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The intracellular signals that mediate skeletal muscle protein loss and functional deficits due to muscular disuse are just beginning to be elucidated. Previously we showed that the activity of an NF-κB–dependent reporter gene was markedly increased in unloaded muscles, and p50 and Bcl-3 proteins were implicated in this induction. In the present study, mice with a knockout of the p105/p50 (*Nfkb1*) gene are shown to be resistant to the decrease in soleus fiber cross-sectional area that results from 10 days of hindlimb unloading. Furthermore, the marked unloading-induced activation of the NF-κB reporter gene in soleus muscles from WT mice was completely abolished in soleus muscles from *Nfkb1* knockout mice. Knockout of the B cell lymphoma 3 (*Bcl3*) gene also showed an inhibition of fiber atrophy and an abolition of NF-κB reporter activity. With unloading, fast fibers from WT mice atrophied to a greater extent than slow fibers. Resistance to atrophy in both strains of knockout mice was demonstrated clearly in fast fibers, while slow fibers from only the *Bcl3*–/– mice showed atrophy inhibition. The slow-to-fast shift in myosin isoform expression due to unloading was also abolished in both *Nfkb1* and *Bcl3* knockout mice. Like the soleus muscles, plantaris muscles from *Nfkb1*–/– and *Bcl3*–/– mice also showed inhibition of atrophy with unloading. Thus both the *Nfkb1* and the *Bcl3* genes are […]

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The intracellular signals that mediate skeletal muscle protein loss and functional deficits due to muscular disuse are just beginning to be elucidated. Previously we showed that the activity of an NF-κB–dependent reporter gene was markedly increased in unloaded muscles, and p50 and Bcl-3 proteins were implicated in this induction. In the present study, mice with a knockout of the p105/p50 (Nfkb1) gene are shown to be resistant to the decrease in soleus fiber cross-sectional area that results from 10 days of hindlimb unloading. Furthermore, the marked unloading-induced activation of the NF-κB reporter gene in soleus muscles from WT mice was completely abolished in soleus muscles from Nfkb1 knockout mice. Knockout of the B cell lymphoma 3 (Bcl3) gene also showed an inhibition of fiber atrophy and an abolition of NF-κB reporter activity. With unloading, fast fibers from WT mice atrophied to a greater extent than slow fibers. Resistance to atrophy in both strains of knockout mice was demonstrated clearly in fast fibers, while slow fibers from only the Bcl3−/− mice showed atrophy inhibition. The slow-to-fast shift in myosin isoform expression due to unloading was also abolished in both Nfkb1 and Bcl3 knockout mice. Like the soleus muscles, plantaris muscles from Nfkb1−/− and Bcl3−/− mice also showed inhibition of atrophy with unloading. Thus both the Nfkb1 and the Bcl3 genes are necessary for unloading-induced atrophy and the associated phenotype transition.

Introduction
Reduced muscular activity due to bed rest, limb immobilization, sedentary lifestyles, or space flight is a widespread phenomenon that leads to significant muscle atrophy, weakness, fatigue, and insulin resistance (reviewed in ref. 1). While it has been known for some time that the loss in muscle protein due to disuse results from decreases in protein synthesis and increases in protein degradation rates (2), we are just beginning to learn about the upstream triggers and signaling pathways that regulate the changes in these target systems that determine protein content. While myostatin (3, 4) and glucocorticoids (5) have been studied for a role in atrophy, and both systems that determine protein content. While myostatin (3, 4) and glucocorticoids (5) have been studied for a role in atrophy, and both systems that determine protein content.

Vertebrate NF-κB represents a family of 5 transcription factors (p65/RelA, c-Rel, RelB, p50, and p52) that contain a Rel homology domain, which confers the dimerization and DNA-binding properties. Active dimers of NF-κB can be formed by various combinations of family members (reviewed in refs. 15, 16). The IkB family members contain a conserved domain of 6–7 ankyrin repeats, which bind the Rel domain of NF-κB and prevent nuclear translocation. Activation of NF-κB occurs when IkBs are phosphorylated by the IkB kinase complex, leading to ubiquitination and degradation of IkB and nuclear translocation of the NF-κB dimer. Known IkBs include p105 and p100 (precursors to p50 and p52, respectively), IkBo, IkBβ, IkBγ, and IkBo (17).

