XIAP Reduces Muscle Proteolysis Induced by CKD

Junping Hu,* Jie Du,† Liping Zhang,† S. Russ Price,* Janet D. Klein,* and Xiaonan H. Wang*

*Renal Division, Department of Medicine, Emory University, Atlanta, Georgia; and †Nephrology Division, Baylor College of Medicine, Houston, Texas

ABSTRACT
X-chromosome-linked inhibitor of apoptosis protein (XIAP) is an endogenous caspase inhibitor. Caspase-3 contributes to the muscle wasting associated with chronic kidney disease (CKD) and other systemic illnesses, but whether XIAP modulates muscle wasting in CKD is unknown. Here, overexpression of XIAP in cultured skeletal muscle cells decreased protein degradation induced by serum deprivation, suggesting that caspase-mediated proteolysis contributes to muscle atrophy. We generated transgenic mice that overexpress human XIAP specifically in skeletal muscle (mXIAP) and evaluated muscle protein degradation induced by CKD. mXIAP mice with normal kidney function exhibited mild skeletal muscle hypertrophy. Muscle weights of mXIAP mice with CKD (mXIAP-CKD) were indistinguishable from wild-type mice, suggesting that overexpression of XIAP in skeletal muscle protects from CKD-induced muscle atrophy. The rate of total protein degradation, proteasome chymotrypsin–like activity, and caspase-3–mediated actin cleavage all were lower in muscle isolated from mXIAP-CKD mice compared with wild-type CKD mice. Concomitant with the reduction in overall proteolysis, mRNA levels of ubiquitin, muscle-specific ring finger 1, and atrogin-1/muscle atrophy F-box were lower in mXIAP-CKD mice, suggesting that decreased expression of the ubiquitin–proteasome pathway components may contribute to the protein-sparing effects of XIAP. In summary, these results demonstrate that XIAP inhibits multiple aspects of protein degradation in skeletal muscle during CKD.


The loss of muscle mass as a result of disease1 and inactivity2 is commonly known as muscle wasting or atrophy. It occurs in chronic kidney disease (CKD),3,4 sepsis,5 cancer,6 diabetes,7,8 trauma,9 burn injury,10 AIDS,11 and heart failure.12,13 Regardless of the triggering event, common mechanisms contribute to the muscle-wasting process. Here we provide information about muscle wasting in CKD.

Malnutrition is a serious complication of CKD, and virtually every survey of dialysis patients has revealed that weight loss with decreased muscle mass is common. These abnormalities are associated with excess morbidity and mortality and often occur before dialysis begins.14–17 Because population screens indicate that the prevalence of CKD will increase sharply over the next decades,18,19 it is important to understand fully the mechanisms that cause muscle atrophy in CKD.

Muscle atrophy in CKD has been attributed to activation of the ATP-dependent ubiquitin–proteasome system that degrades the bulk of protein in cells. In this system, the multisubunit proteasome complex degrades proteins that have been modified by the covalent addition of polyubiquitin chains. A variety of genes involved in this pathway are increased in CKD, including ubiquitin and two muscle-specific E3 ubiquitin ligases, muscle-specific
ring finger 1 (MuRF-1) and atrogin-1 (also called muscle atrophy F-box [MAFbx]). An increase in these ligases has been correlated with muscle atrophy in CKD, diabetes, insulin resistance, fasting, cancer, denervation, muscle immobilization, and hind-limb suspension. Consistent with this, mice with a genetic knockout of MuRF-1 are resistant to atrophy-inducing effects such as denervation.

Other degradation systems are also increased in muscle during CKD, including lysosomal and caspase-3–mediated proteolysis. Caspase-3 facilitates the degradation of several proteins, including actin present in actomyosin. The actin cleavage process yields peptides that include a 14-kD actin fragment that is rapidly degraded by the ubiquitin–proteasome system. This fragment serves as a biomarker of muscle wasting in CKD and other conditions.

