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Muscle wasting from kidney failure—a model for catabolic conditions

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Abstract

Purpose—Muscle atrophy is a frequent complication of chronic kidney disease (CKD) and is associated with increased morbidity and mortality. The processes causing loss of muscle mass are also present in several catabolic conditions. Understanding the pathogenesis of CKD-induced muscle loss could lead to therapeutic interventions that prevent muscle wasting in CKD and potentially, other catabolic conditions.

Major findings—Insulin or IGF-1 resistance caused by CKD, acidosis, inflammation, glucocorticoids or cancer causes defects in insulin-stimulated intracellular signaling that suppresses IRS-1 activity leading to decreased phosphorylation of Akt (p-Akt). A low p-Akt activates caspase-3 which provides muscle proteins substrates of the ubiquitin-proteasome system (UPS). A low p-Akt also leads to decreased phosphorylation of forkhead transcription factors which enter the nucleus to stimulate the expression of atrogen-1/MAFbx and MuRF1, E3 ubiquitin ligases that can be associated with proteolysis of muscle cells by the UPS. Caspase-3 also stimulates proteasome-dependent proteolysis in muscle.

Summary—in CKD, diabetes, inflammatory conditions or in response to acidosis or excess glucocorticoids, insulin resistance develops, initiating reduced IRS-1/PI3K/Akt signaling. In CKD, this reduces p-Akt which stimulates muscle proteolysis by activating caspase-3 and the UPS. Second, caspase-3 cleaves actomyosin yielding substrates for the UPS and increased proteasome-mediated proteolysis. Third, p-Akt down-regulation suppresses myogenesis in CKD. Fourth, exercise in CKD stimulates insulin/IGF-1 signaling to reduce muscle atrophy. Lastly, there is evidence that microRNAs influence insulin signaling providing a potential opportunity to design therapeutic interventions.

Keywords

kidney failure; diabetes; cancer; acidosis; inflammation; glucocorticoid excess

Introduction

Patients with chronic kidney disease (CKD) have an increased risk of mortality and morbidity which is associated with losses of body fat and lean mass (Carrero et al., 2008).

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Two patient groups are at even higher risk of morbidity and mortality, elderly subjects with CKD and those being treated by dialysis. But, the development of muscle wasting is not limited to abnormalities induced by CKD, the pathogenesis of muscle protein losses in CKD is similar to that arising from other chronic diseases (e.g., cancer, diabetes, etc.) (Doyle et al., 1998).

In patients with CKD, the most frequently used marker of depleted protein stores is hypoalbuminemia and some investigators have concluded that this means that patients with CKD suffer from protein malnutrition (Carrero et al., 2008; Getz and Reardon, 2004; Kelly and Garavan, 2005). This is unfortunate because malnutrition is defined as abnormalities caused by an insufficient amount of food eaten or to an imbalance of nutrients. Consequently, if inadequate dietary protein was the cause of signs of protein malnutrition in patients with CKD, then these associations should be reversed by simply increasing the amount of food eaten by these patients (Mitch, 2002). Unfortunately, CKD-induced hypoalbuminemia and lost protein stores are not generally corrected by raising protein intake. For example, estimates of the protein eaten by CKD patients are above values recommended by the World Health Organization (Berry et al., 2013; Karlsson et al., 2012). Second, providing dietary supplements have not corrected hypoalbuminemia or lost protein stores in patients with CKD. Perhaps the signs of protein depletion might be ameliorated by increasing dietary calories but this has not been systematically evaluated. In short, assigning the risks of morbidity and mortality documented in patients with kidney disease to protein malnutrition is not helpful. Instead available evidence indicates that the metabolic abnormalities caused by CKD are the result of mechanisms that are not eliminated by solely altering the diet (Mitch and Goldberg, 1996; Olsburgh et al., 2013; Thomas et al. 2013).

What physiologic abnormalities initiate loss of muscle mass in CKD? In rodents with experimentally-induced uremia, we identified that the metabolic acidosis that generally complicates CKD stimulates the breakdown of muscle proteins resulting in loss of muscle mass (Hu et al., 2013). In this study, uremia was created in rats or mice by subtotal nephrectomy and feeding a high protein diet; the latter resulted in rats with a blood bicarbonate level of 16 mM and the blood urine nitrogen (BUN) above 80 mg/dL, signifying accumulation of unexcreted waste products. Results from uremic rats were compared to those obtained from sham-operated, pair-fed control rats or from pair-fed, uremic rats that had sodium bicarbonate (NaHCO₃) mixed in their diet. In uremic, acidotic rats, there was a sharp increase in the rate of muscle protein degradation compared to results from control rats and this catabolic condition was eliminated by NaHCO₃ to correct acidosis. Subsequently, it was demonstrated that metabolic acidosis stimulates protein degradation in patients with CKD who were not being treated by dialysis and those being treated by hemodialysis or peritoneal dialysis (Graham et al., 1996; Graham et al., 1997; Wu et al., 2012). In one report patients were treated by peritoneal dialysis throughout a year-long evaluation when metabolic acidosis was fully corrected. In non-acidotic patients, body weight and estimated muscle mass increased and there was reduced hospitalization of the non-acidotic patients (Hammad et al., 2007).

