

Regulation of Muscle Protein Degradation: Coordinated Control of Apoptotic and Ubiquitin-Proteasome Systems by Phosphatidylinositol 3 Kinase

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Abstract. Muscle proteolysis from catabolic conditions, including chronic kidney disease, requires coordinated activation of both the apoptotic and ATP-ubiquitin-proteasome systems (Ub-P'some), including upregulation of components of the Ub-P'some system. Activation of the apoptotic system is required because caspase-3 initially cleaves myofibrils, yielding substrates for the Ub-P'some system plus a characteristic 14-kD actin fragment. The authors studied insulin deficiency, a model of accelerated muscle atrophy, to understand how regulation of the apoptotic and the Ub-P'some systems could be coordinated. As expected, phosphatidylinositol 3 kinase activity (PI3K) was suppressed in muscle; in addition to decreased insulin, the mechanism includes IRS-1 phosphorylation at serine-307. Caspase-3 activity was also increased, and the authors linked it to a low PI3K-induced activation of the apoptotic system that includes a conformational change in Bax

and release of cytochrome C. Coordinated atrogen-1/MAFbx expression is required as a critical factor for Ub-P'some system-dependent muscle proteolysis in diabetes and other catabolic states. The mechanism that regulates atrogen-1/MAFbx expression is unknown. Atrogen-1/MAFbx expression increased when the authors suppressed PI3K activity in muscle cells. The forkhead transcriptional factor, a downstream substrate of PI3K, stimulated atrogen-1/MAFbx promoter transcriptional activity markedly. The authors found in diabetic muscle that mRNA of the forkhead transcriptional factor, its nuclear translocation, and binding to the atrogen-1/MAFbx promoter were increased. When PI3K activity is low, both apoptotic and Ub-P'some pathways are activated coordinately to cause muscle proteolysis. This mechanism could increase muscle atrophy in conditions with impaired insulin responsiveness.

Many catabolic conditions, including uremia, diabetes, sepsis, burn injury, etc., cause accelerated loss of muscle mass. Muscle protein loss in rodent models of these conditions results from an initial cleavage of actomyosin followed by accelerated breakdown of actin, myosin, and fragments of these proteins in the ubiquitin-proteasome (Ub-P'some) system (1). Many of the conditions that activate the Ub-P'some system to cause muscle atrophy, including uremia, exhibit insulin resistance, which suggests that a common pathway of muscle proteolysis is present. There is evidence in CAPD patients that activation of the Ub-P'some system contributes to muscle atrophy (2).

Regulation of the Ub-P'some system has been intensively

investigated because its activation is common to several conditions causing muscle atrophy (1). Recent results suggest that an E3 ubiquitin ligase plays a key role for Ub-P'some-mediated muscle protein degradation (3–6). First, catabolic states cause a significant increase in expression of this specific E3 (*atrogin-1* or *MAFbx*) in muscle. Second, its overexpression results in atrophy of muscle cells, while knockout of this gene leads to a 56% reduction in the degree of muscle atrophy that follows denervation (3). Consequently, identification of factors that regulate atrogen-1/MAFbx could provide insight into the mechanisms that accelerate muscle proteolysis.

In two conditions characterized by accelerated muscle protein loss, acute diabetes and chronic renal insufficiency, we found that the initial step in progressive loss of muscle protein is activation of caspase-3, which cleaves actomyosin to its constituent proteins and fragments of these proteins and leaves a “footprint” of caspase-3 action in muscle, a characteristic 14-kD actin fragment (7). The resulting substrates are degraded in the Ub-P'some system. The cellular signaling mechanisms that activate both apoptotic and the Ub-P'some pathways to cause loss of muscle protein remain obscure. One potential mechanism involves insulin/IGF-1 signaling pathways because the predominant effect of insulin on protein turnover in humans is to reduce protein degradation (8). Moreover, insulin resistance is common to metabolic acidosis, uremia, and other catabolic conditions that are associated with activation of the

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Ub-P³some system (9–11). These findings suggest that there may be a link between abnormalities in insulin signaling and activation of protein degradation in skeletal muscle (12). Insulin binding to its receptor stimulates tyrosine kinase activity, leading to phosphorylation of the insulin receptor substrate-1 (IRS-1) and activation of PI3K/Akt. PI3K/Akt reportedly can regulate apoptosis and activate transcription factors (13–15).

We examined the effects of insulin deficiency in rats to determine if coordinated regulation of apoptotic and Ub-P³some systems occurs in mammals. Like uremia, this model exhibits several characteristics, such as increased transcription of genes encoding components of the Ub-P³some system and accelerated muscle proteolysis via this system (16–18). Our goal was to identify signaling abnormalities that would activate both caspase-3 and increase the expression of the key E3 ubiquitin ligase, atrogin-1/MAFbx. Our results suggest that a decrease in PI3K signaling is a master signal that stimulates muscle proteolysis through coordinated regulation of caspase-3 and the expression of atrogin-1/MAFbx.

Materials and Methods

Male Sprague-Dawley rats weighing 150 to 200 g were purchased from Charles River Laboratories (Raleigh, NC); the experimental protocols were approved by the Institutional Animal Review board of the University of Texas Medical Branch at Galveston, Texas. L6 and C2C12 muscle cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Histologic Analysis of Muscle Atrophy in Response to Diabetes

Acute diabetes was induced in anesthetized rats, and they were pair-fed as described (18). For the experiment, the rats were re-anesthetized, and arterial blood was collected to measure blood glucose; the gastrocnemius muscles were removed, immediately frozen in liquid nitrogen, and then stored at -80°C .

