Attenuation of skeletal muscle atrophy via protease inhibition

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Morris, Carl A., Linda D. Morris, Ann R. Kennedy, and H. Lee Sweeney. Attenuation of skeletal muscle atrophy via protease inhibition. J Appl Physiol 99: 1719–1727, 2005. First published June 23, 2005; doi:10.1152/japplphysiol.01419.2004.—Skeletal muscle atrophy in response to a number of muscle wasting conditions, including disuse, involves the induction of increased protein breakdown, decreased protein synthesis, and likely a variable component of apoptosis. The increased activation of specific proteases in the atrophy process presents a number of potential therapeutic targets to reduce muscle atrophy via protease inhibition. In this study, mice were provided with food supplemented with the Bowman-Birk inhibitor (BBI), a serine protease inhibitor known to reduce the proteolytic activity of a number of proteases, such as chymotrypsin, trypsin, elastase, cathepsin G, and chymase. Mice fed the BBI diet were suspended for 3–14 days, and the muscle mass and function were then compared with those of the suspended mice on a normal diet. The results indicate that dietary supplementation with BBI significantly attenuates the normal loss of muscle mass and strength following unloading. Furthermore, the data reveal the existence of yet characterized serine proteases that are important contributors to the evolution of disuse atrophy, since BBI inhibited serine protease activity that was elevated following hindlimb unloading and also slowed the loss of muscle fiber size. These results demonstrate that targeted reduction of protein degradation can limit the severity of muscle mass loss following hindlimb unloading. Thus BBI is a candidate therapeutic agent to minimize skeletal muscle atrophy and loss of strength associated with disuse, cachexia, sepsis, weightlessness, or the combination of age and inactivity.

hindlimb unloading; protease inhibitors; Bowman-Birk inhibitor; protein degradation

SKELETAL MUSCLE ATROPHY IS associated with removal of load-induced signaling, either during disuse or under microgravity conditions. As the primary response to the removal of load appears to be decreased protein synthesis (8, 20, 29), increased degradation (14, 30), and a variable component of apoptosis (1, 13, 32), possible treatment strategies to attenuate muscle atrophy could target restoration of normal signaling of one or more of these processes.

Much recent work has focused on the IGF-I/phosphatidylinositol 3-kinase/Akt signaling pathway and identified its role as a critical regulator of muscle cell size, capable of stimulating muscle hypertrophy (3) or inhibiting muscle atrophy (8, 40). Although transgenic IGF-I overexpression produced muscle hypertrophy, it did not attenuate muscle atrophy associated with hindlimb unloading (HU) (11); however, direct intramuscular injection of IGF-I was recently shown to reduce muscle atrophy in a denervation model (40). Expression of constitutively active Akt, a signaling protein downstream of IGF-I, was able to stimulate hypertrophy and to ameliorate denervation atrophy (8). Also, the levels and activities of other key signaling proteins involved in muscle growth have been shown to significantly change during modified muscle use (17, 18, 36). Together, these results suggest that maintaining growth signaling via gene therapy or pharmacological approaches likely could lead to an amelioration of muscle atrophy.

An alternative and perhaps complementary therapy to counter muscle atrophy could be to target the accelerated protein degradation rate associated with decreased muscle use. The removal of muscle protein, most notably myofibrillar proteins, occurs primarily through activation of the ubiquitin-proteasome pathway (38, 41, 42). However, this pathway is not involved in initial myofibrillar protein cleavage. Calcium-dependent protease inhibitor (calpains), lysosomal-related proteinase (cathepsins B+) and apoptosis (caspases) are all likely involved, although the specific roles and the extent of involvement of each are unclear (1, 13, 19, 38, 43).

Other degradative processes that may be involved in muscle atrophy include intracellular and extracellular protease cascades, such as serine proteases (16, 37, 39) and matrix metalloproteinases (MMPs) (35). Serine proteases are widely expressed and play important roles in many cellular processes requiring regulated protein turnover. In muscle, serine proteases have been identified that are capable of cleaving myofibrillar proteins (16, 37), and chymase, a serine protease, can cleave soluble muscle proteins (12). Serine proteases have also been linked to extracellular matrix remodeling processes (15, 28) and activation of MMPs (10, 26, 35). Serine protease cascades potentially could act as a first step in the initiation of protein degradation. Thus blocking the initiation of such a cascade would likely provide significant benefit in reducing muscle loss.

