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The International Journal of Biochemistry & Cell Biology 37 (2005) 2147–2155
IJBCB
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Review

Cellular signals activating muscle proteolysis in chronic kidney disease: A two-stage process

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Received 29 November 2004; received in revised form 8 March 2005; accepted 22 March 2005

Abstract

Muscle atrophy is a prominent feature of catabolic conditions and in animal models of these conditions there is accelerated muscle proteolysis that is dependent on the ubiquitin–proteasome system. However, ubiquitin system cannot degrade actomyosin or myofibrils even though it rapidly degrades actin or myosin. We identified caspase-3 as the initial and potentially rate-limiting proteolytic step that cleaves actomyosin/myofibrils. In rodent models of catabolic conditions, we find that caspase-3 is activated to cleave muscle proteins and actomyosin to fragments that are rapidly degraded by the ubiquitin system. This initial proteolytic step in muscle can be recognized because it leaves a footprint of a characteristic 14-kDa actin band. Stimulation of caspase-3 activity depends on activation of phosphatidylinositol 3-kinase. When we suppressed this enzyme in muscle cells, protein breakdown increased as did the expression of caspase-3. In addition, there was increased expression of E3-ubiquitin-conjugating enzymes that are involved in muscle proteolysis, atrogin-1/MAFbx and MuRF1. Thus, when phosphatidylinositol 3-kinase activity is low in muscle cells or rat muscle, both caspase-3 and the ubiquitin–proteasome system are stimulated to degrade protein. Additional investigations will be needed to define the cell signaling processes that activate muscle proteolysis in uremia and catabolic conditions.

Keywords: Uremia; Caspase; Ubiquitin; Proteasome; Skeletal muscle

Contents

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doi:10.1016/j.biocel.2005.03.012

1. Introduction

Over the past decade, results from studies of animal models that mimic human diseases have demonstrated that models characterized by loss of muscle mass have common properties. Among these shared properties is an acceleration of muscle protein degradation that is carried out by the ubiquitin–proteasome proteolytic (Ub-P'some) system. The increase in proteolysis is accompanied by increased transcription of genes encoding components of the Ub-P'some system and other genes that are not so clearly linked to muscle protein degradation (Mitch & Goldberg, 1996). Many of these animal models also express increased levels of circulating cytokines and/or insulin resistance (DeFronzo & Beckles, 1979; DeFronzo, Tobin, Rowe, & Andres, 1978; DeFronzo, 1979; Festa et al., 2000; Flakoll, Wentzel, & Hyman, 1995; Lecker, Solomon, Mitch, & Goldberg, 1999; Mitch & Goldberg, 1996). Taken together, these results suggest a final common pathway that accelerates the loss of muscle mass in "catabolic conditions" and raise the possibility that there may be a therapeutic intervention or group of interventions that could successfully intervene to reduce muscle loss. For these reasons, it is important to identify signals that result in muscle atrophy and the mechanisms by which these signals activate proteolysis. We have investigated specific models that exhibit characteristic problems associated with chronic kidney disease (CKD) and the results suggest that signals and mechanisms that are present in CKD are also active in other conditions.