Gastrocnemius muscles were studied because they contain both red and white muscle fibers, and protein turnover in the mixed muscle of the hindquarter exhibits similar responses to those of epitrochlear muscles that exhibit accelerated protein degradation in response to acute diabetes (18,19). Sections of gastrocnemius were examined for muscle atrophy. The muscle was pinned at resting length and embedded in HistoPrep media (Fisher 1 Reagent Lane; Fairlawn, NJ). Cross-sections of muscle were fixed in 4% paraformaldehyde at room temperature; after blocking and washing, the slides were incubated with anti-laminin antibody (Sigma-Aldrich) for 16 h at 4°C . The slides were stained with Avidin-Biotin-Complex method (Vector Lab, Burlingame, CA). Muscle cell area was determined by NIH ImageJ1.30v. In each section, 120 to 180 muscle cells were examined.

Cryosections of rat skeletal muscle (10 μm) were fixed in acetone at -20°C for 15 min and blocked for 30 min at room temperature. The slides were incubated for 16 h at 4°C with an antibody recognizing activated caspase-3 (1:200; Cell Signaling, Beverly, MA). Sections were subsequently incubated for 30 min at room temperature with Alexa Fluor 488 goat, anti-rabbit secondary antibody (1:400; Molecular Probes, Eugene, OR). After washing with PBS containing 0.1% Tween-20, slides were mounted with Vectashield Mounting medium containing propidium iodide (Vector Lab, Burlingame, CA), and microscopic images were captured with Spot RT advanced version 3.5.5 color digital camera

(Diagnostic Instrument, Inc., Sterling Height, MI). The specificity of immunolabeling was confirmed by replacement of primary antibody with rabbit IgG-negative control antibody (DakoCytomation, Carpinteria, CA). Cells positive for activated caspase-3 were quantified as the percentage of cells with activated caspase-3. For each section, 1000 to 1200 cells were counted for positive staining of myofibrils, and results are reported as the mean percentage of positive cells.

Preparation of Muscle for Immunoblotting

Gastrocnemius muscle (100 mg) was homogenized in 1 ml of RIPA buffer (50 mM Tris [pH 7.4]; 1% NP-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 1 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin, and pepstatin; 1 mM Na_3VO_4 ; 1 mM NaF). The mixture was centrifuged ($16,000 \times g$) at 4°C for 5 min, and protein in the supernatant was used to detect pIRS-1^{ser307} and pAkt by Western blotting using antibodies to pAkt (1:500) or pIRS-1^{ser307} (1:1000; Cell Signaling).

The activity of PI3K was measured as described (7). Activated Bax in homogenized muscle was measured as described (7). We measured the 14-kD actin fragment resulting from the activity of caspase-3 using described methods (7).

Cytochrome C in muscle cytoplasm was evaluated by homogenizing fresh muscle in buffer (20 mM HEPES [pH 7.5]; 210 mM mannitol; 250 mM sucrose; 10 mM KCl; 1.5 mM MgCl_2 ; 1 mM EDTA; 1 mM EGTA; 0.05% BSA plus 100 μM PMSF; 1 mM DTT; 10 $\mu\text{l}/10$ ml of protease inhibitor). Cell debris was removed by centrifugation ($800 \times g$) at 4°C for 10 min. The supernatant was centrifuged ($14,000 \times g$) at 4°C for 10 min to remove mitochondria and then again ($100,000 \times g$) at 4°C for 1 h. to obtain the cytosolic fraction. Cytochrome C in muscle cytoplasm was detected by Western blotting using a monoclonal antibody (1:500) for cytochrome C (BD PharMingen, San Diego, CA). The resulting immunoblots were examined for contamination by components of mitochondria; the blots were stripped and probed with 1:500 antibody against cytochrome oxidase subunit IV (Molecular Probes).

Expression of Ub-P³some System Components and the Forkhead Transcription Factor

In gastrocnemius muscle, the mRNA levels for atrogin-1/MAFbx and FKHL1 were measured as described (20). Nuclei from gastrocnemius muscle were isolated as described (20). The presence of the forkhead transcription factor in these nuclei was evaluated by Western blot using an antibody recognizing FKHL1 (Cell Signaling).

Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts from gastrocnemius muscle of diabetic and control rats (20). The extracts were exposed to a [^{32}P]-labeled oligonucleotide corresponding to forkhead consensus binding sites present in the rat atrogin-1/MAFbx promoter located at -340 to -363 and -1714 to -1743 (NCB1 Genome Annotation, NW_047779, containing the complete genomic sequence of rat atrogin-1/MAFbx).

To construct an atrogin-1/MAFbx promoter-reporter, we PCR-amplified rat atrogin-1/MAFbx promoter ($-2400/+1$) using a forward primer (5'GGGAGCTCCCCCTTGAACACAGAGCCC-3) and a reverse primer (5'TTCTCGAGGGTACCGAGAGCGGACGC-3). These were derived from NCB1 Genome Annotation, NW_047779. The promoter fragment was subcloned into *SacI* and *XhoI* sites of PGL2 Basic (Promega, Madison, WI).