The cellular mechanisms mediating protein degradation were subsequently determined to involve the ubiquitin-proteasome system (UPS) (Bailey et al., 1996; Isozaki et al., 1996; Mitch et al., 1994). The importance of the UPS in mediating biological processes was recognized by awarding the Nobel Prize in Chemistry to Avram Hershko, Aaron Ciechanover and Irwin Rose (<http://nobelprize.org/chemistry/laureates/2004/>). Besides increasing protein degradation, the activation of the UPS was accompanied by increased levels of the mRNAs that encode different components of the UPS. An increase in ubiquitin (Ub) following correction of acidosis demonstrated that suppression of the activity of the UPS in muscles (Pickering et al., 2002). How does acidosis activate protein degradation in

the UPS? To address this question, we examined rats with acute acidosis or uremia with chronic acidosis (Bailey et al., 1995). The goal of these experiments was to determine if intracellular pH measured by NMR decreased in response to acidosis. If so, it would imply that hydrogen ions activate a potential receptor stimulating the UPS to initiate the highly coordinated process of protein degradation and the transcription of genes encoding components of the UPS. There were no statistically significant differences in the intracellular pH of uremic rats that had the same characteristics as those uremic, acidotic rats that were exhibiting accelerated breakdown of muscle protein by the UPS (Bailey et al., 1996). This negative result stimulated us to search for other signals that would stimulate protein degradation in muscle.

The stimulus for the loss of muscle mass in CKD is frequently attributed to inflammation because CKD is associated with an increase in circulating levels of inflammatory cytokines (Miyamoto et al., 2011; Stenvinkel et al., 2005). However, the mechanism by which inflammatory cytokines cause losses of muscle mass has been difficult to establish. In rats, it was shown that treatment with IL-6 stimulates muscle protein degradation but the intracellular pathways that cause loss of protein stores and muscle mass was not identified (Goodman, 1994). Subsequently, it was shown that inflammation can cause defects in the intracellular signaling that is initiated by insulin or IGF-1 (Shoelson et al., 2006). A mechanism from inflammation to impaired insulin signaling in muscle to protein degradation was identified during evaluations into the mechanism by which angiotensin II, a stimulus for inflammation causes protein degradation in muscle (Song et al., 2005). Angiotensin II stimulates production of IL-6 and the acute phase protein, serum amyloid A (Zhang et al., 2009). These factors activate the suppressor of cytokine signaling3 (SOCS3) to phosphorylate Serine307 of the insulin receptor substrate-1 (IRS-1). An increase in phosphorylated Serine307 of IRS-1 was shown to lead to the degradation of IRS-1, causing interference with insulin/IGF-1 intracellular signaling. In fact, insulin-induced intracellular signaling is impaired not only by angiotensin II but also by CKD, metabolic acidosis or inflammation, resulting in acceleration of muscle protein breakdown.

CKD Stimulates Proteolytic Mechanisms in Muscle

The majority of intracellular proteins in all tissues are degraded by the UPS (Rock et al., 1994). However, other proteolytic processes contribute to loss of muscle mass, including the uptake and degradation of certain extracellular or cell surface proteins or some cytosolic proteins by endocytosis into autophagic vacuoles that fuse with lysosomes (Lecker and Mitch, 2011; Mitch and Goldberg, 1996). In lysosomes, proteins are degraded by several acid-optimal proteases, including cathepsins B, H, L and D, and other acid hydrolases (Lecker et al., 2004). Besides lysosomal proteases there is a Ca^{2+} -activated (ATP-independent) proteolytic process involving cysteine proteases (Calpains) that degrade cytoplasmic proteins. Another important family of cytosolic proteases is the caspases that are generally involved in the destruction of cell constituents during apoptosis. In addition, caspase-3 plays another role by initiating proteolysis in muscle while influencing proteolysis by the 26S proteasome (Du et al., 2004; Wang et al., 2010).

The ubiquitin-proteasome System (UPS)

Mechanisms affecting proteolysis by the UPS are described by other articles in this issue. Briefly, protein degradation in the UPS begins by concerted actions of enzymes that build a chain consisting of the heat shock protein cofactor, Ub onto substrate proteins, marking them for degradation (Lecker and Mitch, 2011; Reiter et al., 2009). The Ub chain leads to recognition of the protein by the 26S proteasome, a large multicatalytic protease complex that degrades Ub-conjugated proteins to small peptides (Lecker and Mitch, 2011). The initial