Inhibitors of apoptosis proteins are endogenous inhibitors of caspases, and X-chromosome linked inhibitor of apoptosis protein (XIAP) is the most potent member of the group. XIAP directly inhibits both the initiation (e.g., caspase-9) and execution (e.g., caspase-3) phases of the caspase cascade, which is crucial for cell growth, differentiation, and apoptosis. XIAP, however, has other roles in cells. For example, it contains a RING finger domain that functions as an E3 ubiquitin ligase. Previously, we showed that XIAP can alter overall protein degradation, including myofibrillar protein breakdown, in the muscles of diabetic mice by attenuating caspase-3 and reducing proteasome-dependent proteolysis. Others have shown that XIAP can influence cellular functions unrelated to caspase inhibition, such as enhancing Akt signaling that might inhibit protein turnover.

In this study, we tested whether overexpression of XIAP could prevent or diminish muscle wasting in CKD. We developed transgenic mice that overexpress XIAP specifically in skeletal muscle (mXIAP mice). Protein degradation rates were examined in wild-type (WT) and mXIAP mice with CKD to investigate the impact of XIAP on muscle protein loss. We also studied the effect of XIAP on several components of the ubiquitin–proteasome pathway. Our results provide evidence that XIAP regulates muscle mass via a multifaceted mechanism.

RESULTS

XIAP Was Decreased in the Muscle of CKD Mice

Previously, we found that caspase-3 activity was increased in the muscle of CKD mice, suggesting that the level of XIAP may have been altered. To test this possibility, we performed immunoblot analysis of XIAP protein with muscle lysates of CKD and control mice. XIAP was decreased by 45% in CKD mice (Figure 1A). To evaluate whether the XIAP response in CKD was due to an intracellular or extracellular signal, we incubated C2C12 muscle cells with pooled serum from CKD mice (uremic serum) or controls (29 mg/dl) for 16 hours. Addition of uremic serum significantly decreased XIAP abundance in a dosage-dependent manner (Figure 1B).

Overexpression of XIAP Blocked Actin Degradation Induced by Activated Caspase-3

To determine whether accelerated protein degradation in C2C12 muscle cells could be attenuated by XIAP, we incubated myotubes under reduced serum conditions (i.e., serum withdrawal) or treated them with 50 nM staurosporine to activate caspase-3. Both treatments increased the amount of the 17-kD activated caspase-3 protein that results from cleavage of 31-kD pro-caspase-3. Moreover, serum deprivation and staurosporine increased actin degra-
value normalized to GAPDH. Data are means 

densitometry of the mRNA bands as a percentage of control

GAPDH is used as a loading control. The bar graph shows the 

measure ubiquitin, atrogin-1/MAFbx, and MuRF-1 mRNAs.

with total RNA isolated from each group of cells are used to

2% horse serum (Ctrl) or serum withdrawal (sw). Northern blots 

differentiated in 2% horse serum. The cells are then treated with

staurosporine (stau) in 2% horse serum for 24 hours; the last two 

treatments activate caspase-3. XIAP using lentivirus-mediated gene transfer (Figure 2A).28 No-

Figure 2. Overexpression of XIAP prevents proteolysis-related 

response. (A) C2C12 cells are transduced by either Len-XIAP 

(LenX) or control virus (LenC). The cells are treated with 2% horse 

serum (Ctrl), 0.05% serum (serum withdrawal [sw]), or 50 nM 

staurosporine (stau) in 2% horse serum for 24 hours; the last two 

treatments activate caspase-3. The amount of XIAP, actin cleav-

age and caspase-3 are measured by Western blot of cell lysates. 

The experiment is repeated two additional times with similar 

results. (B) C2C12 cells are transduced by either LenX or LenC and 
differentiated in 2% horse serum. The cells are then treated with 

2% horse serum (Ctrl) or serum withdrawal (sw). Northern blots 

with total RNA isolated from each group of cells are used to 

measure ubiquitin, atrogin-1/MAFbx, and MuRF-1 mRNAs. 

GAPDH is used as a loading control. The bar graph shows the 

densitometry of the mRNA bands as a percentage of control 

value normalized to GAPDH. Data are means ± SE (n = 4). *P < 

0.05, **P < 0.01 versus Ctrl; #P < 0.01 versus sw with LenC.

XIAP also affects the upregulation of this system, we examined

the levels of mRNA encoding ubiquitin as well as the atrogin-

1/MAFbx and MuRF-1 E3 ligases in C2C12 myotubes 

transduced with Len-XIAP. All three mRNAs were significantly 

increased >350% in myotubes incubated under serum-

withdrawal conditions for 24 hours (Figure 2B). XIAP 

overexpression significantly blunted the increase in ubiquitin 

and atrogin-1/MAFbx mRNAs and completely prevented the 

increase in MuRF-1 (Figure 2B).