Another IkB family member, Bcl-3, is a protooncogene that is transcriptionally upregulated in some cases of chronic lymphocytic leukemia because of its location next to the breakpoint junction of a t(14;19) translocation. In various cancers, Bcl-3–p50 complexes have been shown to bind to NF-κB cis-elements and are thought to activate transcription (18–21). Generally, p50 and p52 homodimers, which lack transactivation domains, function as transcriptional repressors (22). However, upon binding with Bcl-3, these complexes can activate transcription through the Bcl-3 transactivation domain (23, 24). Thus, Bcl-3 is an unusual member of the IkB family, because it can function as a transcriptional coactivator.

As a direct extension of our previous work, here we tested whether the p105/p50 (Nfkb1) gene or the B cell lymphoma 3 (Bcl3) gene was required for unloading-induced NF-κB activation and muscle atrophy. To do this, we used knockout mice for each gene and determined NF-κB reporter activity, muscle fiber size, and general myosin phenotype after 10 days of hindlimb unloading. Compared with fiber atrophy of soleus muscles from WT mice, fiber atrophy of soleus muscles from both types of knock-
out mice was abolished, while atrophy of the plantaris muscle was inhibited by 67% in these same mice. In the soleus, the sevenfold unloading-induced increase in WT NF-κB reporter activity was eliminated in both strains of knockout mice. In knockout mice there also was a complete inhibition of the typical slow-totarget shift in myosin isoform expression seen in WT mice. Overall, these data suggest that muscular inactivity triggers the activation of Bcl-3 and p50, which in turn may activate genes that are necessary for the induction of muscle atrophy.

Results

Descriptive characteristics of p105/p50–/– (Nfkb1–/–) and Bcl3–/– mice

To investigate a potential role of p50 and Bcl-3 transcription, and p50 and Bcl-3 proteins were implicated in this development. Also, in some of the smaller-fiber-size categories in both knockout mice, there appear to be more fibers from unloaded than from control mice. This is likely a reflection of the 8% (in Nfkb1–/– mice) and 11% (in Bcl3–/– mice) difference in mean fiber area with unloading, which did not reach statistical significance.

To determine whether the inhibition of atrophy occurred in another muscle besides the mixed (50% slow and 50% fast) soleus muscle, the fast plantaris muscle was also measured for changes in fiber size. Even though the mouse plantaris contains predominantly fast myosin (95% fast), it atrophied to an extent similar to that of the mouse soleus with unloading, as has also been shown by others (25–27).

Plantaris muscle sections from WT, Nfkb1–/–, and Bcl3–/– mice exhibited normal morphology based on the H&E stain (Figure 5, A–F). Sections stained for anti-laminin (immunofluorescence) were used to measure fiber cross-sectional area (Figure 5, G–L). The mean fiber size of the WT unloaded plantaris muscles (789 ± 53 μm²) was 27% smaller (Figure 6A) than that of weight-bearing control muscles stained for anti-laminin (Figure 3, G–L), and these sections were used to measure the fiber cross-sectional area. For experiments with mice, between 300 and 500 fibers were measured per muscle. The mean fiber size of the WT unloaded soleus muscles (623 ± 37 μm²) showed a 29% decrease (Figure 4A) compared with that of weight-bearing control muscles (874 ± 39 μm²). In the Nfkb1–/– mice the soleus fiber atrophy was inhibited (control, 815 ± 43 μm²; unloaded, 742 ± 31 μm²). There was a similar inhibition of fiber atrophy in the Bcl3–/– mice after unloading (control, 974 ± 23 μm²; unloaded, 869 ± 62 μm²). There were no statistically significant differences in control mean fiber area between WT and Nfkb1–/– mice or between WT and Bcl3–/– mice.

Plots of the frequency distribution for each set of measurement revealed a clear increase in the percentage of small fibers (i.e., a leftward shift) in the unloaded muscle of WT mice (Figure 4B). When the Nfkb1 gene was knocked out, the shift to smaller fibers was inhibited (Figure 4C).