Ub conjugation process requires ATP and three enzymatic components, the E1 (Ub-activating enzyme) and E2 (Ub-carrier or conjugating proteins) prepare Ub for its conjugation to the substrate protein. The activated Ub can then be conjugated to the substrate protein by a Specific E3 enzymes (Ub-protein ligases) recognize substrate proteins leading the E2 carrier to transfer activated Ub to lysines in the substrate protein and to lysines in Ub. The chain of Ubs is recognized by the 26S proteasome which degrades the substrate protein. The 26S proteasome constitutes about 1-2% of cell mass and is present in the nucleus and cytosol of all cells (Lecker and Mitch, 2011). It is composed of about 60 subunits and like the Ub conjugation process, it requires ATP to perform its functions, including conjugation of Ub to protein substrates, removing Ub from the protein that is recognized for degradation by the proteasome and for degradation of the protein. The 26S proteasome is composed of a central barrel-shaped 20S proteasome coupled to 19S regulatory particles at either or both ends (Mitch and Goldberg, 1996). The 20S proteasome is the site of proteolysis, containing four stacked, hollow rings, each exhibiting seven distinct but related, subunits with β subunit rings containing the active sites for proteolysis on the interior face of the cylinder. The outer, α subunit rings surround a narrow, gated pore through which substrates enter and products exit. Thus, the processes of Ub conjugation of a protein and the functioning of the 19S particle are mechanisms that insure proteolysis is an exquisitely selective process. Knowledge about the regulation of protein degradation and the role of the UPS in degrading proteins has led to thousands of biologically important insights into the biologic function of cells

Does UPS-induced acceleration of protein degradation occur in humans suffering from catabolic conditions? It is difficult to identify the actions of specific proteolytic systems in humans because muscle tissue is not readily available. Still, there is indirect evidence that increased proteolysis in animal models of catabolic diseases also occurs in humans with similar conditions. Specifically, accelerated muscle protein catabolism caused by CKD exhibits cellular mechanisms which are similar to those present in certain other catabolic conditions (e.g., cancer cachexia, starvation, insulin deficiency or sepsis (Mitch and Goldberg, 1996)). Specifically, muscle proteolysis in animal models of these conditions is blocked by inhibitors of the UPS (Pickering et al., 2002; Tiao et al., 1997; Williams et al., 1999). In addition, these models express increased levels of mRNAs encoding certain components of the UPS (Bailey et al., 1996; Mitch et al., 1999; Price et al., 1996). This gene activation property was extended to reveal similar patterns of changes in the expression of about 100 atrophy-related genes (also termed atrogenes) in rodent models of CKD, starvation, cancer or diabetes (Lecker et al., 2004). The presence of mRNAs for Ub and constituents of the UPS have served to document that catabolic illnesses in patients are undergoing muscle protein degradation by the UPS (Pickering et al., 2002; Tiao et al., 1997; Williams et al., 1999).

Dual Roles for Activated Caspase-3 in the Degradation of Myofibrillar Proteins

Approximately two thirds of the protein in muscle is composed of myofibrillar proteins and they are not readily degraded by the UPS. The UPS only slowly digests actomyosin or myofibrils but readily degrades other proteins in the myofibril (actin, myosin, troponin or tropomyosin) (Solomon and Goldberg, 1996). This leads to the conclusion that intact myofibrils and actomyosin undergo an initial cleavage which is needed to provide access so the UPS can degrade the cleaved proteins. Caspase-3 can perform this initial cleavage by cutting actomyosin *in vitro* and in cultured muscle cells, thereby producing substrates which are rapidly degraded by the UPS. This cleavage process of caspase-3 can be identified because it leaves behind a characteristic 14-kDa C-terminal fragment of actin detectable in

the insoluble fraction of muscle proteins (Du et al., 2004). We have found that this action of caspase-3 acts to degrade muscle proteins in rodent models of uremia, diabetes and angiotensin II-induced hypertension. There also is evidence that the 14-kDa actin fragment might develop into a biomarker of muscle proteolysis. This is suggested because the 14-kDa fragment has been found in muscles of patients who develop muscle atrophy from immobilization and the inflammation of osteoarthritis or from burn injury or in patients treated by hemodialysis (Du et al., 2004; Workeneh et al., 2006). For example, the density of the fragment in western blots of the insoluble fraction of muscle proteins was highly correlated ($r = 0.7$) with the measured rate of protein degradation in muscles of patients undergoing hip replacement surgery. In addition, changes in the density of the 14 kDa actin fragment in muscle biopsies of hemodialysis patients identified important responses of increased muscle protein degradation despite a prolonged period of exercise training. Specifically, in patients trained in bicycling, the 14-kDa actin fragment was suppressed, implying a decrease in muscle protein degradation. In contrast, biopsies from dialysis patients did not significantly change the 14-kDa actin fragment, implying that muscle proteolysis was unchanged by the resistance exercise.

Other responses to caspase-3 also act to stimulate muscle protein degradation. We found that activated caspase-3 cleaves Rpt2 and Rpt6 subunits of the 19S proteasome particle. The cleavage activates the 26S proteasome-dependent proteolysis *in vitro* and *in vivo*. Cleaving the Rpt2 and Rpt6 subunits was critical for stimulating the response because there was no increase in 26S proteasome proteolysis when the caspase-3 recognition sites in Rpt2 and Rpt6 were mutated so caspase-3 could not cleave them. Evidence for pathophysiologic relevance of caspase-3-induced cleavage of Rpt2 and Rpt6 is found in mouse models of CKD or insulin resistance (*db/db* mice) because these subunits were at low levels in muscle. In summary, caspase-3 augments muscle protein breakdown by initially cleaving actomyosin and myofibrils to provide substrates. Caspase-3 also cleaves critical subunits of the 19S proteasome to stimulate degradation of muscle proteins by the 26S proteasome.