2. Chronic kidney disease and protein metabolism

In patients with CKD, evidence for a decrease in protein stores is commonly present; virtually every survey of dialysis patients reveals a low serum albumin plus weight loss and decreased muscle mass. These abnormalities are often present before dialysis therapy begins and they contribute to excessive morbidity and mortality (Avram & Mittman, 1994; Ikizler & Hakim, 1996; Kopple, 2001; Qureshi et al., 1998). In CKD patients, the loss of protein stores has been attributed to "malnutrition" but several lines of evidence indicate that this conclusion is incorrect (Mitch, 2002). Malnutrition is defined as disorders that are caused by an inadequate or abnormal diet but the mechanisms causing loss of muscle mass in CKD are more complicated. Regarding hypoalbuminemia, it is clear that malnutrition is not a major cause of this abnormality in CKD patients. Young women with anorexia nervosa have normal values of plasma albumin concentration despite substantial loss of body weight and muscle mass, indicating that more than an inadequate diet is required to cause hypoalbuminemia (Smith, Robinson, & Fleck, 1996). Second, serial examinations of hemodialysis patients reveal that the development of hypoalbuminemia is most closely linked to the presence of inflammation or metabolic acidosis while an inadequate protein intake plays a minor role (Kaysen et al., 2004; Movilli et al., 1998). The mechanisms causing loss of muscle protein are also more complex than the result of an inadequate diet. CKD is associated with several metabolic abnormalities that stimulate protein degradation (Mitch, 2002). For example, metabolic acidosis is a prominent feature of CKD and in infants, children, adults and elderly subjects with normal kidney function, metabolic acidosis causes loss of muscle mass (Frassetto, Morris, & Sebastian, 1997; Kalhoff, Diekmann, Kunz, Stock, & Manz, 1997; McSherry & Morris, 1978; Reaich, Channon, Scrimgeour, & Goodship, 1992). The mechanism by which acidosis causes loss of muscle mass in CKD patients involves stimulation of protein degradation plus the irreversible oxidation of essential, branched-chain amino acids (Graham et al., 1996; Graham, Reaich, Channon, Downie, & Goodship, 1997; Lofberg, Wernerman, Anderstam, & Bergstrom, 1997; Price, England, Bailey, Van Vreede, & Mitch, 1994; Reaich et al., 1993; Stein et al., 1997).

In addition to acidosis, another complication of CKD is insulin resistance and a decrease in insulin activity could increase protein breakdown because suppression of protein degradation is a principal response to insulin in humans (Louard, Fryburg, Gelfand, & Barrett, 1992; Nair, Ford, & Halliday, 1987). Indeed, models of insulin deficiency stimulate the Ub-P'some system in muscle leading to accelerated muscle atrophy (Mitch et al., 1999; Price et al., 1996). A third mechanism causing loss of protein stores is that the dialysis procedure itself can stimulate protein degradation in muscle (Ikizler et al., 2002; Pupim et al., 2002). The increase in muscle protein degradation persists for at least several hours after the dialysis has finished. Finally, there is evidence that CKD or uremia are associated with increased levels of circulating inflammatory cytokines (Pereira et al., 1994) and these cytokines might stimulate catabolism directly or indirectly (Goodman, 1991, 1994).

It should be pointed out, however, that there is no agreement about the effects of uremia and dialysis on protein turnover (Ikizler et al., 2002; Lim, Yarasheski, & Flanigan, 1995). Regardless, it is important to recognize that a small change in protein metabolism will lead to marked losses of protein stores if it persists for weeks or months (see below). In summary, there are several abnormalities associated with CKD or uremia that can stimulate protein degradation directly or indirectly. The result is loss of muscle mass. Importantly, several of these abnormalities (e.g., depressed insulin action, increased cytokine levels, acidosis, etc.) are present in other catabolic conditions and hence could be the stimulus causing protein losses.

3. Mechanisms that cause protein losses

Evidence that kidney failure causes loss of protein stores includes the common presence of hypoalbuminemia plus abnormal anthropometric measurements but the mechanisms by which CKD causes loss of muscle mass are controversial. It is important to note that these mechanisms may be active even when a patient seems relatively stable clinically, resulting in substantial losses of protein stores. This is possible because there is a very high rate of protein turnover even in healthy adults: the daily rates of protein synthesis and degradation are equivalent to the protein contained in approximately 1–1.5 kg of muscle mass (Mitch & Goldberg, 1996). Consequently, even a small decrease in protein synthesis and/or increase in protein degradation will cause substantial losses of protein stores if the defect in protein turnover persists over weeks or months.