The Bowman-Birk inhibitor (BBI) is a well-characterized, nontoxic serine protease inhibitor (22, 23), with the ability to inhibit the activity of numerous proteases, such as chymotrypsin, trypsin, cathepsin G, elastase, and chymase (6, 27, 46). BBI has been investigated in a number of anticarcinogenic and anti-inflammatory studies as purified BBI or as an extract enriched for BBI, the BBI concentrate (BBIC) (reviewed in Ref. 22). BBI inhibits proteolytic activity in lung, kidney, and liver tissue following intraperitoneal injections in mice, with similar dose-dependent inhibition of proteolytic activity observed both in vitro and in vivo (5, 34). Mice provided a diet of 1.0% BBIC exhibited no growth abnormalities and had a significantly extended life span (25) and minimized the overall loss of body weight in an animal model of leukemia (24).

To determine whether inhibition of serine protease activity could attenuate disuse atrophy of skeletal muscle, mice were...
provided food supplemented with BBIC and subjected to a period of HU. The muscles were then analyzed to determine whether BBIC attenuated the muscle mass and strength losses associated with HU. The muscles were assayed to measure serine endopeptidase and the chymotrypsin-like proteasome activity under both weight-bearing and HU conditions and to determine whether these activities were inhibited by BBI. As it has been suggested that serine proteases are able to activate MMP activity, we also tested whether supplementation with BBIC was able to blunt any increased MMP activity.

The present results demonstrate that supplementation with BBIC significantly attenuates skeletal muscle atrophy during HU. An increase in serine protease activity was observed following HU, and the activity was reduced below basal levels in the presence of BBIC. Thus dietary intake of BBI represents a potential new therapy to limit the degree of skeletal muscle atrophy that arises from disuse.

METHODS

Preparation of BBIC

BBIC was purified as previously described (25). This purification procedure maintains the chymotrypsin inhibitor activity but reduces the level of trypsin inhibitory activity, as high levels of trypsin inhibitory activity have been shown to cause a potentially deleterious pancreatic feedback response in rats (22, 25). BBIC has been shown to have the same inhibitory profile as the purified protein BBI (22). BBIC food (1.0%) was prepared by Central Soya (Ft. Wayne, IN) and mixed with Rodent Diet AIN-93G (Bio-Serv, Frenchtown, NJ) to produce the food pellets. The activity of BBIC is defined as chymotrypsin inhibitory units (CI units) (25), with the batch of BBIC used in this study containing ~100 CI units/g. Therefore, the food supplemented with 1.0% BBIC had a potency of ~1 CI unit/g. Control food (Ctrl) was prepared similarly without the addition of BBIC. Before mixing, a quantity of BBIC was repeatedly autoclaved and then mixed with the powdered rodent diet to produce an inactive isocaloric Ctrl food (aBBIC). Repeated autoclaving has been shown to destroy the protease inhibitor activity of BBIC (21). All animals were provided food ad libitum.

Animals

The experiments in this study were approved by the University of Pennsylvania’s Institutional Animal Care and Use Committee. Six-month-old male C57Bl/6 mice were used for this study. The animals were randomly separated into one of three feed groups: BBIC, aBBIC, or Ctrl. The mice were switched to the experimental diets, containing 1.0% BBIC, 1.0% aBBIC, or no additional supplementation (Ctrl) 5–7 days before the beginning of the experimental period for acclimation to the new food. One-half of the animals were hindlimb suspended in individual suspension cages, while the others were placed in individual cages to be used as nonsuspended controls. Thus the animals were randomly assigned to one of six groups: 1) control, nonsuspended (Ctrl-Non); 2) control, hindlimb suspended (Ctrl+HS); 3) BBIC, nonsuspended (BBIC-Non); 4) BBIC, hindlimb suspended (BBIC+HS); 5) aBBIC nonsuspended (aBBIC-Non); and 6) aBBIC, hindlimb suspended (aBBIC+HS).

Hindlimb Suspension

The animals were suspended by using a modified tail suspension technique originally described for rats (33) and adapted in-house for mice. The animals were anesthetized with an intraperitoneal injection of a mixture of ketamine and xylazine, and the body weight was measured. The tails were cleaned and attached to a stainless steel chain by using a strip of adhesive tape (Skin Trac; Zimmer, Warsaw, IN). The chain was attached loosely to a track at the top of the cage until the animal had recovered from the anesthetic. When the mice began to exhibit normal activity, the chain was lifted sufficiently to raise the hindlimbs off the floor of the cage. The suspension system enabled the mice to move freely around the cage while preventing the hindlimbs from touching the floor or walls.