What influences the activity of caspase-3? First, impairment of insulin or IGF-1 can activate both caspase-3 and the UPS (Du et al., 2004; Lee et al., 2004). For example, in mice with streptozotocin-induced acute diabetes, a decrease in phosphatidylinositol 3-kinase (PI3K) activity and phosphorylation of Serine-307 of IRS-1, resulted in impaired insulin signaling. Second, acute diabetes stimulates a conformational change in the pro-apoptotic protein, Bax. This change in Bax stimulates the release of cytochrome C from mitochondria which activates caspase-3 (Lee et al., 2004). The decrease in PI3K activity also stimulates the UPS by suppressing p-Akt and hence, the degree of phosphorylation of forkhead transcription factors (FoxO). Thus, a decrease in the phosphorylation of FoxO transcription factors (p-FoxO) stimulates the expression of the muscle-specific, E3 Ub ligases atrogenin-1/MAFbx and MuRF1. At least in cultured myotubes, a rise in Atrogenin-1/MAFbx is associated with increased protein degradation. However, the mechanism underlying these responses are unclear because the protein substrates recognized by atrogenin-1/MAFbx are MyoD and eIF-1 rather than myofibrillar proteins. Possibly, atrogenin-1/MAFbx exerts a secondary role which stimulates muscle cell protein degradation (Sacheck et al., 2004).

Caspase-3 activity is also regulated by the presence of endogenous inhibitors of apoptosis proteins (IAPs) in muscle. *In vitro* and *in vivo* systems have demonstrated that IAP's, including XIAP acts to suppress caspase-3 activity (Holcik and Korneluk, 2001). In determining the influence of caspase-3 on muscle protein metabolism in rodent models of diabetes and CKD, we found that increased XIAP expression not only attenuates caspase-3 activity but also reduces muscle protein loss (Hu et al., 2010; Wang et al., 2007).

Activation of calpains has been suggested as another protease that catalyzes the initial cleavage of myofibrillar proteins. Calpains are calcium-dependent, cysteine proteases that are active in muscular dystrophy and animal models of sepsis (Wei et al., 2005). The importance of calpains in initiating muscle protein degradation in other conditions, such as CKD is unsettled because inhibition of calcium-activated proteases in muscles from rodents with uremia or other types of atrophy does not block overall protein degradation or the degradation of myofibrillar proteins or the accumulation of the 14-kDa actin fragment in muscle cells (Du et al., 2004).

In summary, the UPS is the major proteolytic mechanism in skeletal muscles and its activation leads to degradation of a variety of proteins in a highly specific manner. The UPS does not act in isolation but instead, it acts in coordination with caspase-3 to initiate cleavage of the complex structure of muscle proteins forming substrates to be degraded by the UPS. In addition, caspase-3 cleaves specific subunits of the 19S proteasome particle to stimulate proteolytic activity by the 26S proteasome. These actions result in highly coordinated processes that degrade muscle proteins (Lecker and Mitch, 2011; Mitch and Goldberg, 1996).

Insulin/IGF-1 Resistance and the Degradation of Muscle Proteins

Results from patients and models of CKD indicate that insulin resistance is frequent in patients with CKD or complications of CKD suggesting impaired insulin signaling could cause muscle protein losses (DeFronzo and Beckles, 1979; DeFronzo et al., 1978; Shoelson et al., 2006; Zhang et al., 2009). We evaluated this possibility in diabetic *db/db* mice and in mice with acute diabetes following streptozotocin injection (Price et al., 1996; Wang et al., 2006). In both models, activities of caspase-3 and the UPS led to accelerated muscle protein degradation. Specifically, we found an increase in levels of the 14-kDa actin fragment in muscles of both types 1 and 2 diabetic mice. Evidence for activation of the UPS included suppression of protein degradation in muscles treated with proteasome inhibitors and increased mRNAs of components of the UPS. As noted, expression of muscle-specific E3 Ub ligases is activated by reduced phosphorylation of members of the forkhead transcription factors (FoxO1 and FoxO3). Dephosphorylation of these transcription factors permits them to translocate into the nucleus and stimulate transcription of atrogin-1/MAFbx and MuRF1. The latter E3 Ub ligase is more clearly linked to myofibrillar protein degradation while substrates of atrogin-1/MAFbx do not include myofibrillar proteins. The mechanism initiating responses of the E3 Ub ligases involves a suppression of IRS-1 and Akt signaling. This response is due to reduced generation of the PI3K-generated, phosphatidylinositol 3,4,5-triphosphate (PIP3) as low levels of PIP3 decrease p-Akt and hence, the phosphorylation of FoxO's, increasing the expression of the E3 Ub ligases, atrogin-1/MAFbx and MuRF1 plus an association with UPS-mediated muscle protein degradation.