3.1. The ubiquitin–proteasome system

What proteolytic systems are responsible for muscle atrophy in catabolic conditions? Results from studies of rodent models of human conditions indicate that the muscle atrophy associated with chronic kidney disease (CKD) and many other conditions (e.g., insulin deficiency, burn injury, cancer, sepsis, etc.) clearly involves stimulation of the Ub-P'some system (Mitch & Goldberg, 1996). The evidence for participation of the Ub-P'some system arises from three findings: first, inhibition of the Ub-P'some system in muscle blocks the accelerated protein degradation that is caused by uremia, diabetes, sepsis, denervation or hyperthyroidism (Bailey et al., 1996; Price et al., 1996; Tawa, Odessey, & Goldberg, 1997). Second, these conditions are characterized by increased levels of the mRNAs encoding components of the Ub-P'some system in muscle (Lecker et al., 2004; Mitch & Goldberg, 1996). Third, overexpression of one component of the Ub-P'some system (i.e., the E3 ubiquitin-conjugating enzyme, atrogin-1, also known as MAFbx, see below) causes accelerated proteolysis in muscle cells (Bodine et al., 2001). Even though it is not possible to conduct such precise measurements in humans suffering from catabolic conditions, there is evidence that the Ub-P'some system is activated in muscle of patients with uremia as well as trauma, sepsis or cancer (Mansoor et al., 1996; Pickering et al., 2002; Tiao et al., 1997; Williams, Sun, Fischer, & Hasselgren, 1999.). Clearly, involvement of this proteolytic system in each of these conditions suggests there could be a common signal in these disorders that activates the Ub-P'some system to cause muscle protein losses.

Because the Ub-P'some system is responsible for degrading the bulk of protein in all cells and also degrades transcription factors and enzymes that regulate the cell cycle and metabolic processes (Ciechanover, 1994; Coux, Tanaka, & Goldberg, 1996; Rock et al., 1994), the biochemical processes of this system have been extensively investigated. Briefly,

ubiquitin is a member of the heat-shock family of proteins and is present in all cells. Ubiquitin is activated by an ATP-requiring reaction using an E1 ubiquitin-conjugating enzyme. In the next step of the proteolytic process, ubiquitin is transferred to one of about 40 members of the E2 family of ubiquitin-conjugating system. Activated ubiquitin is then joined covalently to a substrate protein in a transfer process that involves a specific E3 enzyme. There is a large family of E3 ubiquitin-conjugating enzymes because the E3 enzyme yields specificity of the ubiquitin conjugating reaction; a substrate protein will be recognized by a specific E3 enzyme and ubiquitin is transferred to a lysine in the substrate protein. The ubiquitin conjugating process is repeated until a chain of at least four ubiquitin molecules is joined to the substrate proteins, the minimum needed for increased recognition and degradation of the substrate by the 26S proteasome (Thrower, Hoffman, Rechsteiner, & Pickart, 2000). In another ATP-requiring reaction, the ubiquitins are removed and the substrate protein is degraded.

This property of the E3 enzymes to confer specificity of the ubiquitin conjugating reaction is emphasized because recent reports indicate that two E3 enzymes (i.e., atrogin-1/MAFbx and MuRF) have a special role in the degradation of muscle proteins. Both enzymes are expressed at a high level (atrogin-1/MAFbx expression rises ∼20-fold) in muscle of rats with starvation, uremia, acute insulin deficiency or cancer (Lecker et al., 2004; Lee et al., 2004). Second, when atrogin-1/MAFbx was overexpressed in myotubes, protein breakdown increased sharply. But, when the gene encoding atrogin-1/MAFbx was knocked out in mice, the muscle atrophy that usually follows muscle denervation was inhibited by ∼50% while knockout of the MuRF1 gene suppressed muscle atrophy in this model by 36% (Bodine et al., 2001). These results point out that upregulation of expression of components of the Ub-P'some system in several conditions play a critical role in determining the acceleration of muscle protein loss.

3.2. Caspase-3 and muscle proteolysis

The Ub-P'some system alone cannot explain muscle the protein losses that occur in catabolic conditions. Solomon and Goldberg (Solomon & Goldberg, 1996) reported that the reconstituted Ub-P'some system will rapidly degrade actin or myosin but cannot breakdown actomyosin or myofibrils. Thus, another protease must initially breakdown the complex structure of muscle protein before the Ub-P'some system can degrade it fully. Identifying this initial step in muscle protein breakdown is necessary before we can understand the mechanisms that lead to muscle atrophy. As noted earlier, this initial proteolytic system and the Ub-P'some system should be activated by the same signals and identifying the initial proteolytic system and signals activating it could provide therapeutically important insights. Regarding therapeutics, if an initial step in breaking down the complex structure of muscle were not blocked, catabolic conditions could still cause loss of muscle structure and function even when the Ub-P'some system was inactive.