Muscle Mechanical Measurements

Following the experimental period, the mice were anesthetized, body weight was measured, and the soleus and gastrocnemius (Gast) muscles were removed. One soleus muscle was prepared for mechanical muscle force measurements. The other soleus muscle was weighed and frozen immediately for subsequent biochemical analysis without any ex vivo stimulation. The Gast muscles were weighed and immediately frozen for biochemical analyses. Force measurements were performed as previously described (3). Briefly, the resting length was obtained by adjusting muscle length until achieving maximal twitch tension. Maximal tetanic force was measured by stimulating the soleus muscles with a 100-Hz, 500-ms pulse at supramaximal voltage. Following the tension measurements, the muscle was blotted, weighed, and then rapidly frozen in melting isopentane and stored at −80°C for subsequent histological analysis.

Immunohistochemical Analysis/Fiber Size Determination

Frozen muscle cross sections (10 μm) were cut from the midbelly on a cryostat and stored at −20°C. To determine fiber size, the frozen sections were stained with an antibody against laminin (NeoMarkers, Fremont, CA). The slides were washed in PBS and blocked in 5% BSA in PBS for 1 h at room temperature and then incubated overnight in 5% BSA/PBS containing the laminin antibody at 4°C. A rhodamine-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories) was used as a secondary antibody to visualize staining. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) to slow photobleaching. Microscopy was performed on a Leitz DMR microscope (Leica, Bannockburn, IL), and image acquisition was performed by using a MicroMAX digital camera system (Princeton Instruments). The fiber sizes were calculated by using OpenLab imaging software (Improvision, Waltham, MA).

Proteolytic Assays

Assays to measure the proteolytic activity of the muscle extracts were performed to determine whether 1) HU increased proteolytic activity and whether 2) BBI could attenuate any increased proteolytic activity. To determine whether there were any changes in serine protease activity, we utilized the fluorogenic substrate butoxycarbonyl-Val-Pro-Arg-(4-methyl)-coumarylamine (Boc-VPR-MCA) that has been used previously to monitor serine endopeptidase activity in other tissues in mice and humans (31, 34, 45). The chymotrypsin-like proteasome activity was measured using N-succinyl-Leu-Leu-Val-Tyr-(4-methyl)-coumarylamine (N-Suc-LLVY-MCA). Another assay, gelatin zymography, was used to determine whether supplementation with the BBIC was able to reduce the activation of MMPs previously observed following hindlimb immobilization (35) that may suggest serine protease involvement in increased extracellular remodeling.

For the proteolytic assays, the frozen muscles were homogenized, and the proteins were extracted (1:5 wt/vol) by using a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM DTT. The homogenates were centrifuged (30,000 g for 30 min at 4°C), and the supernatant was collected and stored at −80°C until use. The protein concentration was determined by using the Bradford assay (Bio-Rad) with BSA as a standard.

Proteasome and Serine Protease Activity

To determine whether HU increased either the chymotrypsin-like proteasome or serine protease activity, the rate of hydrolysis of the WordPress.com
fluorescent peptides N-Suc-LLVY-MCA and Boc-VPR-MCA (Sigma, St. Louis, MO) was measured and found to be reduced from unloaded and weight-bearing, nonsuspended animals. The chymotrypsin-like proteasome activity in the muscle extracts (0.5 mg/ml total protein) was determined by measuring the rate of N-Suc-LLVY-MCA cleavage (0.2 mM) in the presence of 0.05% SDS, as described (19, 44). Serine endopeptidase activity was determined via measurement of the rate of Boc-VPR-MCA hydrolysis (100 μM) in the muscle extracts (0.1 mg/ml) in a buffer containing 50 mM Tris·HCl (pH 7.0) and 1 mM DTT, as described previously (45), with minor modifications. The proteolytic activity was measured by using three muscle samples from both suspended and nonsuspended animals. The protease inhibitors MG-132 or purified BBIC (Sigma) were added before addition of the substrate at a final concentration of 40 and 20 μM, respectively. The reaction was initiated by the addition of the fluorescent peptides with the release of free MCA monitored at excitation and emission wavelengths of 380 and 460 nm, respectively, in a plate-reading fluorimeter for 90 min at 37°C.