Besides a decrease in insulin/IGF-1 signaling, PIP3 may be decreased by another mechanism, an increase in the activity of PTEN (the phosphatase and tensin homolog deleted from chromosome 10). This phosphatase has a reciprocal influence on PIP3: an increase in PTEN stimulates muscle protein degradation because it decreases PIP3 and this in turn, decreases p-Akt and p-FoxO to stimulate the expression of atrogin-1/MAFbx and MuRF1 and protein degradation (Figure 1). Earlier we found that an increase in PTEN results in a pathophysiologically relevant acceleration of muscle protein degradation (Hu et al., 2007). Evidence from *db/db* mice or mice with acute diabetes indicates that impaired IRS-1/Akt signaling is considered to be the major signaling pathway increasing cell growth.

Recently, a new mechanism affecting intracellular insulin-initiated signaling was uncovered. Thomas et al reported that an endogenous phosphatase, SIRPα, can be detected in the

membrane of skeletal muscle and it was shown to suppress insulin signaling (Thomas et al., 2013). Even in a mouse model of CKD, SIRP α expression was found to be sharply increased in part by the presence of inflammatory cytokines. This result was due to activation of NF- κ B. The mechanism impairing insulin responses was found to be caused by an interaction between SIRP α and the insulin receptor and IRS-1. The result was a decrease in tyrosine phosphorylation of the insulin receptor and of IRS-1, leading to suppressed levels of p-Akt with activation of protein degradation by the UPS. These results provide another mechanism by which defective insulin-induced intracellular signaling can activate muscle wasting.

Insulin/IGF-1 Signaling and Glucocorticoids

It is not surprising that glucocorticoids (GC) are increased in rodents or humans when muscle protein degradation is increased because GC are “stress” hormones and are produced at higher levels in CKD, diabetes, inflammation and acidosis. It is surprising, however, that the mechanism underlying GC-induced loss of muscle mass depends on the level of GC: when the level is similar to that in rodents responding to “stress”, GC alone does not cause muscle protein losses unless there is another stimulus (e.g., impaired insulin signaling). In this case, caspase-3 and the UPS are activated to breakdown muscle proteins. How do physiologic levels of GC activate muscle protein? Regarding metabolic acidosis, it was demonstrated that adrenalectomized (ADX) rats do not develop muscle protein losses unless they are acidified and treated with sufficient GC to achieve levels present in rodents being stressed. Notably, neither ADX rodents given GC only or acid loading only experience an increase in muscle protein losses. However, the combination of both a high physiologic level of GC and acidosis or acute diabetes and another catabolic condition are both present (Hu et al., 2013; Hu et al., 2009; Mitch et al., 1999). The mechanism was shown to involve a non-genomic response to GC: the stress level of GC activates the glucocorticoid receptor and it interacts with PI3K to reduce its activity (Hu et al., 2009). As discussed, a decrease in PI3K activity leads to a decrease in p-Akt and p-FoxO1 causing muscle protein degradation by the UPS (Figure 1). Note that a different, unexplained response occurs in response to pharmacologic doses of GC: muscle proteolysis acts indirectly to stimulate genes but only for a few days (Auclair et al., 1997; Kayali et al., 1987; Sandri, 2008). How these responses are mediated has not been identified because genes encoding the critical E3 Ub ligases do not possess GC receptor elements (GRE). Alternatively, high doses of GC could involve phosphorylation of the transcription factor, Sp1, through a MEK1-mediated response (Marinovic et al., 2002).

Insulin/IGF-1 Signaling and Myogenesis in CKD

Rates of protein turnover in normal adults are very high (3.7–4.7 g/kg/day) so even a small but persistent percentage decrease in protein synthesis or increase in protein degradation causes major changes in lean body mass (Mitch and Goldberg, 1996). Besides changing in protein turnover, myogenesis contributes to the maintenance of muscle mass through conversion of muscle progenitor cells (satellite cells) into myofibrils. The satellite cells are found under the basal lamina of myofibrils but upon activation, satellite cells proliferate to form myoblasts that differentiate into myotubes and fuse to form myofibrils which act to increase existing myofibers or to repair damaged myofibers. In mice with CKD, we have found that the functions of satellite cells in muscle are impaired; there is decreased proliferation and differentiation in response to injury (Zhang et al., 2010). Not only does this contribute to the development of the muscle atrophy of CKD but it also can cause abnormalities, including a sharp increase in collagen deposition and fibrosis in injured muscles of mice (Zhang et al., 2010). What controls the activity of satellite cells? First, the usual responses to muscle injury, the presence of inflammation or stretch cause proliferation

and differentiation of these cells but these responses are suppressed by CKD. Myogenesis involves expression of the transcription factors, Myf5 and MyoD, resulting in proliferation of myoblasts. Subsequently, another myogenic gene, myogenin, is expressed and coincides with cell differentiation, forming new myofibers. Satellite cells isolated from muscles of mice with CKD not only exhibit decreased levels of MyoD and myogenin but also impaired regeneration of injured muscles. As with other abnormalities caused by CKD, these functional abnormalities are linked to insulin/IGF-1 resistance because satellite cells isolated from mice with CKD responded similarly to responses of satellite cells isolated from mice in which we had knocked out the IGF-1 receptor (Zhang et al., 2010). In both groups of satellite cells, the levels of p-Akt were similarly reduced, suggesting that impaired insulin/IGF-1 intracellular signaling caused the impaired myogenesis (Zhang et al., 2010).