How is muscle structure initially disrupted? Because several catabolic conditions are characterized by high circulating levels of inflammatory cytokines or insulin resistance, we decided to study proteases that are activated by cytokines or a decrease in insulin action. In vitro, we found that caspase-3 cleaves actomyosin yielding a "footprint" of its action, a characteristic, 14 kDa actin fragment (Du et al., 2004). The experiments were extended to cells and animals and we found that caspase-3 breaks down the complex structure of myofibrils in cultured muscle cells and in muscles of rats with uremia or insulin deficiency. Caspase-3 activity yields a 14 kDa actin fragment that can be found in the insoluble fraction of muscle (Fig. 1). In cultured myocytes, this fragment is quickly degraded by the Ub-P'some system but when protein degradation in skeletal muscle is increased, this fragment accumulates and serves as a marker of caspase-3 activity. In fact, we found that this footprint is not only found when there is accelerated protein breakdown in muscles of rats with uremia or insulin deficiency but also in muscles of humans with muscle atrophy from burn injury or aging (Du et al., 2004; Lee et al., 2004; Rondon-Berrios et al., 2004). Finally, we showed that inhibition of caspase-3 slows the rate of muscle protein breakdown occurring in response to insulin deficiency. In summary, two consecutive processes are activated to degrade muscle protein in uremia and other catabolic conditions; caspase-3 initially breaks up actomyosin/myofibrils and the activated Ub-P'some system then degrades the products of this cleavage into small peptides that are degraded to amino acids by peptidases.

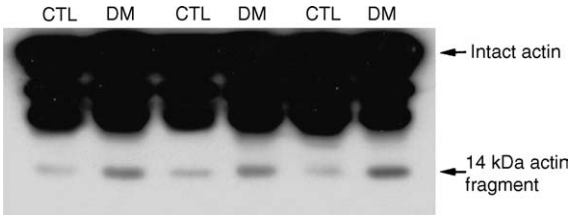

Fig. 1. The 14 kDa actin fragment, a footprint of caspase-3 activity on myofibrils and actomyosin, accumulates in muscle of rats with insulin deficiency. Muscles from rats with uremia or insulin deficiency and from control rats were homogenized in a grinding buffer consisting of 10 ml of Dulbecco's phosphate buffered saline without added calcium or magnesium plus 1 tablet of protease inhibitors (Roche Diagnostics). About 50 mg tissue is added to 1 ml of this buffer (ratio of 20:1 buffer to muscle weight) and the mixture is ground for 5 min while the tube is kept in ice. About 100 μl of the mixture is centrifuged at 3000 × *g* at 4 °C for 5 min and the supernatant is discarded. The pellet is mixed with 100 μl of 2× SDS buffer and mixed well before boiling for 20 min and then centrifuging at 16,000 × *g* for 5 min at room temperature. This releases the fragments from the insoluble muscle fibrils. About 15–20 μl of supernatant is separated on 15% SDS-PAGE using 100 V and detected by western blotting using the Sigma (A-2066) anti-actin antibody as the primary antibody.

3.3. Calcium-activated proteases

It has been suggested that activation of calcium-activated proteases could be the trigger that initiates the breakdown of muscle protein. For example, Huang and Forsberg (Huang & Forsberg, 1998) reported that overexpression of endogenous inhibitors of calpain in skeletal muscle cells suppressed the increase in protein breakdown that is stimulated by serum withdrawal and Tidball and Spenser (Tidball & Spencer, 2002) found that overexpression of calpastatin decreases the degree of muscle atrophy occurring because of disuse. In studies of a model of sepsis in rats, others reported that there is rupture of the Z band in muscle which can be blocked by dantrolene (Williams et al., 1999). On the other hand, we have found that the accelerated muscle atrophy induced by uremia or insulin deficiency or other catabolic conditions cannot be attributed to calcium-activated proteases (Bailey et al., 1996; Mitch & Goldberg, 1996; Price et al., 1996). We also found that inhibiting calpain activity does not block the muscle proteolysis induced by insulin deficiency (Du et al., 2004).

