studied after incubating in the media with inhibitors of lysosomal function and calcium-activated proteases plus 2-deoxyglucose and DNP to deplete ATP; five pairs of CRF and control rats were used to test the influence of incubating with MG 132 on intracellular tyrosine (see Methods). *P < 0.05 vs control by t test.
eliminating acidosis would change the increase in ATP-dependent proteolysis in CRF. For this study, acidotic CRF rats, CRF rats fed the NaHCO$_3$ supplement, and pair-fed control rats were compared. Protein degradation was measured in muscles incubated with methylamine and E64 in calcium-free KRB containing cycloheximide, glucose, insulin, and branched-chain amino acids to block lysosomal and calcium-activated proteases, while muscles from the contralateral limb were incubated in the same solution plus 2-deoxyglucose and 0.5 mM DNP but no glucose. In this experiment, proteolysis in muscles from acidotic CRF rats was higher than in muscles of CRF rats fed NaHCO$_3$ (Fig. 1). The rate of protein degradation in muscles of nonacidotic CRF rats was slightly but not statistically higher than the rate in pair-fed control rats. When ATP production in muscle was blocked, these differences were eliminated.

In a final experiment, MG132, an inhibitor of the proteasome (32), was used to document involvement of the ubiquitin–proteasome pathway in this catabolic response to CRF. In these studies, the mean serum bicarbonate of acidotic CRF rats was 13.2 mM vs 24.1 mM for the pair-fed control rats ($P < 0.05$). Again, acidic CRF rats had the highest rates of muscle protein degradation, and addition of MG132 eliminated the difference in rates of protein degradation between the two groups (Fig. 2). Elimination of the excessive proteolysis of CRF was not an artifact because of accumulation of tyrosine in the intracellular pool of muscle (Table II), and we found no evidence for toxic effects of MG132 on protein turnover. This conclusion is based on measurements of protein synthesis in the presence of MG 132; protein synthesis (37.5±3.8 nmol phenylalanine/g per h) in muscles of seven normal, fasted rats was similar to values measured previously (28) and did not change significantly (−6.4±3.9 nmol phenylalanine/g per h) in the contralateral muscle incubated with MG132. We also measured the ATP content in muscles after incubation. It averaged 3.43±0.32 in muscles of five control and 3.98±0.49 μmol/g in muscles of four CRF rats, which is within the range of values measured in epitrochlearis muscle by Nesher et al. (33). By ANOVA, there was no significant difference in the ATP content when MG 132 was present (3.54±0.66, control; 2.51±0.09 μmol/g, CRF).

Changes in mRNAs of the ubiquitin–proteasome system in CRF. The results of hybridizing muscle mRNA with cDNAs for ubiquitin, the C3 and C9 subunits of the proteasome, and GAPDH are shown in Fig. 3. Previously, we showed that CRF does not change GAPDH mRNA levels in rat muscle (29), and, after accounting for variations in RNA loading and transfer using GAPDH mRNA levels, we found that CRF induced...
a 324% rise in mRNA for ubiquitin (n = 5 for each group of rats). There was no increase in ubiquitin mRNA in muscles of CRF rats fed the NaHCO₃ supplement (Fig. 3). Similar results were obtained with cDNAs of the proteosome subunits (C3, 137%; C9, 251%; P < 0.05 vs control values); we found no increase in mRNA for the C2 subunit of the proteasome.

To evaluate whether the CRF-induced increase in the levels of mRNAs encoding components of the ubiquitin–proteasome pathway involves an increase in gene transcription, nuclear run-on experiments were performed using nuclei isolated from muscle and liver of CRF or pair-fed control rats. There was no significant difference in the quantity of GAPDH or γ-actin mRNAs transcribed from nuclei isolated from muscles or livers of CRF and control rats. In contrast, CRF was associated with an increase in transcribed ubiquitin and C3 proteasome subunit mRNAs in muscle. A representative blot is shown in Fig. 4. In liver, CRF was not associated with an increase in the transcription of GAPDH, γ-actin, ubiquitin, or C3 genes (data not shown).

Discussion

Our results provide evidence that the metabolic acidosis of CRF stimulates ATP-dependent proteolysis in muscle and, more specifically, proteolysis involving the proteasome, because the inhibitor of proteasome activity blocked this response. Moreover, metabolic acidosis in CRF is associated with an increase in the muscle content of mRNAs encoding ubiquitin and subunits of the proteasome. These responses were reversed by feeding chow mixed with NaHCO₃ to correct the acidosis of CRF, pointing to acidification as a stimulus for these responses. Finally, we investigated the mechanisms for the higher levels of mRNAs encoding components of the ubiquitin–proteasome system and found coordinated increases in transcription of these genes in muscle. It is tempting to speculate that the higher levels of mRNAs encoding elements of this proteolytic system occurring in muscle in other catabolic conditions (e.g., sepsis, cancer, fasting, etc.) also arise from increased transcription, but this has not been shown experimentally. Thus, our results indicate that the metabolic acidosis of CRF activates a specific catabolic pathway in muscle and the transcription of genes encoding components of the pathway.

These responses could compromise the ability of the organism to maintain muscle mass (34).