Inflammation and Losses of Muscle in CKD

Inflammation is often suggested as a trigger of muscle protein losses because several illnesses causing loss of lean body mass result in high circulating levels of inflammatory cytokines (Kimmel et al., 1998). However, this association does not identify how inflammatory cytokines stimulate the loss of muscle mass. To address this question, we examined how cytokines might be associated with muscle protein losses. Using an Affymetrix GeneChip® Mouse Exon 1.0 ST Array, we found differential expression of 130 genes ($P < 0.05$) in gastrocnemius muscles of mice with CKD and control mice: 19 genes were strongly up-regulated and were associated with inflammation or immune responses. In patients with CKD, circulating levels of cytokines included increases in the expression of IL-6 and TNF α (compared to healthy, matched controls). We also found that an increase in IL-6 was linked to decreased levels of IRS-1 and p-Akt which in turn led to increased proteolysis in the UPS (Zhang et al., 2009). It is not known if other cytokines stimulate similar changes in proteolysis.

Insulin/IGF-1 Signaling and Exercise in CKD

Exercise is beneficial for general health and can play a preventative role in certain disease states because it: 1) increases muscle protein synthesis; 2) decreases protein degradation; and 3) improves muscle progenitor cell function. There is considerable information that the lack of exercise adversely affects muscle mass and function. For example, reduced muscle activity (disuse) as occurs in prolonged bed rest, limb immobilization or spaceflight causes muscle atrophy. On a more positive side, the intensity of physical activity is inversely associated with mortality; an average energy expenditure of about 1000 kcal per week is associated with a 20% -30% reduction in “all-cause” of mortality (Paffenbarger et al., 1986; Paffenbarger et al., 1993). Endurance exercise (e.g., bicycling) increases cross-sectional areas and numbers of myofibers but resistance training initially causes tears in the sarcolemma and basal lamina and it appears that this “myo-trauma” initiates the release of growth factors, including IGF-1. Since IGF-1 activates satellite cells to regenerate myofibers, this type of exercise can lead to hypertrophy. Muscle atrophy from disease rather than disuse generally develops following muscle damage or from systemic diseases i.e., uremia). Results from trials of aerobic exercise and resistance training can prevent disuse atrophy while its effects on systemic disorders have not been defined (Karacabey, 2005).

Myostatin and the Muscle Wasting in CKD

Myostatin is a member of the TGF- β family of secreted proteins and is predominantly expressed in skeletal muscle (cardiac muscle and adipose tissue have low levels of myostatin). In skeletal muscle, myostatin is produced as a prepromyostatin which is cleaved to produce promyostatin, consisting of myostatin and a propeptide. The propeptide produces an inactive, “latent complex” by binding to myostatin. The latent myostatin can then be

activated by proteolysis or after stimulation by free radicals. Liberation of myostatin allows it to bind ActRIIB, the high-affinity type-2 activin receptor, on muscle membranes. Subsequently, the myostatin-ActRIIB interaction results in activation of the Type-1 activin receptor serine kinases, ALK4 or ALK5. These kinases phosphorylate Smads 2 and 3 to stimulate gene transcription. Notably, other TGF- β family members (e.g., activin A and GDF11) also bind to ActRIIB, stimulating the same intracellular signaling pathway.

Myostatin plays a pivotal role in regulating skeletal muscle mass and its functions. For example, deletion of the myostatin gene produces mice that exhibit a dramatic increase in the size and number of skeletal muscle fibers (Szabo et al., 1998). Moreover, transgenic mice which overexpress a dominant negative myostatin receptor, or the propeptide or other proteins that block myostatin yield phenotypes bearing huge amounts of muscle (Han and Mitch, 2011). This has been shown in mice, cattle, sheep, dogs, horses and a human with a loss-of-function myostatin mutation resulting in enormous muscles and excellent athletic performances. For example, in competitive racing events, whippet dogs bearing a single copy of the mutated myostatin are among the fastest dogs (Han and Mitch, 2011). But, whippets with two copies of the same mutation develop massive muscles and run only slowly. Myostatin polymorphism in thoroughbred horses also results in a strong association between decreased expression of myostatin and exercise tolerance. Thus, myostatin deficiency produces muscle hypertrophy and improves physical performance.

Myostatin and the myostatin/activin signaling pathway are upregulated in conditions that cause muscle wasting. For example, high levels of myostatin are present in muscles of aging subjects, in patients undergoing prolonged bed rest and in muscles of patients with AIDS, chronic kidney disease (CKD) or heart failure (Han and Mitch, 2011). Myostatin in muscle is also increased in animal models of cancer cachexia, CKD, glucocorticoid administration, burn injury, mechanical unloading and space flight and serum levels of Activin A rise in response to cancer, kidney failure or heart failure (Verzola et al., 2011). Finally, experimental administration of myostatin or Activin A to mice causes a ~30% decrease in muscle mass, defining its role as a catabolic factor in muscle. These responses document two facts: first, both myostatin and activin A influence muscle size; and second, an excess of myostatin results in loss of muscle mass. This raises the question: can their influence be overcome by a treatment strategy that is non-toxic?