To evaluate the regulation of atrogin-1/MAFbx transcription by forkhead, we co-transfected L6 muscle cells with the 2.4-Kb atrogin-1/MAFbx promoter-luciferase reporter with an equal amount of pCMV-FKHL1 or pCMV-GFP as described (20,21). Luciferase

activity was measured after 24 h. As a control, pGL2 basic was cotransfected with pCMV-FKHRL1.

Insulin Signaling and Protein Degradation in Muscle Cells

L6 skeletal muscle cells were cultured as described (7). To evaluate the influence of PI3K on protein degradation in muscle cells, we used a dominant-negative p85 subunit of PI3K in an adenovirus vector that also contains the GFP gene (7). Control cells were infected with adenovirus containing only the gene for GFP so the efficiency of infection can be examined by the presence of GFP expression. Protein degradation in cells prelabeled with L[U-¹⁴C] phenylalanine was measured as described (20).

Statistical Analyses

Values are presented as means ± SEM. Results were analyzed using *t* test when results from two experimental groups were compared or using ANOVA when data from three groups were studied. For data analyzed by ANOVA, pair-wise comparisons were made by the Student-Newman-Keuls test.

Results

Accelerated Muscle Atrophy and Abnormal Insulin Signaling

Since insulin resistance is present in uremia or metabolic acidosis as well as other catabolic states, we studied how acute

insulin deficiency in rats affects proteolytic pathways in muscle. As in uremia or metabolic acidosis, rats with insulin deficiency have suppressed PI3K activity in muscle, lose weight, and exhibit accelerated muscle proteolysis via activation of the Ub-P^osome pathway (16,17,18, 22,23). For these reasons and because insulin deficiency is more easily created and less expensive compared with models of metabolic acidosis or uremia, we studied how acute insulin deficiency affects apoptotic and Ub-P^osome pathways in muscle (16,22). We confirmed that these rats lose weight and develop muscle atrophy; in the gastrocnemius muscle, we found that the cross-sectional area of muscle fibers (Figure 1, A and B) from four acutely diabetic rats ($1390.6 \pm 109.2 \mu^2$) was significantly less than that of four pair-fed control rats ($2227.4 \pm 65.3 \mu^2$; $P < 0.001$). The loss of muscle mass was associated with accumulation of the characteristic 14-kD actin fragment (Figure 1C).

We previously linked the initial mechanism for muscle proteolysis to activation of caspase-3 (7). By immunohistochemical analysis, we found that the amount of activated caspase-3 in muscle of diabetic rats exceeded that in muscle of control rats ($0.56 \pm 0.17\%$ diabetic versus $3.48 \pm 0.43\%$ control; $P < 0.001$; Figure 2).

There also was a decrease in PI3K activity and in the level

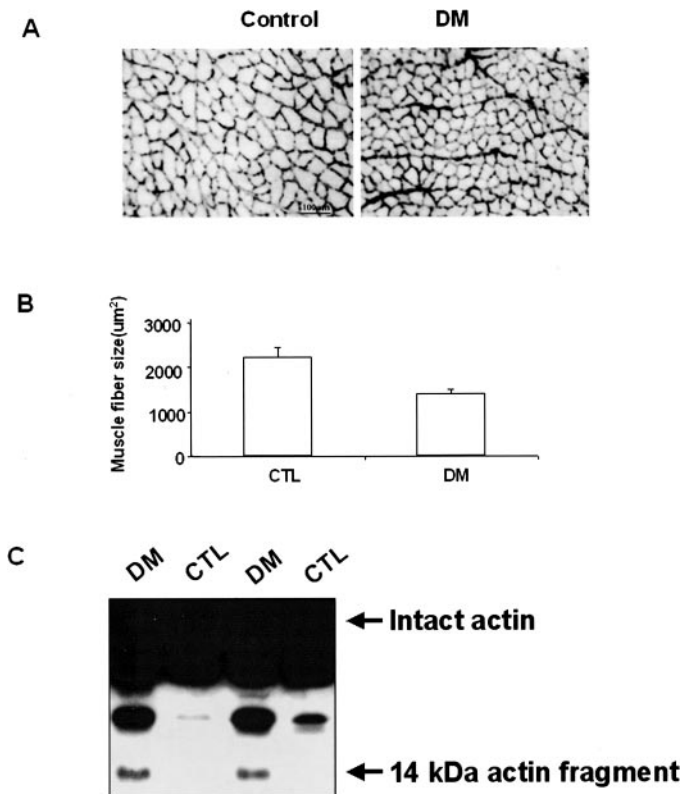


Figure 1. The muscle atrophy caused by insulin deficiency includes actin cleavage. (A and B) Insulin deficiency causes a significant ($P < 0.001$) decrease in the size of myofibers compared with sizes of those in muscle of control rats. (C) This loss of muscle mass from insulin-deficiency diabetes is associated with accumulation of the characteristic 14-kD actin fragment that results from cleavage of myofibrils by caspase-3.