The lack of Bcl3 also inhibited the typical leftward shift of the fiber-size distribution (Figure 4D). The shape of the frequency distribution in the Bcl3–/– mice was slightly different from that of either the WT or the Nfkb1–/– mice. This mild effect on fiber-size distribution may be due to the absence of Bcl-3 protein during muscle development. Also, in some of the smaller-fiber-size categories in both knockout mice, there appear to be more fibers from unloaded than from control mice. This is likely a reflection of the 8% (in Nfkb1–/– mice) and 11% (in Bcl3–/– mice) difference in mean fiber area with unloading, which did not reach statistical significance.

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Figure 1

Protein expression of p105, p50, and Bcl-3 from WT, Nfkb1 knock-out (−/−), and Bcl3 knockout (−/−) mouse soleus muscle lysates by immunoblotting. There was no protein expression of Bcl-3 or of p105/p50 in extracts from the Bcl3–/– or Nfkb1–/– mice, respectively.

Figure 2

NF-κB reporter activity in control and unloaded muscle from WT versus knockout mice. WT mice showed a sevenfold activation of the NF-κB–dependent reporter in unloaded (n = 25) compared with weight-bearing control muscles (n = 24). The NF-κB–dependent activation seen in WT mice was eliminated in the Nfkb1 (control, n = 20; unloaded, n = 20) and Bcl3 (control, n = 6; unloaded, n = 6) knockout mice. *Mean value differs significantly from control (P < 0.05). RLU, relative luciferase units.
However, mean fiber area of Nfkb1−/− mice only atrophied 9% due to unloading; this represents a 67% attenuation of atrophy (control, 958 ± 63 μm²; unloaded, 853 ± 40 μm²). Mean fiber area in the Bcl3−/− mice only atrophied 10% because of unloading; this represents a 63% inhibition of atrophy (control, 954 ± 42 μm²; unloaded, 845 ± 62 μm²). To further describe the changes in fiber areas, frequency distributions were plotted. Fibers from unloaded WT mice showed a shift to a larger percentage of smaller fibers compared with fibers from the weight-bearing controls (Figure 6B). However, both the Nfkb1−/− (Figure 6C) and the Bcl3−/− (Figure 6D) mice showed a much smaller difference in fiber-size distribution due to unloading compared with WT mice. The difference in the shapes of the frequency distributions between the soleus and the plantaris muscle is due to the differences in motor unit composition (28).

Myosin isoform expression in control and unloaded muscles from WT versus knockout mice. During muscle unloading it has long been noted that slow-twitch rodent muscles such as the soleus express more fast myosin isoforms and have faster shortening velocities than control muscles (reviewed in ref. 1). Since knockout of the Nfkb1 and Bcl3 genes largely inhibited atrophy, we also determined whether the absence of knockout mice showed a much smaller difference in fiber-size distribution due to unloading compared with WT mice. The difference in the shapes of the frequency distributions between the soleus and the plantaris muscle is due to the differences in motor unit composition (28).

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**Figure 3**
Soleus muscle cross sections in control and unloaded WT, Nfkb1−/−, and Bcl3−/− mice. (A–F) Morphology of representative sections using H&E stain. (G–L) Representative cross sections from anti-laminin–stained soleus muscles. The anti-laminin–stained sections were used for measurement of mean fiber area. Scale bars: 50 μm.

**Figure 4**
Fiber size in control and unloaded soleus muscles from WT versus knockout mice. (A) Mean percentage reduction in soleus fiber cross-sectional area in WT (control, n = 17 muscles; unloaded, n = 15 muscles), Nfkb1−/− (control, n = 10 muscles; unloaded, n = 10 muscles), and Bcl3−/− mice (control, n = 6 muscles; unloaded, n = 6 muscles) after 10 days of unloading. Mean fiber cross-sectional area decreased by 29% in WT mice and was not significantly different in control versus unloaded muscles of Nfkb1−/− or Bcl3−/− mice. (B–D) Frequency distribution of fiber cross-sectional area in control and unloaded WT (B), Nfkb1−/− (C), and Bcl3−/− mice (D). *Mean value differs significantly from control (P < 0.05).
these genes would obviate the characteristic increase in fast myosin expression due to unloading. Muscle sections were immunostained using an antibody recognizing the fast myosin heavy chains (fMHCs) IIa and IIb (Figure 7, A–F). Unloaded WT mice exhibited a typical phenotypic shift to a faster muscle (Figure 7G). Expression of fMHC significantly increased by 12% in control compared with unloaded muscles. These results are consistent with recent reports of mice that underwent hindlimb unloading for 10 days (29) as well as other similar studies (reviewed in ref. 30). However, knockout of the Nfkb1 gene completely abolished the shift to a faster muscle (Figure 7G). Similarly, in the Bcl3–/– mice, the shift to a faster phenotype with unloading was blocked. The phenotype of both knockout strains is slightly different from that of WT. Soleus muscles from Nfkb1 knockout mice showed a 12% greater percentage of fibers expressing fMHC, while muscles from Bcl3–/– mice expressed a 16% lower percentage of fMHC. However, independent of initial myosin isoform composition, the shift to a faster phenotype was abolished.

