Bowman-Birk inhibitor concentrate prevents atrophy, weakness, and oxidative stress in soleus muscle of hindlimb-unloaded mice

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Arbogast S, Smith J, Matuszczak Y, Hardin BJ, Moylan JS, Smith JD, Ware J, Kennedy AR, Reid MB. Bowman-Birk inhibitor concentrate prevents atrophy, weakness, and oxidative stress in soleus muscle of hindlimb-unloaded mice. J Appl Physiol 102: 956–964, 2007. First published November 16, 2006; doi:10.1152/japplphysiol.00538.2006.—Antigravity muscles atrophy and weaken during prolonged mechanical unloading caused by bed rest or spaceflight. Unloading also induces oxidative stress in muscle, a putative cause of weakness. We tested the hypothesis that dietary supplementation with Bowman-Birk inhibitor concentrate (BBIC), a soy protein extract, would oppose these changes. Adult mice were fed a diet supplemented with 1% BBIC during hindlimb unloading for up to 12 days. Soleus muscles of mice fed the BBIC-supplemented diet weighed less, developed less force per cross-sectional area, and developed less total force after unloading than controls. BBIC supplementation was protective, blunting decrements in soleus muscle weight and force. Cytosolic oxidant activity was assessed using 2′,7′-dichlorofluorescin diacetate. Oxidant activity increased in unloaded muscle, peaking at 3 days and remaining elevated through 12 days of unloading. Increases in oxidant activity correlated directly with loss of muscle mass and were abolished by BBIC supplementation. In vitro assays established that BBIC directly buffers reactive oxygen species and also inhibits serine protease activity. We conclude that dietary supplementation with BBIC protects skeletal muscle during prolonged unloading, promoting redox homeostasis in muscle fibers and blunting atrophy-induced weakness.

CONDITIONS THAT LESSEN GRAVITATIONAL loading over days to weeks, e.g., prolonged bed rest or long-duration spaceflight, cause wasting and weakness of the postural muscles. This process can be reproduced experimentally in rodents by hindlimb unloading. This intervention causes loss of weight in antigravity muscles that is detectable within 48 h and is attributed to an imbalance between protein synthesis (8) and protein degradation (36). In addition, prolonged unloading induces “contractile dysfunction,” which we define as a decrement in maximal force developed per unit cross-sectional area by unfatigued muscle. These parallel mechanisms, muscle atrophy and contractile dysfunction, contribute additively to the weakness caused by prolonged unloading.

Oxidative stress may contribute to these changes. Muscle atrophy and weakness are linked to oxidative stress in conditions that range from limb immobilization (21) to chronic obstructive pulmonary disease (27) to sepsis (3). Recent evidence suggests a similar association in muscle after prolonged unloading. In adult rats subjected to hindlimb unloading for 28 days, Lawler et al. (23) reported elevation of biochemical markers of oxidative stress in the soleus, the archetypal antigravity limb muscle. Their data indicate that antioxidant scavenging capacity is decreased and antioxidant enzyme levels are diminished in unloaded muscle, whereas lipid hydroperoxide levels are elevated. More recently, Matuszczak et al. (28) found that administration of the antioxidant allopurinol had protective effects during hindlimb unloading, blunting contractile dysfunction in soleus muscles. Similarly, unloading of the respiratory skeletal muscles via continuous mechanical ventilation has been shown to induce oxidative stress (35) and contractile dysfunction (4), which can be inhibited by systemic administration of the antioxidant Trolox (4). In combination, the existing data suggest that unloading causes oxidative stress, that oxidative stress contributes to muscle weakness, and that antioxidant supplementation can oppose these events.

On the basis of this model, the present study examined the effects of gravitational unloading on redox homeostasis and contractile function of mouse soleus muscle and evaluated dietary supplementation with Bowman-Birk inhibitor (BBI) concentrate (BBIC) as a putative countermeasure. BBI is a well-characterized soy protein (6) that has anticarcinogenic (41), chemoprotective (19), and radioprotective (10, 15) effects via mechanisms of action that include serine protease inhibition (6) and antioxidant properties (14, 15, 39). In support of the latter mechanism, BBI has been shown to lessen production of reactive oxygen species by nonmuscle cell types (14, 39) and to diminish the systemic oxidative stress caused by whole body irradiation (15). Recently, Morris et al. (31) showed that dietary supplementation with BBIC prevents loss of function in unloaded muscle, an outcome consistent with an antioxidant action. To address this issue, we conducted a series of experiments that tested three hypotheses: 1) Dietary supplementation with BBIC prevents atrophy and contractile dysfunction of mouse soleus muscle caused by gravitational unloading. To confirm the findings of Morris et al., soleus mass, isometric contractile properties, and fatigue characteristics were assessed after 12 days of gravitational unloading with or without dietary supplementation with BBIC. 2) Unloading causes a time-dependent increase in intramuscular oxidative activity that correlates with muscle atrophy. To test this hypothesis, we subjected adult mice to hindlimb unloading for 1, 3, 6, or 12 days. At each time point, the soleus muscle of hindlimb-unloaded...
mice was tested for changes in cytosolic oxidant activity and muscle mass relative to freely ambulating control animals. 3) BBIC has antioxidant effects on skeletal muscle, blunting the rise in intracellular oxidants caused by unloading. To test this hypothesis, we evaluated the effect of BBIC dietary supplementation on cytosolic oxidant activity in soleus muscles of mice subjected to 12 days of hindlimb unloading.