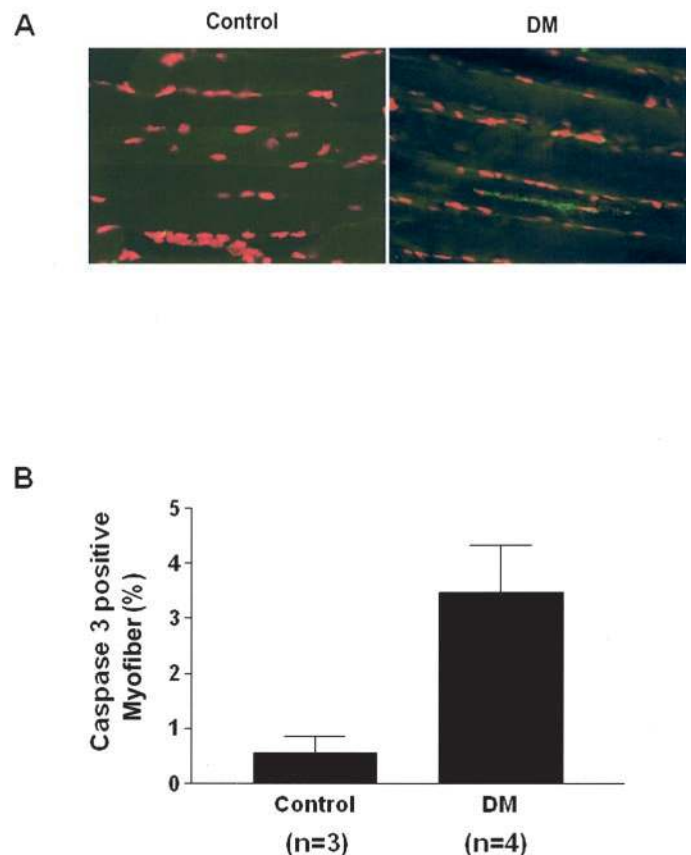


Figure 2. Caspase-3 is activated in muscle of diabetic rats. (A) Immunohistochemical evidence for the presence of activated caspase-3 was assessed in muscle of control and diabetic (DM) rats. (B) The percentage of cells staining positively for activated caspase-3 was calculated by counting the number of cells with activated caspase-3 present in 1000 myofibers.

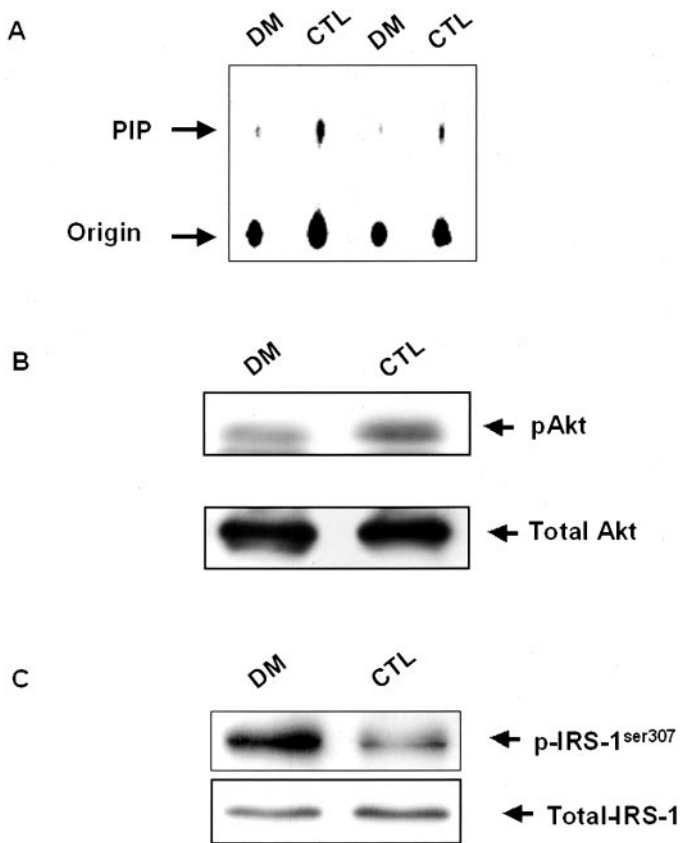


Figure 3. Insulin deficiency causes a decrease in PI3K activity in muscle. The activity of IRS-1-associated PI3K in muscle was measured in diabetic (DM) and control (CTL) rats. (A) DM was associated with significantly lower amount of phosphatidylinositol phosphate (PIP) production. (B) There also was less of the phosphorylated form of Akt in muscle of DM rats as determined by Western analysis of pAkt. (C) DM increased the level of IRS-1 that is phosphorylated on serine 307 (p-IRS-1^{Ser307}).

of phosphorylated Akt compared with results in muscle of control rats (Figure 3, A and B). To evaluate a mechanism for this response, we measured the level of phosphorylation of serine 307 in IRS-1 (p-IRS-1^{Ser307}); it was increased by diabetes (Figure 3C). This response is associated with insulin resistance in models of type II diabetes (24).

Activation of Apoptotic Pathways in Animal Muscle and Muscle Cells by a Low PI3K Activity

We evaluated how caspase-3 could be activated and studied the pro-apoptotic protein, Bax, because it can cause cytochrome C release into muscle cytoplasm (14). As shown in Figure 4A, there were greater amounts of activated Bax. This would lead to disruption of the mitochondrial inner membrane potential to release cytochrome C to activate caspase-3 (14). To determine if this sequence occurs, we measured the release of cytochrome C into muscle cytoplasm. The increase we found (Figure 4B) could not be attributed to contamination by mitochondria because there was no cytochrome C oxidase IV in the cytoplasm (Figure 4B).