Atrophy of type I versus type II fibers in control and unloaded soleus muscles from WT versus knockout mice. The atrophy of the soleus muscle during hindlimb unloading is due to a decrease in fiber size with no change in fiber number (31). The decrease in cross-sectional area of type II (fast) fibers in the unloaded mouse soleus muscle is...
The majority of work that has been performed on NF-κB has involved the canonical p65-p50 heterodimer; however, our data support the requirement of Bcl-3 and p50 in muscle-disuse atrophy, because of the inhibition of NF-κB activity and muscle fiber atrophy in mice with a knockout of either gene. Importantly, our previous work showed increased nuclear κB–binding complexes containing Bcl-3 and p50, but not p65, in nuclear extracts from unloaded rat muscle (10). While p50 lacks a transactivation domain and homodimers can act as transcriptional repressors (22), Bcl-3–p50 complexes can activate transcription (24). Physiological examples equal to, and in some cases greater than, the atrophy seen in type I (slow) fibers within the first 2 weeks of the non–weight-bearing condition (reviewed in ref. 2). To determine whether there was a difference in atrophy in type I versus type II fibers due to unloading, we measured fiber type–specific cross-sectional area in WT mice. Fibers expressing fast myosin atrophied to a greater extent (33%) than slow fibers (17%) (Figure 8). To determine whether the inhibition of atrophy in the knockout mice was due to a lack of atrophy in slow and/or fast fibers, we assessed type I and type II fiber area in soleus muscles from Nfkb1−/− and Bcl3−/− mice. In Nfkb1−/− mice, fast fibers atrophied only 10% and slow fibers atrophied 15%. Thus the inhibition of atrophy was marked in fast fibers, while atrophy occurred to a normal extent in slow fibers. In the Bcl3−/− mice, there was a 12% decrease in fiber size in fast fibers and a lack of atrophy in slow fibers. The inhibition of atrophy in these mice was due to reduced atrophy in both fast and slow fibers.

While mouse soleus muscles have near-equal numbers of fast and slow fibers, fibers from plantaris muscles express predominantly (95%) fast myosin (data not shown). Thus the plantaris muscle atrophy seen with hindlimb unloading (27%) in WT mice (Figure 6A) can be attributed almost entirely to fast fibers. Thus the lack of atrophy in the plantaris muscles from Nfkb1−/− and Bcl3 knockout mice is due largely to the lack of atrophy of fast fibers.
Involving Bel-3–p50 complexes have also been identified. Nasopharyngeal carcinoma is associated with Bel-3–p50 complexes bound to the epithelial growth factor receptor promoter (18). Squamous cell carcinomas are associated with increased Bel-3 expression and Bel-3–p50 binding to κB sites. In addition, overexpression of Bel-3 in thymocytes (19) or in various B cell lines (20) leads to increased formation of nuclear Bel-3–p50 complexes.

Several possible pathways lead to the formation of Bel-3–p50 complexes in the nucleus. Increased Bel-3 expression alone can lead to increased formation of Bel-3–p50 complexes in thymocytes (19) and in B cell lines (20), without the processing of p105. Thus, it is possible that increased expression of Bel-3 in unloaded muscle is driving the formation of Bel-3–p50 complexes and that these are involved in the transactivation of specific κB site–dependent genes. It is also possible that unloading induces the processing of p105. Inducible proteolysis of p105 by TNF, IL-1β, or phorbol ester increases the formation of Bel-3–p50 complexes, but p105 is completely processed; this implies the dimerization of newly released p50 proteins and either subsequent or concurrent sequestration by Bel-3 (32). However, the upstream triggers for the involvement of Bel-3 and p50 in the process of muscle–disuse atrophy have yet to be determined.