The first set of experiments included muscles in which lysosomal and calcium-dependent proteolytic pathways were inhibited so the relative contribution of ATP-independent and ATP-dependent proteolytic systems could be evaluated. The decrease in protein degradation associated with inhibition of lysosomal function and calcium-activated proteases was greater in muscles of CRF compared with SO pair-fed rats. By unpaired Student’s t test, this difference was not statistically significant, but assessing the contribution of lysosomes and calcium-activated proteases by evaluating differences in proteolytic activity could obscure some influence of CRF on these pathways. However, results in Figs. 1 and 2 and Table I indicate that the major contributor to excess proteolysis is activation of the ATP–ubiquitin–proteasome pathway. Since the residual rates of protein degradation in muscles of CRF and control rats did not differ significantly after inhibition of ATP production, we conclude that CRF stimulates ATP-dependent proteolysis. These results suggest that even a modest degree of acidification in CRF is deleterious because this pathway was stimulated even in CRF rats with an average serum bicarbonate of 20 mM.

The initial experiments did not identify which pathway is involved in the accelerated ATP-dependent proteolysis, although our earlier results in acidemic rats suggested it was likely to be the ubiquitin–proteasome system (15). The addition of an inhibitor of proteasome function, MG 132, provides more direct evidence for activation of this system because it eliminated the CRF-induced increase in muscle protein degradation. Like other peptide aldehyde inhibitors, MG 132 readily crosses cell membranes and inhibits the proteolytic activity of both 20S and 26S proteasomes (the latter form degrades ubiquitin–protein conjugates) (32, 35–37). The specificity of the reaction with an inhibitor (including the reaction between MG 132 and the proteasome) is always a potential problem, but this class of compounds has been used to demonstrate that the proteasome is involved in regulating the cell cycle, the processing of the cystic fibrosis transmembrane conductance regulator, and in antigen processing in macrophages (32, 36–38). In muscles from control rats, MG 132 reduced protein degradation sharply, which is consistent with the suggestion that the 20S and 26S proteasome complexes are responsible for the turnover of the bulk of protein in cells (e.g., normal muscle) (32). The apparent reduction in the rate of protein degradation below the level achieved by ATP depletion could represent degradation in the 20S proteasome that can function without ATP since this class of inhibitor will block both the 20S and 26S form of the proteasome (32, 36, 37).

Increased levels of mRNAs encoding ubiquitin and components of the proteasome in muscles of CRF acidicotic rats compared with pair-fed CRF rats given NaHCO₃ or SO control rats provide additional evidence for activation of the ubiquitin–proteasome pathway (Fig. 3). These responses cannot be attributed to dietary differences, as all groups were pair fed and gained weight (albeit at different rates). Furthermore, gene transcription for ubiquitin and the C3 proteasome subunit was increased in muscle while that for γ-actin and GAPDH was unchanged; this was consistent with the CRF-induced changes in mRNAs. Similar changes in gene transcription were not detected in liver from CRF rats, suggesting that this catabolic response appears to be specific for muscle and...
not part of a generalized response to stress. Additional support for this conclusion is given by our finding that muscle DNA and RNA contents are unaltered by CRF (29).

Do these changes in protein degradation, the mRNA levels of ubiquitin and subunits of the proteasome, and gene transcription occur in all types of skeletal muscle? Unfortunately, the amount of muscle and the methods required to measure these responses in the same muscle prevented us from arriving at an easy answer. However, we predict that these changes occur in most types of skeletal muscle for the following reasons: (a) The epitrochlearis muscle we used to measure protein degradation and the gastrocnemius used for RNA analyses contain both red and white types of muscle fiber (33, 39), and protein degradation in mixed-fiber muscles responds similarly to fasting and acute uremia (28, 40); (b) we found that mRNAs of ubiquitin and proteasome subunits in the predominantly white-fiber EDL and mixed-fiber gastrocnemius muscles increase in response to acidemia and glucocorticoids (41); and (c) in response to another catabolic condition, starvation, protein degradation and mRNAs of components of the ubiquitin–proteasome system are increased in EDL and in the red-fiber soleus muscle of rats (30, 42).

Taken together, our results suggest that the loss of muscle mass associated with CRF is related to up-regulation of the ATP–ubiquitin–proteasome–dependent pathway. What is the signal for these responses? Our previous work suggests that metabolic acidosis in CRF does not cause a sustained alteration in the intracellular pH of muscle (43). On the other hand, we did find that induction of acidosis by feeding NH4Cl to adrenalectomized rats is not sufficient by itself to activate protein degradation or increase mRNAs of the ubiquitin–proteasome pathway in muscle (14, 41). In both cases, glucocorticoids must be provided, but the same dose of glucocorticoids given to nonacidotic adrenalec tomized rats does not cause these responses. Presumably, glucocorticoids are also involved in the response to CRF since the steady-state production of corticosterone (measured as its excretion) by CRF rats with or without supplemental NaHCO3 exceeds that of pair-fed control rats (16). Moreover, in CRF patients, Garibotto et al. (44) found a strong positive correlation between muscle protein degradation and plasma levels of cortisol but a negative correlation with serum bicarbonate, suggesting that both acidosis and glucocorticoids are needed to stimulate muscle protein degradation.

In summary, these results provide evidence that CRF activates the ubiquitin–proteasome proteolytic pathway. The primary catabolic stimulus was shown to be a response to acidification rather than the accumulation of other waste products. Several catabolic conditions (e.g., burns, sepsis, cancer, etc.) are associated with increased levels of mRNAs encoding proteins of the ubiquitin–proteasome system. Understanding the mechanisms for activation of the pathway and increased transcription of these genes could lead to more effective therapy for conditions like CRF, which are associated with loss of muscle mass.

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