Generation of Transgenic XIAP Mice

To investigate further the effects of XIAP on muscle loss, we gen-

erated transgenic mice that overexpress XIAP selectively in skele-
tal muscle (mXIAP). The strategy for generating the mXIAP mice is depicted in Supplemental Figure 1. mXIAP (TgMCK-XIAP) mice 

have two transgenes that were derived from TgCre-MCK mice and 

Tglox-XIAP mice. The first transgene (TgCre-MCK) is Cre-MCK 

(causes recombination muscle-specific promoter of creatine ki-

nase), which encodes a Cre-recombinase under the control of a 

creatinine kinase promoter that is active only in skeletal muscle. The 

second bicistronic transgene (Tglox-XIAP) encodes human XIAP 

under the control of a modified constitutive β-actin promoter. 

A green fluorescence protein (GFP) gene and stop codon, flanked 
on either side by loxP cis-elements, were inserted between the 

β-actin (CX-1) promoter and XIAP gene to prevent XIAP expres-

sion. In skeletal muscle, the Cre-recombinase recognizes the loxP 

elements and deletes the intervening GFP and stop codon, thereby 

enabling expression of the XIAP gene. Genotyping results are 

shown in Supplemental Figure 2. mXIAP mice exhibit significant limb and trunk muscle hypertrophy (Figure 3A). The cross-sec-

tional areas of muscle fibers from mXIAP mice were significantly greater than those in WT mice. Immunoblot analysis confirmed 

that XIAP was overexpressed in hind-limb muscles of mXIAP 

mice, compared with control littermates (Figure 3B). The one 

notable exception was the lack of increase in XIAP in the predom-

inantly oxidative-fiber soleus muscle.

XIAP Attenuated Protein Degradation and Actin 

Cleavage Induced by CKD

To determine whether overexpression of XIAP affected muscle 

atrophy in vivo, we measured overall body and wet muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle 

weights before and 1 month after inducing CKD (Table 1). Body 

weights of WT-CKD mice were decreased by 19% com-
pared with the WT control mice, whereas mXIAP-CKD mice 

exhibited a 9% reduction in body weight compared with the 

mXIAP control mice. Consistent with the muscle
Protein degradation was measured in isolated plantaris and EDL muscles for direct evaluation of the effect of XIAP on the accelerated protein turnover induced by CKD (Figure 4). The rate of total proteolysis was 37 and 36% higher in the plantaris and EDL muscles, respectively, of WT-CKD mice compared with the same muscles from WT control mice. In the same muscles, heterologous overexpression of XIAP did not alter the basal proteolysis rate, but it completely prevented the increase in protein degradation as a result of CKD. In the soleus muscle of mXIAP mice, protein degradation was elevated in response to CKD (data not shown). These findings correlate with the abundance of XIAP in the individual muscles.

Actin cleavage was evaluated by immunoblot analysis (Figure 5A). The amount of the characteristic 14-kD actin cleavage fragment was 280% higher in the gastrocnemius muscle of WT-CKD versus WT-control mice. The actin fragment was significantly lower in the mXIAP-CKD mice than in WT-CKD mice, indicating that XIAP attenuated the degradation of actin. Caspase-3 activity also was significantly higher in the gastrocnemius muscle of WT-CKD versus WT-control mice, and the induction of caspase-3 activity by CKD was reduced 80% by heterologous expression of XIAP (Figure 5B).

Effects of XIAP on Activation of the Ubiquitin–Proteasome Pathway by CKD

Because our previous work indicated that the ubiquitin–proteasome pathway is activated in the muscles of rats with CKD, we evaluated two parameters that are indicative of pathway activity. Consistent with previous studies, the mRNAs encoding ubiquitin, atrogin-1/MAFbx, and MuRF-1 were significantly increased during CKD (Figure 6). Overexpression of XIAP blunted each of those responses, with the most notable effect being on MuRF-1 expression, which was not different from levels in WT-control mice. The chymotrypsin-like activity in proteasomes partially purified from CKD mouse muscles was significantly higher than in the preparation isolated from WT-control mice (Table 2); the same activity in the proteasome preparation from mXIAP-CKD mouse muscles was equal to that in either WT- or mXIAP-control mice, with the exception of the soleus muscle.