4. Cellular signals activating muscle protein degradation

Stitt et al. (Stitt et al., 2004) reported that activation of the phosphatidylinositol 3 kinase (PI3K)/Akt pathway suppresses the activation of both atrogin-1/MAFbx and MuRF1. Activation of these E3 enzymes depended on inhibition of the forkhead family of transcription factors (FOXO) and specifically, the FOXO1 factor. They also showed that activation of Akt by injecting IGF-1 into the muscles of mice led to reduced muscle protein degradation that follows stimulation by glucocorticoid treatment or muscle denervation. Sandri et al. (Sandri et al., 2004) and Sacheck et al. (Sacheck, Ohtsuka, McLary, & Goldberg, 2004) also reported that activation of the PI3K/Akt in muscle cells will reduce the expression of atrogin-1/MAFbx in cultured myocytes by a FOXO-dependent mechanism but in their studies, FOXO3 seemed to be the key factor regulating expression of atrogin-1/MAFbx. When they blocked FOXO3 activation, the expression of atrogin-1/MAFbx and the muscle atrophy that was stimulated by starvation was suppressed.

We studied the response to and consequences of insulin deficiency in muscle of rats; there was suppression of both PI3K and Akt activities in muscle and an increase in atrogin-1/MAFbx of almost 20-fold (Lee et al., 2004). We showed that the increase in atrogin-1 expression could be attributed to interaction of the FOXO transcription factor with the atrogin-1 promoter. In summary, all three reports lead to the conclusion that suppression of the PI3K/Akt cellular signaling pathway will stimulate activity of the Ub-P'some system leading to muscle atrophy.

As noted muscle protein degradation requires two stages; activation of caspase-3 and the Ub-P'some system. This led us to explore whether suppression of the PI3K/Akt pathway would stimulate caspase-3 activity to breakdown the complex structure of muscle. In rats with accelerated muscle proteolysis from insulin deficiency (Price et al., 1996; Mitch et al., 1999), we found that the pro-apoptotic factor, Bax, is activated without an increase in Bax production (Lee et al., 2004). We then showed there was a link between Bax activation and the PI3K pathway because we found that inhibition of the PI3K pathway in cultured muscle cells activated Bax. The mechanism for activation of caspase-3 activity involved Bax-induced release of cytochrome C from

mitochondria leading with an increase in the cytosolic level of cytochrome C. Finally, we found that caspase-3 is activated in muscle of rats with insulin deficiency and in muscle there was the expected increase in the level of the footprint of caspase-3 activity, the 14 kDa actin fragment (Fig. 1).

In exploring the importance of the PI3K signaling pathway in regulating muscle protein turnover, we recently evaluated this cellular signaling pathway by studying mice with partial deficiency of PTEN, the phosphatase that inactivates the active product of PI3K (Hu, Du, & Mitch, 2004). As expected, partial PTEN deletion leads to an increase in activated Akt. In addition, partial deficiency of PTEN sharply limits the accelerated muscle proteolysis that occurs with insulin deficiency.

Glucocorticoids (GC) are also involved in the activation of muscle protein degradation and recent evidence emphasizes that they influence muscle protein metabolism through complex mechanisms. In response to a chronic infusion of angiotensin II (angII), rats not only become hypertensive but also develop anorexia and increased muscle protein degradation (Brink et al., 2001). As with other catabolic conditions, we found that angII-induced loss of muscle protein is related to activation of caspase-3 and the Ub-P'some system in muscle (Song et al., 2005). Interestingly, the increase in muscle protein degradation could be blocked by two strategies: inhibiting caspase-3 activity and blocking the glucocorticoid receptor. An involvement of GC in producing accelerated muscle proteolysis was sought because angII stimulates GC production and GC are necessary for the accelerated protein degradation that occurs in other catabolic conditions.