Gelatin Zymography

To determine whether there was increased extracellular matrix remodeling, as measured by MMP-2/9 activity, gelatin zymography of the muscle extracts, from Ctrl- and BBIC-fed mice, was performed following previously described methods (35) with modifications. The muscle samples (50 μg total protein) were mixed with 2× nonreducing sample buffer and loaded onto an 8% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gels were washed twice for 20 min in 2.5% Triton X-100 in PBS and equilibrated at room temperature in the developing buffer [50 mM Tris·HCl (pH 7.5), 200 mM NaCl, 5 mM CaCl2] for 30 min and then incubated overnight at 37°C in fresh developing buffer. Following incubation, the gels were stained with 0.5% Coomassie brilliant blue twice for 20 min and then destained in 50% methanol/7% acetic acid. Quantification of the in-gel protease activity was performed by using a scanner and densitometry software (Kodak 1D, Eastman-Kodak, Rochester, NY).

Western Blotting

Analyses of protein levels were determined by using standard SDS-PAGE and Western blotting methods on the muscle extracts. Antibodies against desmin (Santa Cruz Biotech, Santa Cruz, CA) and talin (Sigma) were used to determine whether changes in proteolytic activity promoted cleavage of these proteins.

Statistical Analysis

Statistical significance was determined by applying the raw data to either an unpaired t-test or a one-way ANOVA, with Bonferroni post hoc analysis, where applicable. The data are shown as means ± SE, unless otherwise noted. P < 0.05 was considered statistically significant.

RESULTS

Body Weights and Muscle Mass

To determine whether consumption of BBIC was able to attenuate muscle mass loss during HU, we first compared the soleus muscle mass of Ctrl mice to a group of BBIC-treated mice following 3, 7, and 14 days of HU (Fig. 1A). Following 3 days of hindlimb suspension, no significant difference between the Ctrl+HS and BBIC+HS soleus mass was observed [8.0 ± 0.3 mg (n = 4) and 8.9 ± 0.4 mg (n = 4), respectively]. However, after 7-day HU, the soleus mass of the BBIC-treated mice was significantly greater than that of the Ctrl mice [8.6 ± 0.4 mg (n = 4) vs. 7.2 ± 0.3 mg (n = 4); P < 0.05], and, following 14-day HU, the muscle weights were BBIC+HS, 7.9 ± 0.3 mg (n = 4) vs. Ctrl+HS, 6.6 ± 0.3 mg (n = 4); P < 0.05.

To determine whether the active components of BBIC were responsible for the observed attenuation of muscle loss, mice were provided food supplemented with the autolysed, inactive protease inhibitor, aBBIC, and compared against both BBIC+HS and Ctrl+HS groups following 14-day hindlimb suspension. The body weights and the soleus and Gast muscle weights were measured in all weight-bearing and HU animals. The body weights of the nonsuspended mice in all feed groups did not change during the 2-wk experimental period, increasing by <2%. Following 14 days of unloading, the body weights were reduced in all treatment groups (Ctrl+HS, −12.7 ± 2.4%; BBIC+HS, −9.1 ± 2.7%; aBBIC+HS, −11.4 ± 2.2%). Food intake was monitored in the BBIC- and aBBIC-treated mice, with the aBBIC-treated mice consuming 3.2 ± 0.2 g/day, whereas the BBIC-Non group consumed 3.1 ± 0.2 g/day. However, food intake in the BBIC+HS mice averaged −0.5 g/day less (2.7 ± 0.2 g/day) than the other three groups.

The Gast and soleus muscle weights of the weight-bearing, nonsuspended animals were similar for all treatment groups (Table 1), suggesting that BBIC supplementation does not produce any observable hypertrophy in unperturbed muscles. However, dietary supplementation with BBIC slowed the loss of muscle mass, with significant reductions observed in both the Gast and soleus muscles (Fig. 1B; Table 1). The soleus...
BBIC reduces muscle atrophy