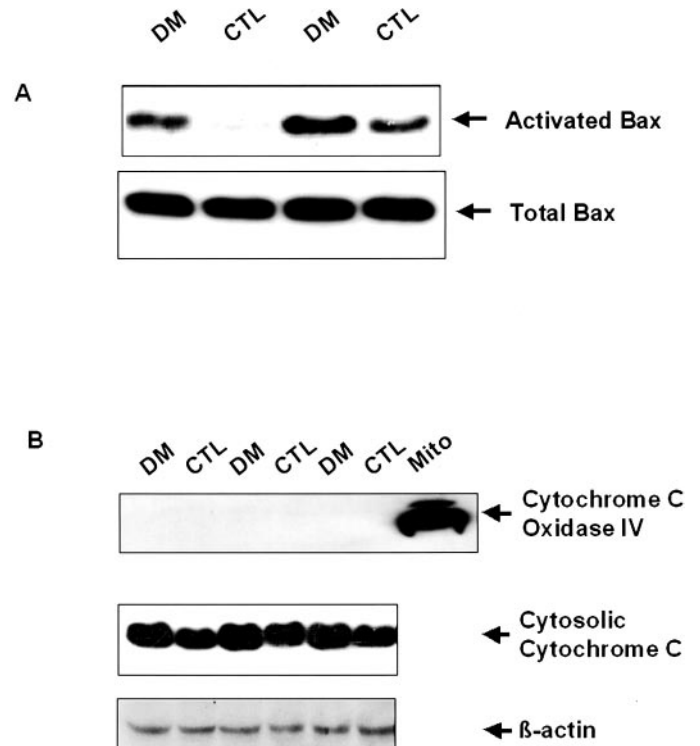


Figure 4. Insulin deficiency activates apoptotic signals in muscle. Activated Bax was immunoprecipitated with antibodies specific for this form of Bax. The amount of activated Bax was then detected by Western blot analysis. (A) The amount of Bax in the conformation that promotes apoptotic pathways was higher in muscle of diabetic (DM) compared with control (CTL) rats. This change was not due to differences in the total amount of Bax present. The increase in activated Bax in muscle of DM rats was associated with a higher level of cytochrome C in muscle cytoplasm. In each of three pairs, there was a higher level of cytochrome C in the cytoplasm of muscle of DM rats. This was not due to contamination of the cytosolic preparation by mitochondria, because there was no cytochrome C oxidase IV detected by Western blot.

To assess whether there is a causal relationship between suppressed PI3K and activated Bax, we incubated C2C12 muscle cells in serum-deprived media and measured activated Bax by Western blot (7). Serum deprivation increased the level of activated Bax, and addition of 10 ng/ml insulin or 10% FBS blocked this response (Figure 5). However, when the cells were treated with the PI3K inhibitor, LY294002, the ability of insulin or FBS to suppress the activation of Bax was low. Presumably, the lower level of activated Bax when serum-free treated cells were incubated with the PI3K inhibitor might be due to accelerated apoptosis in this condition (unpublished observations; J. Du and W.E. Mitch). Thus suppression of PI3K leads to activation of Bax.

These results provide a mechanism whereby low PI3K/Akt activity in intact muscle will increase the level of activated Bax to release cytochrome C and activate caspase-3. Caspase-3 then cleaves myofibrils to yield substrates for the Ub-P^osome system. This sequence explains our earlier results showing that

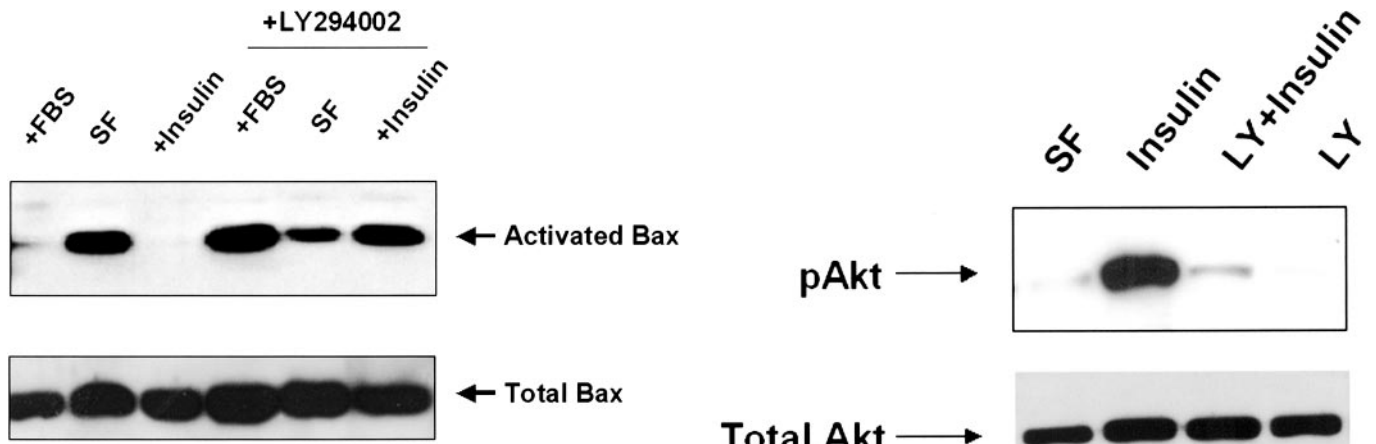


Figure 5. Suppression of PI3K activity in muscle cells leads to activation of Bax. C2C12 muscle cells were cultured in 10% FBS or serum free (SF) media with or without addition of 10 ng/ml insulin. The experiments were repeated in the presence of 50 μ M LY294002, the PI3K inhibitor. The amounts of activated and total Bax were measured. Serum-free media increased the activation of Bax, and the presence of LY294002 prevented 10% FBS or insulin from suppressing the activation of Bax.