Earlier studies have provided evidence that GC play a prominent role in mediating muscle protein breakdown. First, treatment of animals or cultured cells with high doses of GC increases muscle protein degradation via processes that involve the Ub-P'some system (Sacheck et al., 2004; Sandri et al., 2004; Stitt et al., 2004). Second, elimination of GC by removing the adrenal glands or treating animals with an inhibitor of the GC receptor prevents the stimulation of the Ub-P'some system and the increase in muscle protein breakdown that is induced by acidosis, insulin deficiency, starvation or sepsis (Hall-Angeras, Angeras, Zamir, Hasselgren, & Fischer, 1991; May, Kelly, & Mitch, 1986; May, Bailey, Mitch, Masud, & England, 1996; Mitch et al., 1999; Price et al., 1994; Wing & Goldberg, 1993). These experiments show that GC have a permissive effect on stimulating muscle protein degradation since both activation of the Ub-P'some system and the degradation of muscle protein are restored if high but physiologic doses of GC are given to the adrenalectomized animals with acidosis or insulin deficiency. The conclusion is that GC are necessary but not sufficient to stimulate the Ub-P'some system or muscle protein degradation. The mechanism by which GC exerts the permissive response is unknown.

5. Muscle atrophy caused by kidney disease

To return to kidney disease, there is abundant evidence that patients with CKD or uremia have decreased protein stores. There also is evidence from animal models of uremia that caspase-3 is activated in muscle and that the Ub-P'some system participates in the muscle atrophy that occurs in animals and patients with kidney failure. Indirect evidence indicates that the cell signaling processes present in other catabolic conditions are also active in muscle of uremic animals. For example, uremia stimulates GC production that exerts a permissive action in increasing muscle protein degradation (May, Kelly, & Mitch, 1987). Second, we find that the activity of PI3K/Akt is low in muscle of uremic rats (Bailey, Price, & Mitch, 2002). The mechanism that suppresses muscle PI3K activity in uremia has not been identified but at least two factors associated with uremia could be responsible, insulin resistance and metabolic acidosis (Franch et al., 2004; Nandi, Kitamura, Kahn, & Accili, 2004). Finally, increased cytokine levels are present in many CKD patients and could cause muscle protein losses. Presumably cytokine-induced proteolysis would involve the same pathways because animal models of sepsis, etc., exhibit increased protein breakdown in the Ub-P'some system (Mitch & Goldberg, 1996).

6. Conclusions and future directions

Muscle protein degradation in catabolic conditions is regulated by a complicated interplay between organs that include neuroendocrine tissues, adrenal glands, adipose tissue, the liver and muscle in addition to

circulating factors such as cytokines, angiotensin II, glucocorticoids and growth factors. In kidney diseases and other catabolic conditions, muscle atrophy occurs because there is stimulation of two proteolytic pathways that function in tandem: caspase-3 and the Ub-P'some system. The mechanisms regulating these responses are complex and involve intracellular signaling processes such as PI3K, Akt, and transcription factors such as those in the forkhead family or NF-κB.

An important goal that stimulates the study of mechanisms that cause muscle atrophy is that understanding mechanisms will lead to the development of therapeutic strategies. For example, there is evidence that stimulation of the IGF-1 pathway can improve muscle mass in models of muscle wasting that include aging or muscular dystrophy (Barton, Morris, Musaro, Rosenthal, & Sweeney, 2002; Barton-Davis, Shoturma, Musaro, Rosenthal, & Sweeney, 1998). These results suggest that it is possible that other growth factors would exert similar benefits. There also is evidence that strategies that lead to stimulation of the PI3K/Akt cellular signaling pathway could prevent progressive muscle wasting. Other strategies that could be developed involve blocking caspase-3 or the E3 ubiquitin-conjugating enzymes, atrogin-1/MAFbx or MuRF1. The prediction would be that inhibiting the activity of these enzymes would prove useful in preventing or combating muscle protein losses. Until these strategies are clinically available, less exotic means can and should be used to prevent muscle loss in CKD patients, including elimination of metabolic acidosis and treating infections or diseases that could release inflammatory cytokines.

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