Table 1. Muscle mass data

<table>
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<tr>
<th></th>
<th>Absolute Muscle Mass, mg</th>
<th>Muscle/Body Weight, mg/g</th>
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<tbody>
<tr>
<td></td>
<td>WB</td>
<td>HU</td>
</tr>
<tr>
<td>Soleus</td>
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<tr>
<td>Control</td>
<td>10.5±0.6</td>
<td>6.4±0.4</td>
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<tr>
<td>+BBIC</td>
<td>10.6±0.4</td>
<td>7.9±0.2*</td>
</tr>
<tr>
<td>+aBBIC</td>
<td>10.9±0.5</td>
<td>7.1±0.1</td>
</tr>
<tr>
<td>Gast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>158.3±4.4</td>
<td>124.5±5.0</td>
</tr>
<tr>
<td>+BBIC</td>
<td>158.7±4.2</td>
<td>142.6±3.0*</td>
</tr>
<tr>
<td>+aBBIC</td>
<td>159.5±2.3</td>
<td>131.2±5.1</td>
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</table>

Values are means ± SE. Muscle weights and muscle-to-body weight ratios are given. WB, weight bearing; HU, hindlimb unloaded; BBIC, Bowman-Birk inhibitor concentrate; aBBIC, autoclaved BBIC; Gast, gastrocnemius. *The soleus and Gast muscle weights of the BBIC + hindlimb-suspended (HS) animals (n = 7) were significantly different from those of both control (Ctrl) + HS (n = 7) and aBBIC + HS (n = 8) animals (P < 0.05). †The muscle-to-body weight ratios of the BBIC + HS group was significantly different from that of Ctrl + HS and aBBIC + HS (P < 0.05).

Muscle weights of the BBIC + HS mice (7.9 ± 0.2 mg, n = 9) were significantly greater than in both Ctrl + HS and aBBIC + HS mice (6.4 ± 0.4 mg, n = 7; and 7.2 ± 0.1 mg, n = 8, respectively), with no difference determined between the Ctrl + HS and aBBIC + HS groups following the 14 days of hindlimb suspension. The soleus muscle weights of the Ctrl + HS and aBBIC + HS animals decreased by 39 ± 5 and 35 ± 3% (Fig. 1B), compared with the Ctrl-Non and aBBIC-Non, respectively. The percent atrophy in the BBIC + HS soleus muscles was limited to 26 ± 4%, significantly attenuating mass loss by 25–35%.

Similar results were observed in the Gast muscles (Fig. 1B; Table 1). There was no observable difference in Gast muscle weights of the BBIC-Non mice and the Ctrl-Non or aBBIC-Non mice. As in the soleus muscles, BBIC significantly attenuated the loss of the Gast muscle mass compared with Ctrl and aBBIC-fed mice (Table 1). The percent muscle loss for the BBIC + HS mice (10.1 ± 0.5%) was significantly reduced when compared against either Ctrl + HS (21.4 ± 1.0%) or aBBIC + HS mice (17.7 ± 1.0%) (Fig. 1B). While the aBBIC + HS group lost slightly less mass than the Ctrl + HS group, this may have been due to residual BBI activity.

As body weight changes and fasting can greatly affect muscle mass, the individual muscle weights relative to the body weight were determined (Table 1). A significant attenuation in muscle atrophy of the soleus, normalized to body weight, was determined when the BBIC-fed mice (11 ± 1% loss) were compared with both the Ctrl mice (22 ± 2%) and aBBIC-fed mice (20 ± 3%). In the Gast muscles, the normalized percent atrophy was reduced from 7.5 ± 10% in the Ctrl + HS and aBBIC + HS mice to no observable loss in the BBIC + HS animals.

If an active compound within the BBIC-supplemented food is an inhibitor of muscle atrophy during unloading, then a correlation between BBIC intake and muscle weight should be apparent. As described above, the daily food intake of the BBIC- and aBBIC-treated mice was monitored. A significant correlation between the muscle weights and daily food intake was determined for the BBIC + HS animals (r = 0.785; P = 0.038; n = 7) but not for the aBBIC + HS group (r = 0.131; P = 0.81; n = 6), suggesting the active BBIC is responsible for limiting the loss of muscle mass. Mice eating >3.0 g/day of BBIC food lost only 7.2 ± 0.6% of their muscle mass, compared with 19.4 ± 1.8% in the aBBIC-fed mice, when normalized to body weight.