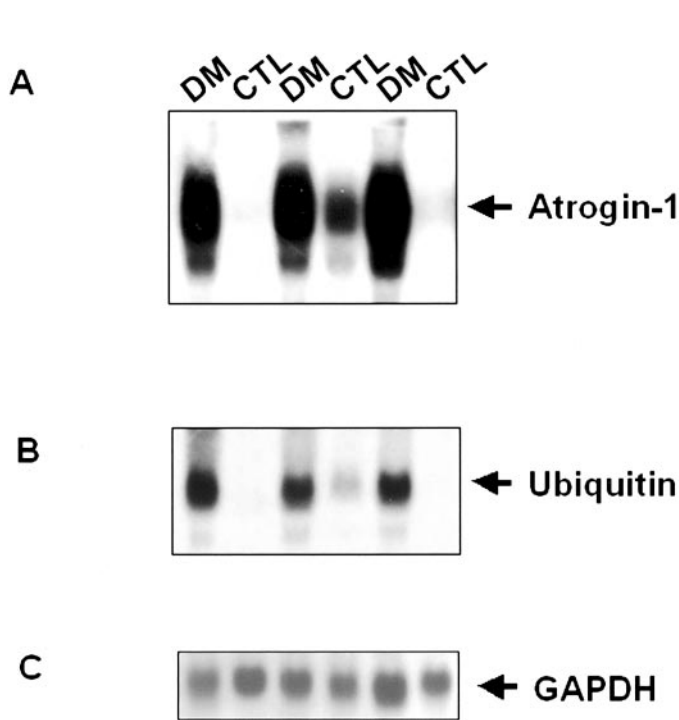


Figure 6. Diabetes is associated with an increase in the mRNA of atrogenin. The mRNA for atrogenin (A) was increased in muscle from diabetic (DM) compared with pair-fed control (CTL) rats.

inhibition of PI3K causes caspase-3–dependent actomyosin cleavage in cultured muscle cells (7).

Coordinated Activation of the Ub-P’some System by a Low PI3K Activity

The Ub-P’some system in muscle is markedly activated in response to insulin deficiency (18). As shown in Figure 6, we

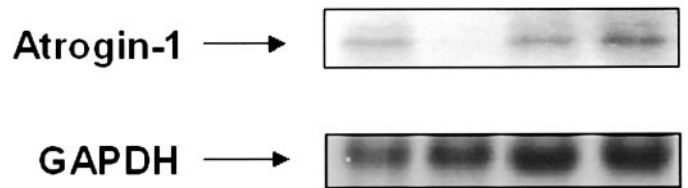


Figure 7. Inhibition of PI3K increases atrogenin expression in muscle cells. C2C12 muscle cells were cultured in serum-free media, and the mRNA level of atrogenin was measured. The experiment was repeated in cells treated for 6 h with 10 ng/ml insulin, with LY294002 (LY), or with LY294002 plus insulin. Serum-free media increased atrogenin mRNA, and insulin suppressed this response unless LY274002 was present to inhibit PI3K activity

confirmed an increase in the levels of mRNAs encoding the E3 ubiquitin conjugating enzyme, atrogenin-1/MAFbx, and ubiquitin (5,18). Does reduced PI3K activity lead to coordinated regulation of the Ub-P’some system?

Identification of Forkhead Transcription Factor as a Regulator of the Ub-P’some System

We studied C2C12 muscle cells treated with LY 294002 to evaluate whether increased levels of these mRNAs could be linked to reduced PI3K activity. As shown in Figure 7, adding insulin to serum-deprived cells led to an increase in the amount of activated Akt (pAkt) and suppression of the expression of the mRNA of atrogenin-1/MAFbx. There was a marked decrease in the amount of pAkt when we added the PI3K inhibitor LY294002 to serum-deprived cells. The same response occurred even if insulin was added. Inhibition of PI3K also increased the mRNA of atrogenin-1/MAFbx. A similar response occurred when insulin was added with the PI3K inhibitor. Thus, low PI3K activity increases the expression of the atrogenin-1/MAFbx gene.