Effects of XIAP on Inactivation of Akt and Phospho-Akt by CKD

Akt is an insulin/IGF-1–activated protein kinase that can regulate both caspase-3 activity and expression of atrogin-1/MAFbx and MuRF-1. In CKD and/or metabolic acidosis, cell signaling through the insulin/IGF-1/phosphatidylinositol-3-kinase (PI3-K)/Akt pathway in muscle is impaired. To investigate whether XIAP may act, in part, by altering Akt signaling, we evaluated the levels of total and phospho-Akt (Figure 7). The levels of both were reduced in WT-CKD versus WT-control mice. In mXIAP-CKD mice, the changes in Akt were prevented.

DISCUSSION

Muscle wasting is a serious complication of CKD that is a strong predictor of morbidity and mortality. Loss of muscle mass is frequently observed in other catabolic diseases as well. It is now widely accepted that muscle atrophy results from the activation of multiple proteolytic systems, including the ubiquitin–proteasome pathway. We previously found that caspase-3 contributes to the atrophy process by cleaving actin in myofibrils in CKD and diabetes. To examine the contribution of caspase-mediated proteolysis further, we studied transgenic mice that selectively express XIAP in skeletal muscle to test whether overexpression of an endogenous protein inhibitor of the caspas, including caspase-3, would reduce atrophy. Our findings support this hypothesis.

Although the uremic animal model has been extensively studied, little is known about the regulation of caspase-3...
and XIAP in CKD. In this study, the level of XIAP protein was decreased when caspase-3 activity and the overall rate of protein degradation in muscle was increased by CKD. Notably, the loss of XIAP protein could be reproduced in cultured myotubes by addition of aliquots of CKD serum to the media, suggesting that an extracellular factor induced the response. Although our studies did not directly address the mechanism causing the decrease in XIAP, it is unlikely that the response was due to the concurrent increase in caspase-3 activity, because in the XIAP-overexpressing C2C12 cells, activation of caspase-3 activity by staurosporine did not cause a decrease in XIAP (Figure 2A). It is possible that the decline in XIAP is related to the overall increase in protein degradation or that a CKD-related “factor” regulates XIAP by altering its expression. The exact identity of this factor is unknown. Possibilities include glucocorticoids, cytokines, and hormones, all of which are altered in CKD and could contribute to the muscle atrophy. Further studies will be needed to elucidate the mechanism causing the reduction in XIAP in CKD.

Our findings are broadly applicable to other models of muscle atrophy. Caspase-3 has been linked to muscle wasting in a variety of conditions.\(^{12,13,37–41}\) XIAP has been reported to be low and caspase-3 activity high in muscles of insulin-deficient mice\(^ {29}\) and in denervated rat skeletal muscle.\(^ {39}\) Consistent with our findings, Vazeille et al.\(^ {42}\) found that the rates of ubiquitination and levels of ubiquitin–protein conjugates, chymotrypsin-like proteasome activity, and caspase-3 activity all were rapidly increased during immobilization-induced muscle atrophy in rats. Furthermore, they found that the responses normalized during a recovery period. In the same muscle, XIAP was low during atrophy and its level was restored after reuse.

XIAP has been reported to exert multiple roles in cells.\(^ {29,30}\) Its best known action is to suppress apoptosis by directly interacting with and inhibiting caspases-3, -7, and -9.\(^ {43}\) XIAP contains a highly conserved RING domain at its C-terminus, which enables it to act as an E3 ubiquitin ligase. Via this RING domain, XIAP is able to catalyze the ubiquitination and degradation of other proteins, including caspase-3.\(^ {44}\) Because caspase-3 activity was lower when XIAP was overexpressed in INS-1 cells, XIAP probably has a broader role in regulating overall protein degradation than just suppression of caspase activities is one aspect of the mechanism by which XIAP attenuated protein degradation in CKD-mXIAP mice; however, our data also indicate that XIAP has a broader role in regulating overall protein degradation than just directly inhibiting the caspase proteases. This conclusion is based on three other XIAP-induced changes. First, the level of MuRF-1 was reduced in the muscle of mXIAP-CKD mice versus WT-CKD mice. Second, raising the level of XIAP reduced the chymotrypsin-like activity of the proteasome (X.H.W., in revision). Third, the amount of Akt was increased in the muscle of mXIAP mice versus WT mice.