MATERIALS AND METHODS

Experimental animals. This study conformed to the guiding principles on care and use of laboratory animals of the American Physiological Society and the National Institutes of Health. All experiments were approved in advance by the Institutional Animal Care and Use Committee of the University of Kentucky Medical Center. Male ICR mice (30–35 g body wt; Harlan, Indianapolis, IN) were maintained on a reversed 12:12-h light-dark cycle with controlled temperature (21 ± 1°C) and humidity. Food and water were provided ad libitum. At 1 wk after arrival, the animals were placed in individual cages for a 3-day acclimation period; then they were randomly assigned to either of two experimental groups: freely ambulating controls and hindlimb-unloaded animals. In each group, the animals were further separated into two groups according to diet. The standard rodent diet AIN-93G (Bio-Serv, Frenchtown, NJ) was fortified with 1% BBIC (Central Soya, Ft. Wayne, IN). As described elsewhere (20), BBIC contains proteins and carbohydrates and is 10% BBI by weight. The biological activity of BBIC is attributed to protease inhibition via its BBI content (19). As negative control, a second diet was prepared from BBIC that had been repeatedly autoclaved to destroy biological activity (18). Control food was prepared similarly without the addition of BBIC. Animals were pretreated with BBIC, heat-inactivated BBIC, or control diet for 5 days and then assigned to one of four groups: 1) freely ambulating animals fed the control diet, 2) freely ambulating animals fed the 1% BBIC diet, 3) hindlimb-unloaded animals fed the control diet, and 4) hindlimb-unloaded animals fed the 1% BBIC diet.

Hindlimb unloading. Hindlimb muscles were unloaded using methods described previously (28). Briefly, Elastoplast tape (Beiersdorfer-Jobst, Rutherford College, NC) was used to wrap the tail of each animal. A metal clip on the tape was attached to a nylon monofilament line via a stainless steel swivel. The distal end of the nylon line was attached to an overhead support and shortened to suspend the animal in a 45° head-down tilt position. The swivel enabled the animal to explore the cage (360° range of motion) and obtain food and water freely. Animals were observed daily for changes in appearance and activity. Each animal was weighed, food and water intakes were recorded, and the angle of hindlimb suspension was adjusted if necessary. At the appropriate time, each animal was deeply anesthetized in the hindlimb-unloaded condition. The animal was removed from the unloading device, the soleus muscle was excised with the animal under surgical anesthesia, and the animal was euthanized.

Muscle preparation. Soleus muscle was isolated with origin and insertion intact. Each muscle was transferred to a dissecting dish containing oxygenated Krebs-Ringer solution at room temperature (in mmol: 137 sodium chloride, 4 potassium chloride, 1 magnesium chloride, 1 potassium phosphate, 12 sodium bicarbonate, and 1 calcium chloride), equilibrated with 5% CO₂-95% O₂ (pH 7.30 at 21°C). pH 7.30 at 21°C. Silk sutures (2-0) were tied to either tendon for use in securing the muscle during functional studies (see below).

Contractile protocol. Individual muscles were transferred to a temperature-controlled muscle bath (Radnoti Glass Technology, Monrovia, CA) containing oxygenated Krebs-Ringer solution at room temperature. Sutures were used to tie the proximal tendon to a glass rod and the distal tendon to a force transducer (model BG 100G, Kulite Industries, Leonia, NJ) mounted on a micrometer, which was used to adjust muscle length. The muscle was positioned between platinum plate stimulating electrodes and stimulated to contract isometrically using electrical field stimulation (supramaximal voltage, 0.2-ms pulse duration). Force transducer output was recorded using an oscilloscope (model 54601B, Hewlett Packard, Palo Alto, CA) and a chart recorder (model BD-11E, Kipp and Zonen, Delft, The Netherlands). In each experiment, muscle length was adjusted to optimize twitch force [optimal length (Lₒ)]. Bath temperature then was increased to 37°C, and 30 min were allowed for thermequilibration. The force-frequency relationship was determined using contractions evoked at 2-min intervals with stimulus frequencies of 1, 15, 30, 50, 80, 120, 160, 250, and 300 Hz and a tetanic train duration of 500 ms. Maximal (300-Hz) tetanic contractions were stimulated between lower-frequency contractions to monitor the change in maximal force over time, an index of stability. At 1 min after completing the force-frequency protocol, we induced acute fatigue by stimulating repetitive submaximal tetanic contractions (40 Hz, 0.5 train/s, 500-ms trains) for 5 min. After each experiment, Lₒ was measured using an electronic caliper, and the muscle was removed, blotted dry, and weighed. Cross-sectional area was calculated according to Close (9).