Fiber size in BBIC- and aBBIC-treated mice

BBIC was found to significantly attenuate mass loss associated with HU (Fig. 1). To determine whether the maintained muscle mass was due to maintenance of muscle fiber size, the individual fiber areas were measured in BBIC- and aBBIC-treated mice. For the measurements, soleus muscle cross sections were stained with laminin and used to calculate the fiber cross-sectional area (CSA). Two nonoverlapping regions were visualized on individual muscle sections from three different mice, resulting in a total of >600 individual fiber areas being measured for both BBIC + HS and aBBIC + HS mice. As shown in Fig. 2, BBIC treatment significantly attenuated the decrease in fiber size following unloading. The mean fiber size of aBBIC + HS mice (578 ± 33 µm²) was reduced by ~37% compared with the aBBIC-Non (920 ± 22 µm²). BBIC treatment limited the reduction in fiber area to ~26% (weight bearing, 931 ± 51 µm² vs. unloaded, 689 ± 36 µm²). The fiber size decreases shown compare well with the muscle mass...
data (Fig. 1; Table 1), suggesting BBIC is inhibiting the loss of muscle fibers rather than other nonmuscle components.

**Contractile Measurements**

It is possible that the maintenance of fiber size and mass could lead to reduced force output by inhibiting critical protein clearance pathways and accumulation of nonfunctional proteins. Thus we performed contractile measurements to determine whether BBIC treatment preserved muscle strength as well as mass and fiber area. The calculated CSA (9) of the Ctrl+HS and aBBIC+HS mice was reduced by 36 and 34%, respectively (Table 2). As shown for muscle mass, the addition of BBIC to the food attenuated the HU-induced reduction in CSA to 26%.

Proportional to the greater muscle mass and CSA, the total tetanic force produced by the BBIC+HS muscles (144.1 ± 4.3 mN; n = 7) was significantly greater than the Ctrl+HS (120.9 ± 7.9 mN; n = 6; P < 0.05) and of borderline significance compared with the aBBIC+HS muscles (129.7 ± 5.4 mN; n = 6; P = 0.055; Table 2). To account for the variations in muscle size, specific force was calculated as both force per milligram of muscle and force per centimeter squared of CSA. The specific force calculations indicate similar forces, regardless of the method of normalization. The force per milligram muscle was 18.9 ± 1.9, 18.0 ± 0.7, and 18.5 ± 1.3 N/mg muscle, whereas the force per CSA values was 15.3 ± 0.8, 14.4 ± 0.5, and 14.1 ± 0.7 N/cm² for the Ctrl+HS, BBIC+HS, and aBBIC+HS muscles, respectively (Table 2). The results suggest that BBIC is maintaining functional muscle mass and enabling overall greater force production by the muscle.

**Western Blots**

Western blots of desmin and talin were performed to determine whether calpain-dependent protein cleavage was reduced in the BBIC+HS mice. Analysis of 3-, 7-, and 14-day HU, BBIC-treated mice exhibited no difference in either desmin or talin cleavage compared with weight-bearing Ctrl mice, suggesting BBIC does not influence calpain-mediated protein degradation (data not shown).

**Inhibition of Proteolytic Activity by BBIC**

**Proteolytic activity.** To determine whether BBI was capable of directly interfering with the chymotrypsin-like activity of the proteasome, the fluorescent peptide, N-Suc-LLVY-MCA, was mixed with Gast muscle extracts from either weight-bearing or HU mice. As shown in Fig. 3A, BBI does not directly inhibit the chymotrypsin-like activity of the proteasome in muscle extracts. Also, we did not observe an increase in the rate of chymotrypsin-like proteolytic activity following 14 days of tail suspension. The presence of BBI did not affect the proteolytic activity, either in weight-bearing or HU muscles (Fig. 3B), while the proteasome inhibitor MG-132, as expected, significantly reduced proteasome activity in both the suspended and nonsuspended muscle homogenates by >80%.

Another fluorescent peptide substrate (N-Boc-VPR-MCA), previously used to monitor serine endopeptidase activity and shown to be responsive to tissue perturbations, was used to measure proteolytic activity in the muscle extracts. As shown in Fig. 3, C and D, the serine proteolytic activity was significantly increased, by 1.7-fold, following 14 days of HU. The addition of BBI significantly inhibited the protease activity in the muscle extracts from both the suspended and nonsuspended animals (77.5 ± 10 and 81.0 ± 11%, respectively) compared with the untreated samples. Incubation with MG-132 did not affect the serine protease activity in the nonsuspended muscles, whereas in the suspended muscles MG-132 reduced the serine endopeptidase activity by 8.9 ± 6%. The observed increase in serine proteolytic activity in the sustained muscle samples and the subsequent inhibition by purified BBI protein, a potent serine protease inhibitor, suggests that serine protease cascades play a role in the process of muscle atrophy.