The one surprising outcome of this study was that XIAP completely blocked the induction of MuRF-1 by CKD. Muscle atrophy is significantly attenuated in MuRF-1–deficient mice.\(^ {21}\) Re-
their selective ubiquitination may facilitate thick filament disassembly. At this stage, we do not know whether XIAP has a direct effect on MuRF-1; however, we believe that the effect of XIAP on inhibition of muscle proteolysis is not only due to suppressed caspase-3, because MuRF-1 is also blunted because both caspase-3 and MuRF-1 proteins are indeed involved in the initial steps of myofibrillar disassembly.

In CKD, signaling through the insulin/IGF-1/PI3-K/Akt signaling pathway is defective. This leads to activation of the FoxO transcription factors and increased expression of atrogin-1/MAFbx and MuRF-1. Given the changes in gene expression and their relationship to PI3-K/Akt signaling, we tested whether Akt signaling was altered in muscle of CKD-control and CKD-mXIAP mice and compared them with the normal control mice. We found that both Akt and activated (phospho) Akt were decreased in the CKD mouse, but the levels of Akt protein and phospho-Akt in the CKD-mXIAP mice were comparable to those in control mice. This improved response in the presence of XIAP overexpression could be linked to the decreased levels of atrogin-1/MAFbx and MuRF-1 expression, because increased Akt phosphorylation should cause inhibition of FoxO and reduced transcription of these E3 ligases. Reduced FoxO activity could also account for the decrease in ubiquitin mRNA, because we recently found that FoxO indirectly regulates ubiquitin transcription. Interestingly, Akt also phosphorylates XIAP at Ser-87. The modification prevents the ubiquitination and subsequent degradation of XIAP; therefore, XIAP could be creating a positive-feedback loop to maintain its protein level and promote muscle cell survival by increasing Akt signaling.

An unexpected finding in these studies was the relatively low level of XIAP expression in the soleus muscle of mXIAP mice. As a result, the cross-sectional area of the soleus was not preserved in CKD-mXIAP mice, and the CKD-induced increase in protein degradation was not prevented. The aberrant XIAP expression in soleus may result from the strain of Cre-transgenic mice that were used to produce the mXIAP mice. The Cre recombinase is under the control of an MCK promoter with two enhancers (1 and 2). A gene cassette positioned between the MCK promoter and the XIAP cDNA contains a stop codon and GFP coding sequence that is flanked by loxP elements. Expression of XIAP is induced when the Cre-recombinase deletes the stop codon/GFP cassette (Supplemental Figure 1). In our mXIAP mice, GFP expression was higher in the soleus than in the heart muscles than other hind-limb muscles that we tested. This indicated that removal of the intervening stop codon was inefficient in these tissues and could explain the low expression of XIAP. Others who used a similar strategy encountered low expression of heterologous genes in the soleus and suggested that the MCK regulatory regions may be less active in pre-steadily slow-twitch muscle compared with predominantly fast-twitch muscles such as the EDL and tibialis anterior. Furthermore, Dunant et al. showed that the MCK promoter was
more active in glycolytic than oxidative muscle fibers. Because muscle phenotypes are determined by a variety of factors including developmental origins, differences in hormonal sensitivity, patterns of contraction, and the type and degree of innervations, one or more of these factors may be responsible for the low level of XIAP transgene expression in the soleus muscle.

In conclusion, evidence is provided that the reduction in XIAP in muscle during CKD is a contributing factor causing accelerated protein degradation and atrophy. We also found that XIAP acts to suppress overall protein degradation by a multifaceted mechanism that involves inhibition of caspase-3 activity, reduction of chymotrypsin-like proteasome activity, and decreased levels of ubiquitin–proteasome pathway mRNAs, including MuRF-1 and atrogin-1/MAFbx. Because the uremic muscle-wasting model shares pathologic characteristics (e.g., increased caspase-3 and ubiquitin proteasome–dependent proteolysis, decreased XIAP) with other catabolic conditions (e.g., cancer, heart failure, denervation), our results may be widely applicable to other catabolic diseases associated with muscle atrophy.