Three strategies have been used to block the muscle wasting potential of myostatin: 1) administration of antibodies, including a peptibody (a genetically engineered myostatin-neutralizing peptide fused to Fc); 2) administration of the myostatin propeptide which binds myostatin or soluble ActRIIB receptors to suppress the influence of myostatin. These myostatin inhibitor strategies increase muscle mass in normal animals including cynomolgus monkeys that were treated to over express follistatin-isoform FS344, a myostatin-sequestering protein. The treated monkeys experienced an increase in muscle mass and strength (Han and Mitch, 2011). There also are examples in which the muscle wasting occurring in different forms of cancer or CKD can be blocked by inhibiting myostatin.

Beneficial Responses to Blocking Myostatin in CKD

Chronic kidney disease (CKD) is among a group of disorders (e.g., diabetes, starvation and some forms of cancer) that are characterized by increases in circulating markers of inflammation, an increase in glucocorticoid production, impaired insulin/IGF-1 signaling and loss of muscle protein (Han and Mitch, 2011). These conditions also exhibit similar patterns of gene expression in muscles suggesting that a common mechanism leads to losses of muscle mass in each condition. Notably, these disorders characteristically exhibit insulin/IGF-1 resistance, increased glucocorticoids, circulating levels of inflammatory cytokines as

well as losses of muscle proteins. These characteristics of CKD include a decrease in p-Akt caused by the development of insulin resistance (Bailey et al., 2006). As described earlier, the decrease in p-Akt leads to increased accelerated muscle proteolysis via the UPS and caspase-3 (see above).

To evaluate whether myostatin stimulates muscle wasting in CKD, Zhang et al (Zhang et al., 2011) studied a mouse model of CKD (subtotal nephrectomy followed by feeding a high protein diet to mimic the serum abnormalities found in patients with CKD) (Bailey et al., 2006). This model produces mice with BUN values >80 mg/dL and metabolic acidosis as well as insulin/IGF-1 resistance. The experimental protocol was to pair mice with CKD for body weight and BUN and then pair-feed them for 4 weeks. During this period, one mouse of each pair was injected subcutaneously with the anti-myostatin peptibody every other day; the paired mouse was injected with the diluent. Treatment with the anti-myostatin, peptibody produced a remarkable protection against the muscle wasting induced by CKD. Specifically, the peptibody reduced the myostatin level in muscles and blocked the loss of body weight that occurred in the paired mouse injected with the diluents. The mechanism preventing loss of muscle mass included an increase in protein synthesis and a decrease in protein degradation plus improvements in satellite cell function. Finally, treatment with the myostatin antibody led to an increase in p-Akt thereby providing a mechanism for the suppression of muscle protein degradation and it is known that an increase in p-Akt stimulates protein synthesis in muscle (Mitch and Goldberg, 1996). Unlike results with a decoy ActRIIB receptor which blocked the muscle losses associated with cancer in which inflammatory cytokines were unaffected, inhibition of myostatin in mice with CKD led to suppression of the circulating levels of inflammatory cytokines and especially, IL-6. To examine how this response occurred, we treated cultured C2C12 muscle cells with TNF- α . This resulted in increased production of myostatin. Next, we treated C2C12 muscle cells with myostatin and found that the cells increased their production of IL-6. These results suggest that a high level of TNF- α as frequently occurs in CKD patients will stimulate myostatin production in muscle. This response in turn, stimulates the production of IL-6. Since an excess of IL-6 not only suppresses insulin/IGF-1 signaling leading to a decrease in p-Akt, there will be increased protein degradation in muscle as well as decreased protein synthesis, causing muscle atrophy (Shoelson et al., 2006; Zhang et al., 2011).

Insulin/IGF-1 Signaling and microRNA

The mechanism for the link to impaired insulin signaling involves a change in the microRNA (Wang, 2013). To identify CKD-associated problems that could cause loss of muscle mass, we developed a microRNA profile of CKD muscle using a microRNA analysis array. We found that CKD was associated with significantly different levels of 12 microRNAs in muscle (Wang et al., 2011). MicroRNAs regulate gene expression in a sequence-specific manner by binding to the 3'-UTR of targeted mRNA. This yields translational repression or cleavage of mRNA at the post-transcriptional level. In general, a decrease in the level of a specific microRNA removes its inhibitory effect on the targeted mRNA, resulting in up-regulation of its protein product.

Several microRNAs can regulate insulin/IGF signaling pathway: muscle-specific miR-1 and miR-133a influence muscle cell growth and differentiation because they directly target IGF-1 (Elia et al., 2009; Hua et al., 2012). This is relevant because microRNA-induced changes in IGF-1 could down-regulate the IRS-1/Akt pathway leading to activation of muscle protein degradation as discussed earlier. Evidence for this hypothesis includes the reports that activated IGF-1 acts reciprocally to regulate miR-1 expression via the FoxO3a transcription factor (Figure 2). For example, FoxO3a stimulates the promoter to increase transcription of miR-1 (Elia et al., 2009). The sequence of events is complex, however, as

some investigators report that the expression of the IGF-1 receptor is directly regulated by miR-133 since over-expression of miR-133 in C2C12 muscle cells significantly suppressed the expression of the IGF-1 receptor at the post-transcriptional level (Huang et al., 2011). This resulted in suppression of Akt phosphorylation. In addition, miR-125b directly negatively modulates IGF-II (insulin-like growth factor 2) and miR-125b is negatively controlled by the mammalian target of rapamycin (mTOR) (Ge et al., 2011).