While the target genes of NF-κB in muscle atrophy have not been elucidated, some downstream effects of NF-κB activation include the induction of genes involved in the ubiquitin-proteasome pathway. UbCH2/E220k, a ubiquitin-conjugating gene involved in the ubiquitin-proteasome pathway, has been shown to be a target of NF-κB in TNF-α–treated muscle cells (33). Our microarray data from control and unloaded rat muscle showed the upregulation of many genes involved in ubiquitin-proteasome–mediated degradation (34), and several of these genes have multiple NF-κB sites in their regulatory regions, including the E3 (i.e., the ubiquitin-protein ligase) MAFlbx/atrogen-1. While atrogen-1 was also activated in a cell culture condition where myotubes were fed only PBS for 6 hours, it did not show increased NF-κB binding in a gel-shift assay (35). This suggests that PBS-fed (“starved”) cells do not model the in vivo atrophy process that results from unloading. Nevertheless, genes involved in ubiquitination and proteasomal degradation may be NF-κB targets during disuse atrophy, but this has yet to be determined experimentally.

Significant attention has been paid to the requirement of the ubiquitin-proteasome pathway for muscle atrophy (36). Much of this work focuses on the role of the ubiquitin ligases, in part because they have the greatest tissue and substrate specificity (37). Two groups simultaneously identified the muscle-specific F-box–containing ubiquitin-protein ligase MAFlbx/atrogen-1 and found it to be markedly upregulated with atrophy due to disuse (38) and in cachexia (39). When MAFlbx knockout mice are subjected to muscle denervation, gastrocnemius atrophy is attenuated by 56% at 14 days (38). Another E3 found to be increased by disuse atrophy (unloading, immobilization, or denervation) was a RING finger–containing ubiquitin ligase named MuRF1 (muscle RING finger 1). MuRF knockout mice showed a 36% attenuation of muscle atrophy after 14 days of gastrocnemius denervation (38).

Regarding other signaling pathways that may be involved in regulating atrophy, recent work implicates inhibition of the Akt growth pathway in the progression of disuse atrophy. The proteins of the Akt cascade of kinases involved in growth control have been shown to be dephosphorylated with unloading, consistent with the decreased protein synthesis rate. This includes Akt (7), mTOR (8), and p70s6 kinase (7, 9), as well as the transcription initiation factor Phas-1 (7). Overexpression of active Akt in denervated muscle prevents atrophy (7, 40), whereas inhibition of Akt produces mild atrophy in normal whole muscle and muscle cells (40, 41). When Akt is dephosphorylated, one of its targets, the forkhead box O (FOXO) family of transcription factors, is activated via dephosphorylation and then nuclear transport. This activity has been linked to the proteolysis associated with dexamethasone– and fasting-induced atrophy (35, 42). However, the requirement of FOXO during disuse atrophy is, at this time, only speculated (42).

A potential linkage between FOXO and NF-κB signaling in muscle can be found in the literature. Muscle atrophy caused by immobilization (43) or unloading (44) is associated with oxidative stress and the generation of ROS. Components of the ubiquitin-proteasome system have been found to be transcriptional targets of ROS signaling in cultured myotubes (45, 46). Furthermore, ROS (via H2O2 treatment) induced activation of both the NF-κB (47) and the FOXO signaling pathway (48) in muscle cells. In the case of NF-κB, the signaling was associated with protein loss, and FOXO expression has been shown to decrease fiber size, via atrogen-1, in muscle (55). Therefore it appears possible that the increased ROS in unloading may trigger either the NF-κB or the FOXO signaling pathway or both, perhaps leading to increased proteolysis through the ubiquitin-proteasome pathway, but this has yet to be directly tested.

A unique characteristic of muscle atrophy due to unloading (2, 29) and other disuse conditions (49–52) is the concomitant increase in fast myosin expression. This was reconfirmed in the present study in the WT unloaded soleus, but it was abolished in muscles of both Nfkb1–/– and Bel3–/– mice. While the mechanism of the disuse-induced increase in fast myosin expression is not understood, there is evidence for a role of the transcriptional coactivator PGC-1α (53) and the MEF2 family of transcription factors (reviewed in ref. 54) in the determination of muscle fiber type. Thus it is conceivable that knockout of either the p105/p50 or the Bcl3 gene could affect the genes involved in the regulation of the shift in fast myosin expression.