CONCISE METHODS

Cell Culture
Mouse skeletal muscle C2C12 cells (ATCC, Rockville, MD) were studied between passages 3 and 9. Cells were cultured in DMEM with 10% FBS, 10% cow serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Eighty percent confluent cells were differentiated in 2% horse serum. Cells were passaged every 2 days. HEK293H cells (Invitrogen, Carlsbad, CA) were cultured in 150-mm dishes with high-glucose DMEM containing 10% FBS, 4 mM l-glutamine, 0.1 mM MEM nonessential amino acids, and 1% penicillin/streptomycin solution.

Animals and CKD Mouse Model
The experiments were approved by the Institutional Animal Care and Use Committee of Emory University (protocol 141-2008) and were in compliance with the standards for the care and use of laboratory animals of the Institute of Laboratory Animal Resources, National Academy of Sciences (Bethesda, MD). To develop muscle-specific overexpressing XIAP mice (mXIAP), we used a Cre/LoxP system. The strategy for the design of the transgene constructs and their use is depicted in Supplemental Figure 1. The mXIAP (Tg^{MCD-XIAP}) mice have two transgenes that are bred from the Tg^{cre-MCK} (Cre-recombinase transgenic) mice and Tg^{flox-XIAP} mice. The presence of the two transgenes was verified by PCR screening of mouse genomic DNA using REDExtract-N- Amo Tissue PCR kit (Sigma-Aldrich, St. Louis, MO). The detailed is in the Supplemental Methods section.

CKD was induced in mice in a one-step procedure. A right kidney was removed through a right flank incision and weighed. The two poles of the left kidney were excised and weighed. The weight of the excised left kidney was equal to two thirds of the right kidney. Hemostasis was achieved by cautery and pressure. Initially, mice were fed 14% Protein Rodent Maintenance Diet Chow (Harlan Teklad, Madison, WI) ad libitum; after 7 days, mice that underwent sham surgery were pair-fed with a weight-matched CKD mouse using a high-protein diet (40% protein) for 2 weeks. For obtaining blood and tissues, mice were anesthetized with 13 mg/kg xylazine/64 mg/kg ketamine. Dissected EDL, soleus, and plantaris muscles were incubated in vitro for measurement of the rate of protein degradation. Because muscle neither synthesizes nor degrades tyrosine, and tyrosine does not accumulate in the intracellular pool of muscle, release of tyrosine to the media marks total protein degradation. Protein

Muscle Protein Degradation
The rate of protein degradation was measured as the release of free tyrosine from incubated muscles in the presence of cycloheximide. Because muscle neither synthesizes nor degrades tyrosine, and tyrosine does not accumulate in the intracellular pool of muscle, release of tyrosine to the media marks total protein degradation. Protein
degradation in cultured myotubes was measured using cells prelabeled with L-[U-14C] phenylalanine as described previously.7,56

**Lentivirus Production**

Len-XIAP and Len-control were produced as described previously.28 Briefly, HEK293H cells (80% confluent) were co-transfected with three plasmids using calcium phosphate, including 5 μg of a packaging plasmid (pCMVΔR8.91), 2 μg of an envelope plasmid, and 3 μg of an expression vector (a plasmid encoding human XIAP, pCCL.CMV.XIAP, or pCCL.CMV.CTL for control). The conditioned medium containing the virus was collected at 48 and 72 hours. Virus was concentrated by centrifugation at 50,000 g for 1.5 hours. Pellets from eight dishes (150 mm) were pooled and concentrated by a second ultracentrifugation for another 90 minutes. The final pellet was resuspended in a 50-μl volume of sterile Hank’s solution containing 4 g/ml polybrene (Sigma-Aldrich). The virus titer was determined by serial dilution.