**Zymography.** To determine whether BBIC supplementation could influence extracellular matrix remodeling, MMP-2/9 activity was measured in Gast muscle extracts of nonsuspended and hindlimb-suspended mice given either Ctrl or BBIC-supplemented food. The muscle extracts from these mice were homogenized and run on nondenaturing gels containing 1% gelatin. Figure 4 shows the quantification of gelatinase activity. The proteolytic activity was measured, and the band intensities from the BBIC-Non, BBIC+HS, and Ctrl+HS homogenates were compared against Ctrl-Non mice. Comparison of the groups, relative to Ctrl-Non, revealed increased gelatinase activity in the Ctrl+HS animals only. The BBIC+HS muscles did not exhibit elevated levels of proteolytic activity compared with the Ctrl-Non animals. Gels developed in an EDTA-containing buffer displayed no bands, suggesting that the

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**Table 2. Contractile properties of soleus muscles from control and aBBIC- and BBIC-treated mice**

<table>
<thead>
<tr>
<th></th>
<th>Nonsuspended</th>
<th>Hindlimb Suspended</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BBIC</td>
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<tr>
<td>Cross-sectional area, mm²</td>
<td>1.24±0.05</td>
<td>1.36±0.05</td>
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<tr>
<td>Twitch, mN</td>
<td>37.0±4.6</td>
<td>50.4±3.3</td>
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<tr>
<td>Tetanus, mN</td>
<td>212.0±19.9</td>
<td>248.9±12.2</td>
</tr>
<tr>
<td>Specific force, N/cm²</td>
<td>17.1±2.0</td>
<td>18.3±0.7</td>
</tr>
<tr>
<td>Specific force, N/mg</td>
<td>20.2±1.8</td>
<td>23.5±1.1</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.5±1.7</td>
<td>31.4±1.4</td>
</tr>
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</table>

Values are means ± SE. Body weights, cross-sectional area, and force measurements in nonsuspended and 14-day suspended mice are given. *The cross-sectional area of the BBIC+HS group was significantly different from that of Ctrl+HS and aBBIC+HS (P < 0.05). The tension measurements were performed on 1 limb from each animal (n = 6 for the 3 nonsuspended groups; n = 7 for BBIC+HS; n = 6 for aBBIC+HS and Ctrl+HS). †The tetanic tension in the muscles of the BBIC+HS animals was significantly greater than that in the Ctrl+HS mice (P < 0.05). The specific force, measured relative to cross-sectional area and muscle weight, was similar for all 3 HS treatment groups.
observed gelatinase activity was due to MMPs (data not shown).

**DISCUSSION**

Significant progress has been achieved in identifying key elements associated with skeletal muscle changes in growth signaling (8, 18, 36, 40), protein degradation (7, 19, 38, 41–43), and apoptosis (1, 13, 32), concomitant with muscle atrophy. There has been success in attenuating the overall response to atrophy, namely loss of muscle mass and strength, via genetic manipulation of these pathways (7, 8, 18, 43). However, biochemical dissection of these pathways has not completely defined all of the required elements to maintain muscle mass and function during modified muscle use. Furthermore, the genetic manipulations that have been successful at maintaining muscle mass are not readily translated into therapeutic strategies.

An alternative strategy to slow disuse atrophy is to inhibit the elevated protein degradation pathways, rather than boost the diminished growth signaling. This approach is more immediately tractable from a therapeutic standpoint, as a number of pharmacological agents exist that target various classes of proteases. In this study, we specifically examined whether dietary supplementation with a well-characterized serine protease inhibitor was sufficient to modulate the degree of atrophy associated with muscle unloading. Our results indicate that dietary supplementation with BBIC significantly attenuated the degree of muscle atrophy following HU in mice. The maintenance of muscle mass was functional, with the specific force in BBIC-treated animals similar to those treated with inactive aBBIC or standard mouse feed. Also, we have identified a novel proteolytic activity that is elevated following a period of hindlimb muscle unloading and inhibited by a well-characterized serine protease inhibitor. The results suggest that BBIC attenuates the observed muscle atrophy by reducing the activity of unloading-induced serine protease activity, slowing the decrease in fiber size and thereby maintaining the overall mass of the muscle.