Interestingly, Tidball and Spencer (29) also found no change in fast myosin expression in soleus muscles subjected to unloading for 10 days that were overexpressing calpastatin and that showed a 30% attenuation of atrophy. Thus, an alternative explanation for the lack of change in myosin expression with unloading where atrophy is also inhibited is that the regulation of atrophy and the change in myosin phenotype may be tightly linked.

Previously it has been shown that the decrease in cross-sectional area of type II (fast) fibers in the unloaded mouse soleus muscle is equal to, and in some cases greater than, the atrophy seen in the type I (slow) fibers within the first 2 weeks of the non–weight-bearing condition (reviewed in ref. 2). Results from the present study show that the extent of atrophy of slow fibers from WT mice was half that of the fast fibers. The inhibition of fiber atrophy in the Nfkb1–/– mice was a result of the lack of atrophy of the fast fibers, while the slow fibers atrophied to the same extent in Nfkb1–/– and WT mice (~16%). However, the inhibition of fiber atrophy in the Bel3–/– mice was due to a lack of atrophy of slow fibers and a significant reduction in the atrophy of the fast fibers (Figure 8). While mice lacking either the Nfkb1 or the Bel3 gene show inhibited muscle atrophy, there may be differences in the way each gene regulates atrophy. Given the slight difference in myosin phenotype and the slight difference in slow and fast fiber atrophy in the 2 types of knockout mice, it would not be surprising if there were somewhat different regulatory mechanisms of muscle atrophy in the Nfkb1–/– versus the Bel3–/– mice.
In summary, the results of this study demonstrate that knockout of the Nfkb1 or the Bcl3 gene inhibits muscle atrophy, phenotype change, and activation of NF-κB activity due to hindlimb-unloading disuse. Atrophy was inhibited in both a mixed mouse muscle (the soleus, 50% fast myosin) and a predominantly fast muscle (the plantaris, 95% fast myosin). Taken together, these results demonstrate the requirement of NF-κB signaling in disuse muscle atrophy and the associated phenotype change. Further work is needed to elucidate the upstream triggers of this signaling and the downstream genes targeted by NF-κB.

Methods

Animals and hindlimb unloading. Normal 8-week-old female B6;129, Nfkb1-/- (Nfkb1-/-) B6;129, and Bcl3-/- (Bcl3-/-) B6;129 mice were purchased from The Jackson Laboratory. All mice were housed in the Boston University Animal Care Facilities under pathogen-free conditions. The mice were randomly assigned to control or hindlimb-unloaded groups. To induce muscle atrophy by disuse (i.e., reduced muscle tension), the rodent’s hindlimbs were suspended off the cage floor (by 1 mm) using elastic tail casts, as described previously (55), for 10 days. The use of animals in this study was approved by the Boston University Institutional Animal Care and Use Committee.

Muscle preparation and analysis. At the end of the experimental treatments, control and unloaded mice were anesthetized with ketamine/xylazine (100 mg/kg), and soleus muscles from right and left hindlimbs were removed, quickly weighed, and either processed immediately for protein assays (see below) or embedded in tissue-freezing medium and frozen in isopentane for sectioning and subsequent morphological and immunohistochemical analysis.

Somatic gene transfer using intramuscular DNA injection. NF-κB-GL3 contains a trimeredized NF-κB site from the Igκ light chain enhancer. These sequences, gifts from S. Ho (UCSD, San Diego, California, USA), were each inserted into the IL-2 minimal promoter (56) driving expression of luciferase in pGL3-basic. Plasmid DNA injections into skeletal muscle were removed, quickly weighed, and either processed immediately for protein assays (see below) or embedded in tissue-freezing medium and frozen in isopentane for sectioning and subsequent morphological and immunohistochemical analysis.

Western blot analysis. Muscle lysates were prepared by grinding with a tissue homogenizer in passive lysis buffer (Promega Corp.) and centrifuging at 5,500 g for 20 minutes at 4°C. Twenty microliters of the supernatant was used to determine firefly luciferase activity using a luciferase assay kit (Promega Corp.) and a Turner Designs Inc. luminometer (model TD-20/20). Activity values reflect total muscle luciferase activity.

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