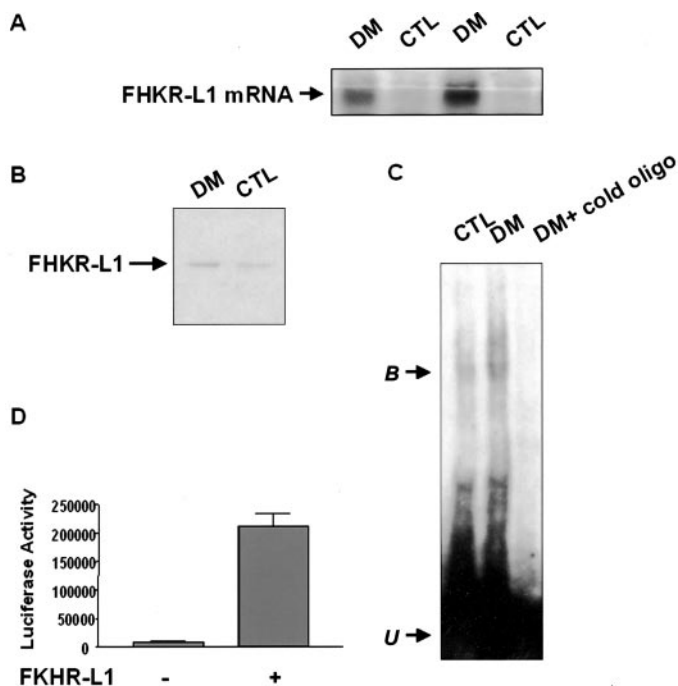


Figure 8. The forkhead transcription factor (FKHRL1) is increased in muscle of diabetic rats and stimulates transcriptional activity of the atrogin promoter in muscle cells. The levels of FKHRL1 mRNA (A) in muscle and the FKHRL1 protein in muscle nuclei (B) were both increased in muscles of diabetic (DM) compared with control (CTL) rats. (C) The results of an EMSA performed using nuclear extracts from muscle of CTL and DM rats with a labeled probe corresponding to the region -340 to -363 in the promoter of atrogin that has homology with a forkhead binding site. The binding of proteins isolated from nuclei of DM rat muscle to this probe was increased but eliminated by including an excess of unlabeled probe in the incubation. (D) The results of co-transfecting L6 muscle cells with a plasmid containing an atrogin promoter-luciferase reporter construct and/or a plasmid that leads to expression of FKHRL1. Luciferase activity was assayed 24 h after transfection. Transfection with plasmid that expresses FKHRL1 (FKHRL1) or with the atrogin promoter-luciferase reporter plasmid alone (RA 1) did not increase transcriptional activity of the atrogin promoter. In two independent experiments, we found that transfection with both plasmids led to a marked increase in atrogin transcriptional activity.

We measured changes in the forkhead transcription factor FKHRL1, a downstream substrate of PI3K, to identify a mechanism by which reduced PI3K activity increases atrogin-1/MAFbx expression. Insulin deficiency increased the levels of FKHRL1 mRNA in muscle (Figure 8, A and B), and the FKHRL1 protein in muscle nuclei increased (Figure 8B). To explore the relevance of these changes to atrogin-1/MAFbx expression, we isolated nuclei from muscle and analyzed an EMSA (20) using a labeled probe corresponding to a homologous forkhead binding site (-340 to -363) in the atrogin-1/MAFbx promoter. Insulin deficiency induced binding of nuclear proteins to the forkhead site in the promoter of atrogin-1/MAFbx (Figure 8C). There was no shift when an excess of

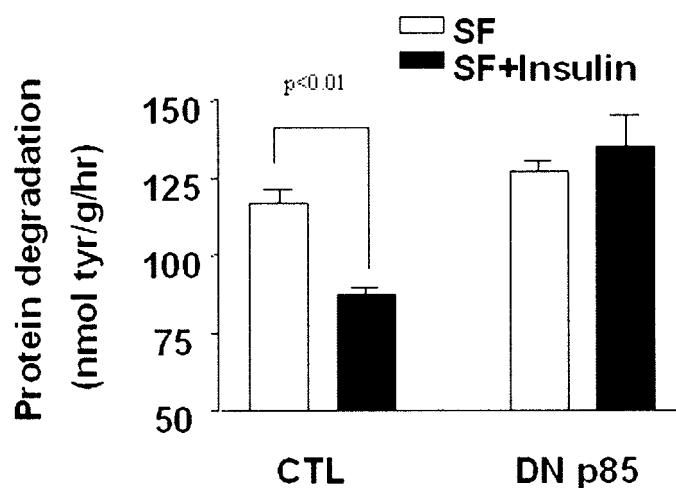


Figure 9. PI3K activity regulates protein degradation in muscle cells. Rates of protein degradation (nmol Tyr/g/h) were measured in control L6 muscle cells (CTL) that were infected with an adenovirus expressing GFP. Other cells were infected with an adenovirus expressing GFP and a dominant negative inhibitor of PI3K activity (DN p85). The cells were incubated in serum-free (SF) media or in SF media supplemented with 10 ng/ml insulin. In control cells, insulin suppressed protein degradation in muscle cells. Insulin did not suppress protein degradation when PI3K activity was inhibited.

the unlabeled probe was added, suggesting specificity of the binding.

We evaluated the contribution of FKHRL1 to the regulation of atrogin-1/MAFbx by co-transfecting 2.4 Kb of the rat atrogin-1/MAFbx promoter-luciferase construct and FKHRL1 cDNA. Expression of FKHRL1 in L6 muscle cells increased atrogin-1/MAFbx transcriptional activity by approximately 20-fold (Figure 8D).

Protein Degradation in Response to Inhibition of PI3K in Muscle Cells

To evaluate if reduction of PI3K activity leads to upregulation of apoptotic and Ub-P^osome systems and stimulation of muscle protein degradation, we used an adenovirus containing the DNA for GFP and a dominant negative inhibitor of PI3K, AdTrack p85 Δ iSH2. As reported (7), we confirmed that incubation of L6 muscle cells in serum-free media resulted in a low level of PI3K activity and that adding insulin sharply increased the activity of PI3K. Moreover, in L6 cells infected with an adenovirus containing the DNA of a dominant negative PI3K construct, AdTrack p85 Δ iSH2, there was only a limited response of PI3K activity when insulin was added (7). In companion studies, we found that incubation of L6 muscle cells in serum-free media stimulated total protein degradation compared with results obtained from cells treated with insulin (Figure 9). In cells in which the adenovirus inhibited PI3K activity (DN p85), insulin did not suppress protein degradation. These results link the suppression of PI3K activity in muscle to expression of atrogin-1/MAFbx and activation of caspase-3 to increased protein degradation.