**Immunohistology, Immunoblot Analysis, and Antibodies**

Muscle tissues were cryosectioned at 10 μm for immunohistochemistry staining. Frozen sections were fixed in 4% paraformaldehyde for 10 minutes and quench-fixed in 50 μM NH₄Cl for another 10 minutes. Cross-sectional area was determined in frozen sections of plantaris muscles using an anti-laminin antibody to detect the muscle membranes. The area of at least 500 individual myofibers per muscle was measured using the Micro-suite Five Biologic Software (Olympus, Melville, NY).8 The levels of specific proteins were detected in the soluble fraction of the skeletal muscle homogenates by standard Western blotting.56 Muscles were homogenized in RIPA buffer except when we measured the 14-kD actin fragment, for which the muscles were homogenized in hypotonic buffer.1 The primary antibodies used were anti-XIAP (AF822, 1:1000 dilution; R&D System, Minneapolis, MN), anti-laminin antibody (1:100 dilution; Sigma-Aldrich); anti–caspase-3 (1:500 dilution; Cell Signaling, Danvers, MA), anti-GFP (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and anti–glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; 1:1000 dilution; Chemicon, Temecula, CA).

**Northern Blots**

Total RNA from the muscle was isolated using TriReagent (Molecular Research, Cincinnati, OH) and used in Northern blots for ubiquitin, atrogin-1/MAFbx, MuRF-1, and GAPDH as described previously.7,56 Autoradiographic band intensities were quantified by densitometry (Bio-Rad Gel Doc system) using the corresponding GAPDH to correct for variations in RNA loading and transfer.

**Caspase-3 Activity**

Caspase-3 activity in cultured cells was measured using the CaspACE Assay System according to the manufacturer’s protocol (Promega, Madison, WI). The protein content of supernatants was quantified with the RC-DC Protein Assay kit (Bio-Rad, Hercules, CA). We measured the activity of caspases using a fluorogenic substrate (Ac-DEVD-AMC), with or without a caspase-3 inhibitor (Ac-DEVD-CHO). The incubation temperature for fluorogen (7-amino-4-methylcoumarin [AMC]) release is 37°C for 1 hour. The difference between the substrate cleavage activity levels in the presence and absence of caspase inhibitor was used to calculate the contribution of caspase-3 activities.

**Chymotrypsin-like Proteasome Activity**

Proteasome was partially purified by differential centrifugation as described previously.28 Equal amounts

---

**Table 2.** Chymotrypsin-like proteasome activity in mouse muscle (nmol AMC/μg protein per h)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sham Gastrocnemius</th>
<th>Sham EDL</th>
<th>Sham Soleus</th>
<th>CKD Gastrocnemius</th>
<th>CKD EDL</th>
<th>CKD Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.8 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>10.5 ± 0.8*</td>
<td>9.3 ± 0.9*</td>
<td>8.5 ± 0.6*</td>
</tr>
<tr>
<td>mXIAP</td>
<td>3.9 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>4.0 ± 0.1</td>
<td>5.3 ± 0.5</td>
<td>4.7 ± 0.6</td>
<td>7.1 ± 0.5*</td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 6). *P < 0.05 versus WT.
of proteasome protein were used for measuring chymotrypsin-like proteasome activity. Proteasome activity was determined as the release of AMC from the fluorogenic peptide substrate LLVY-AMC (N-Suc-Leu-Leu-Val-Tyr-AMC).27 A proteasome inhibitor, 200 μM lactacystin (Calbiochem, San Diego, CA), was included with some samples. Fluorescence was measured in a fluorometer (Shimadzu, Columbia, MD) using a 380-nm excitation wavelength and a 460-nm emission wavelength. The difference between the substrate cleavage activity levels in the presence and absence of lactacystin was used to calculate the contribution of chymotrypsin-like proteasome activity.

Statistical Analysis

Data were expressed as means ± SE. To identify significant differences between two groups, comparisons were made by using the t test, whereas analyses of multiple groups were performed by the Kruskal-Wallis one-way ANOVA. P < 0.05 was considered significant.

ACKNOWLEDGMENTS

This work was supported in part by a Norman S. Coplon Extramural Research Grant from Satellite Health; the University Research Committee of Emory University; and National Institutes of Health grants DK62796 to X.H.W., DK062081 to J.D.K., DK50740 and DK61521 to S.R.P., and HL70762 to J.D.

Dr. Young Zhang kindly provided pBSCX1-LEL plasmid vector and technical support.

DISCLOSURES

None.

REFERENCES


www.jasn.org BASIC RESEARCH