These complicated interactions can lead to activation of the UPS since miR-486 can reduce the expression of FoxO1 and PTEN and their activities. (Xu et al., 2012). In fact, electroporation of a miR-486 mimic into muscle was found to block dexamethasone-stimulated protein degradation of muscle (protein synthesis was unchanged suggesting specificity of the interactions). The mechanism for the suppression of miR-486 included down-regulation of PTEN resulting in an increase p-Akt with phosphorylation of FoxO1. The latter led to suppression of the activation of MuRF1 and reduced muscle protein degradation (Xu et al., 2012). Dey et al. reported that miR-486 increased myoblasts differentiation by inhibiting Pax7 but its impact on myogenesis is unclear (Dey et al., 2011). There are some data about miR-486 influencing the treatment of Duchenne's muscular dystrophy: the mouse model of this disorder exhibited impaired muscle regeneration when miR-486 was over expressed *in vivo*. Whether this negative result reflects changes in cell cycle kinetics or muscle fiber regeneration is unknown (Alexander et al., 2011).

Several reports suggest that miR-23a influences the expression of atrogen-1/MAFbx and MuRF1. One group of investigators found that miR-23a suppressed the translation of the Ub E3 ligases, atrogen-1/MAFbx and MuRF1, to suppress glucocorticoid-induced skeletal muscle atrophy (Wada et al., 2011). In other studies, we found that miR-23a is decreased in muscles of mice with CKD (Wang et al., 2011), associated with an increase in the level of both atrogen-1/MAFbx and MuRF1 (Wang et al., 2009). Another report addressed the consequences of cardiac hypertrophy. The experiments were undertaken because miR-23a is a pro-hypertrophic miRNA and its knockdown attenuation of cardiac hypertrophy. In exploring the molecular mechanism resulting in upregulation of miR-23a, it was found that MuRF1 is a target of miR-23 since MuRF1 translation was suppressed by miR-23a (Lin et al., 2009). Additional studies are needed to understand the complexities of microRNAs on muscle protein metabolism.

In our microRNA profile of CKD muscle, we found that a decrease of miR-29 leads to an increase in the Yin Yang1 protein (YY1) (Wang et al., 2011). This is relevant because YY1 can play a negative role in myogenesis by repressing the synthesis of various genes, including skeletal alpha-actin (α -actin), muscle creatine kinase (MCK), and myosin heavy chain IIb (MyHCIIb). In fact, we found that CKD reduces miR29 and this in turn raised the expression of YY1 resulting in decreased myoblasts differentiation. For this reason, miR29 has been reported to function as a positive regulator of muscle cell differentiation (Wang et al., 2008).

Conclusion

Muscle wasting induced by CKD is due to activation of the UPS proteolytic system as demonstrated when inhibitors of the UPS blocked muscle catabolism and in muscle, there are high levels of the mRNAs encoding components of the UPS. These criteria were used to determine that an activated UPS causes protein losses in rodent models of catabolic diseases and in patients with catabolic conditions. The UPS system does not act in isolation but requires both glucocorticoids and caspase-3 for the stimulation of proteolytic activity. The underlying abnormality, however, is impairment in the intracellular signaling that is initiated by resistance to insulin or IGF-1. Thus, muscle proteolysis in CKD and in other conditions

characterized by insulin resistance, inflammation (which causes insulin resistance) and excess glucocorticoids results in a recognizable pattern. Recent information suggests that loss of muscle mass is possible by blocking myostatin or use of microRNAs that modify this pattern of insulin resistance, inflammation and excess glucocorticoids. Controlled clinical trials are required to test this possibility rigorously.

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Figure 1. MicroRNAs regulate IGF/PI3K/Akt signaling to change muscle protein metabolism
Muscle-specific microRNAs, miR-1, miR-206 and miR-133 down-regulate IGF/PI3K/Akt signaling by directly targeting IGF-1. Activated IGF-1 reciprocally regulates miR-1 expression via changes in the activation of FoxO transcription factors as FoxO can bind to the miR-1 promoter, increasing miR-1 transcription. IGF-II is a target of miR-125b while miR-133 can bind to the 3'-UTR of the IGF-1 receptor (IGF-1R), resulting in decreased abundance of the IGF-1 receptor (IGF-1R). miR-486 and miR-17-92 downregulate PTEN, increasing the level of p-Akt which phosphorylates FoxO resulting in its inactivation and this in turn limits muscle protein wasting. miR-23a suppresses translation of both atrogen-1/MAFbx and MuRF1 by interacting with their 3'-UTR's; this can inhibit muscle atrophy.

microRNA

- Factors that stimulate muscle growth
- Factors that inhibit muscle growth
- | Decrease or inhibit
- Increase or stimulate
- | Indirectly decrease or inhibit