Discussion

Our results show that suppression of PI3K activity is a key event that triggers muscle protein degradation. When we suppressed the activity of this enzyme, we found that there is an increase in total protein degradation (Figure 9) accompanied by a rise in the activity of two proteolytic pathways causing proteolysis. There was activation of a specific sequence of events in the muscle of rats developing muscle atrophy because of a low insulin level. The sequence consists of upregulation of apoptotic signals related to low PI3K/Akt activity with activation of Bax, leading to cytochrome C release and ultimately to activation of caspase-3, which cleaves actomyosin-providing substrates for the Ub-P^{some} system. We also identified a mechanism for the increase in the activity of the Ub-P^{some} activity (18), including an increase in expression of Atrogin-1/MAFbx. These results in intact muscle extend our earlier report of results in cultured cells (7).

How could PI3K activity be suppressed in catabolic states? A possible mechanism is insulin resistance due to phosphorylation of serine 307 on IRS-1; this phosphorylation impairs IRS-1 docking with PI3K to decrease PI3K/Akt activity (25,26). Because such changes have been linked to TNF α responses, the mechanism we describe could apply to many conditions associated with high TNF α levels, including chronic kidney disease (24,27).

A critical aspect of the muscle cell signaling abnormalities associated with suppressed PI3K activity is activation of caspase-3. Caspase-3 activation in muscle (Figure 2) is important because the Ub-P^{some} system does not degrade actomyosin, yet loss of actomyosin is characteristic of muscle atrophy (Figure 1B) that occurs in catabolic conditions (1,28). The cleavage of actomyosin can be identified by the accumulation of the 14-kD actin band in muscle, a response that occurs in rats with chronic uremia or insulin deficiency (7). Others have reported that stimulation of PI3K activity will suppress apoptotic pathways (29,30). In rat skeletal muscle, we found changes in signaling proteins that can explain the link between low PI3K activity and activation of apoptotic pathways. Low PI3K and hence Akt activity (Figure 3) were associated with an increase in activated Bax (Figure 4B and 5), a pro-apoptotic factor (14). Activated Bax promotes the release of cytochrome C from mitochondria (Figure 4A), resulting in activation of caspase-3 (14). Identifying how insulin deficiency activates Bax provides a key mechanism that explains why we found increased activity of caspase-3 in association with muscle fibers (Figure 2) and an increase in the 14-kD actin fragment in muscle.

In rat muscle with metabolic acidosis, uremia, or insulin deficiency, the Ub-P^{some} system is activated (16,18,22), which contributes to muscle protein loss (Figure 1, A and B). Interestingly, we found higher levels of atrogin-1/MAFbx, the E3 enzyme that is closely associated with activation of protein degradation in muscle of rats with uremia as well as insulin deficiency (4,5). Members of the forkhead family of transcriptional factors are downstream

substrates of PI3K (21,31); when PI3K/Akt activity is low, forkhead transcriptional factors are dephosphorylated and are found in the nucleus, where they activate transcription. Upon PI3K activation, Akt/SGK phosphorylates forkhead factors, thereby promoting their exit from the nucleus and resulting in inhibition of forkhead-dependent transcription (21,31). Our results provide a mechanism by which insulin deficiency activates the forkhead transcription factor (Figure 8) and increases FKHRL1 mRNA expression, translocation of FKHRL1 to the nucleus, and its binding to forkhead elements present in the atrogin-1/MAFbx promoter. This sequence would ultimately lead to a 20-fold increase in atrogin-1/MAFbx promoter transcriptional activity (Figure 8). The higher levels of atrogin-1/MAFbx mRNA (Figures 6A and 7) in muscle of diabetic rats was also approximately 20-fold higher than that in muscle of control rats, indicating that an increased forkhead transcriptional activity may mediate most if not all of increased expression of atrogin-1/MAFbx in catabolic states. These results have significant physiologic relevance because others report that the forkhead transcription factor is increased in the muscle of mice subjected to starvation or glucocorticoid administration; both conditions can activate the Ub-P^{some} system, and glucocorticoids are necessary for this activation in both acidosis and insulin deficiency (23,32–34).

Our results identify mechanisms by which suppression of PI3K activity could coordinately activate apoptotic and Ub-P^{some} systems to cause protein breakdown in muscle. Muscle atrophy results from an imbalance in protein synthesis and degradation, and the role of PI3K in muscle atrophy is often attributed to a decrease in protein synthesis. For example, Bodine *et al.* (6) found that muscle hypertrophy was associated with upregulation of the Akt/mTOR pathway and suggested that the PI3K pathway mediates muscle hypertrophy. This group also reported that the PI3K/Akt pathway is suppressed in conditions causing muscle atrophy. Besides suppressing protein synthesis, our findings show that a reduced PI3K activity contributes to muscle atrophy via coordinated regulation of apoptotic and Ub-P^{some} systems, ultimately leading to protein degradation. It is tempting to speculate that the identified pathways account for the excessive muscle protein loss that occurs in conditions associated with insulin resistance, including metabolic acidosis, uremia, and conditions that increase glucocorticoids (11,16,27,35–39).

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