It is important to note that the amelioration of atrophy in the BBIC+HS animals was not due to any initial hypertrophy of the muscles, as the BBIC animals exposed to normal loading showed no increase in muscle mass compared with either Ctrl-Non or aBBIC-Non animals. This enabled direct comparison of the BBIC+HS muscle weights, both absolute and relative to body weight, with those of the aBBIC+HS and Ctrl+HS. Following 3 days of HU, there was no difference between the Ctrl-fed and BBIC-fed muscle weights. However, after 7 and 14 days, the muscle weights were significantly
greater in the BBIC-treated HU animals. The maintenance of muscle mass was paralleled by maintenance of force production. The specific force, measured as force per CSA and force per milligram muscle wet weight, was similar for all HU groups.

Investigation into the role of protein degradation during disuse atrophy has focused on three primary proteolytic pathways: the Ca\(^{2+}\)-dependent proteolysis (calpains), ATP-dependent proteolysis (ubiquitin-proteasome degradation), and lysosomal proteolysis (cathepsin B+L). The ubiquitin-proteasome pathway appears responsible for the majority of muscle protein degradation (19, 36, 41, 42). However, intact myofibrillar proteins are not substrates of the proteasome (19, 38), so the proteosome-dependent activity was not inhibited by purified BBI (16, 37).

Following HU, components of the ATP-dependent ubiquitin-proteasome system have been shown to be upregulated (19, 39, 41). The level of chymotrypsin-like proteosome activity was previously shown to be minimally elevated after 14 days of hindlimb suspension with significant increases only observed following 21 days of unloading (19). We measured the proteosome-dependent cleavage of the fluorogenic substrate N-Suc-LLVY-MCA and did not observe an increase in the chymotrypsin-like proteosome activity following 14 days of suspension. However, as expected, the proteosome inhibitor MG-132 significantly reduced the peptidase activity. The proteosome-dependent activity was not inhibited by purified BBI in either nonsuspended or suspended muscle extracts. BBI does not directly inhibit proteosome activity, but it is not clear whether or not BBI inhibition influences flux through the ubiquitin-proteosome pathway by inhibiting upstream protein degradation pathways, thus decreasing proteosome substrates.

Also, neither talin nor desmin degradation was influenced by the presence of BBIC, suggesting little to no effect on the calcium-dependent proteolytic pathway by the serine protease inhibitor. It would thus be of interest to examine calpain inhibitors in combination with BBI, since a calpain inhibitor (leupeptin) has been reported to attenuate muscle loss associated with denervation (2).

Although the three well-characterized degradative pathways described above clearly are major sources of observed muscle atrophy, our data suggest that there are other serine proteases that make important contributions to the atrophy process. These may include previously described serine protease cascades (39), as others have identified myofibrillar serine proteases capable of directly degrading myosin and actin (16, 37).

In the present study, we have identified serine endopeptidase activity that is significantly elevated in HU muscle extracts (Fig. 3).

While we have not elucidated the specific serine proteases responsible for the component of the atrophy response that we have attenuated, Stevenson et al. (39) identified differential expression of several serine proteases and serine protease inhibitors following HU. A potential target of BBI could be the serine protease, mast cell chymase, which is usually released with trypsinase and tumor necrosis factor-\(\alpha\) during activation and degradation of cell membranes. BBI/BBIC is a potent inhibitor of chymase (46), which has been shown to degrade soluble muscle proteins (12), to directly cleave components of the extracellular matrix (e.g., fibronectin) and to activate MMPs (28).

In vitro studies have found that BBI is capable of inhibiting activation of pro-MMP-9 (4), suggesting BBI/BBIC could reduce protease activity in the extracellular space. Our results suggest that some component of this serine protease activity must be an initiating event in the stimulation of MMP activity to bring about extracellular matrix changes (Fig. 4).

It is currently unclear if these serine protease activities that are elevated in disuse atrophy function upstream of, or in parallel with, the three primary degradative pathways. What is clear is that BBI significantly inhibited the elevated serine endopeptidase activity, leading to an amelioration of muscle loss and preservation of function during a period of disuse. Importantly, while there was a significant reduction in the serine protease activity of BBIC-treated, nonsuspended mice muscle extracts, there is no evidence that the inhibitory activity affects normal function. As BBIC is nontoxic, orally bioavailable, and is currently being evaluated in human trials, it is likely that human trials on the effects of BBIC in disuse atrophy of skeletal muscle could be performed in the near future. If functional muscle loss can be prevented via simple dietary supplementation, this could lead to more rapid recovery from prolonged bed rest or limb immobilization (casting).

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DISCLOSURES
The University has submitted patent applications.

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