Cytosolic oxidant activity. Individual soleus muscles were transferred to a bath containing oxygenated Krebs-Ringer solution at 37°C. The muscle was pinned at near-optimal length (Lₒ) as determined by contractile studies of the contralateral muscle (see below). Oxidant activity was determined by use of the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR), as described previously (2). Briefly, DCFH-DA dissolved in ethanol was added to buffer (final concentration 50 μM) containing the isolated muscle. DCFH-DA diffuses into muscle fibers, where cytosolic esterases cleave the diacetate moiety to yield 2′,7′-dichlorofluorescin (DCFH), an electrically charged product retained in the cytosol. DCFH reacts with muscle-derived oxidants to yield the fluorescent derivative 2′,7′-dichlorofluorescein (DCF; 480-nm excitation, 520-nm emission). The change in emission intensity over time is proportional to DCF accumulation, an index of net oxidant activity in the cytoplasm. Emissions were quantified using an epifluorescence microscope (model TE 2000S, Nikon USA, Melville, NY) and a charge-coupled device camera (CoolSNAP-ES, Roper Scientific Photometrics, Tucson, AZ) controlled by a computer using data acquisition software (Metamorph 6.1, Universal Imaging, Downingtown, PA). Data were obtained from a 0.27-mm² site on the muscle and stored for later quantification. Emissions from DCFH-free buffer or muscle fibers are not detectable under these conditions, and background correction is not required. To study the effect of unloading, we measured intracellular oxidant activity in soleus muscle after 1, 3, 6, and 12 days of unloading. In a subsequent protocol, the effect of BBIC dietary supplementation on oxidant activity was measured after 12 days.

BBIC interaction with reactive oxygen species and nitric oxide derivatives. The capacity of BBIC to directly scavenge reactive oxygen species was tested in vitro using the cytochrome c reduction assay (29). The reaction medium contained 10 μM oxidized cytochrome c (Sigma), 1% (wt/wt) BBIC, and 1.25 μM hypoxanthine (Sigma), 5 mM phosphate-buffered saline. Xanthine oxidase (0.02 U/ml) was added to induce superoxide anion production. Cytochrome c reduction was measured spectrophotometrically as the difference between the peak absorbance at 550 nm and the average of values measured at 540 and 560 nm. BBIC effects on nitric oxide (NO) activity were tested in vitro using 4-aminomethyl-2′,7′-dihalo fluorescence detection (DAF-FM), a selective fluoride probe that reacts with NO to yield a stable benzotriazole derivative. DAF-FM diacetate (5 μM; Molecular Probes) was preincubated with esterase (5 U/ml; Sigma) to generate DAF-FM. BBIC (1% w/v) was tested for its capacity to inhibit DAF-FM reaction with NO released from 100 μM N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosodihydrazino)ethane (NOC-12; EMD Bioscience, La Jolla, CA). Hemoglobin (50 μM; Sigma) served as a positive control for NO quenching. Simultaneous reactions were performed in triplicate using a 24-well plate; reaction product emis-
sions were measured using our epifluorescence microscope-based system (see above).

Proteolytic and serine protease activities. Proteolytic activity was determined according to Morris et al. (31) using soleus muscle samples from three animals per experimental condition. Frozen samples were homogenized, and the proteins were extracted (1:18 wt/vol) using a commercial extraction buffer (T-per, Pierce, Hercules, CA). The homogenates were centrifuged at 1,000 g for 5 min at 4°C. The supernatant was collected and stored at −80°C for later analysis. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as a standard. Protease activities were measured in muscle extracts (0.1 mg/ml), with derivatives of methylcoumarin (MCA) used as enzyme-substrate. Cymotrypsin-like proteasome activity was determined according to Morris et al. (31) using soleus muscle samples from three animals per experimental condition. Frozen samples were homogenized, and the proteins were extracted (1:18 wt/vol) using a commercial extraction buffer (T-per, Pierce, Hercules, CA). The homogenates were centrifuged at 1,000 g for 5 min at 4°C. The supernatant was collected and stored at −80°C for later analysis.

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Table 1. Animal growth and behavior

<table>
<thead>
<tr>
<th></th>
<th>Body Weight, g</th>
<th>Water Intake, ml/day</th>
<th>Food Intake, g/day</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 12</td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Control</td>
<td>30.8 ± 0.8</td>
<td>33.5 ± 0.8</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>BBIC</td>
<td>29.9 ± 0.9</td>
<td>33.2 ± 1.0</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Unloaded</td>
<td>30.1 ± 1.1</td>
<td>28.8 ± 0.6*</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>Unloaded + BBIC</td>
<td>30.1 ± 1.2</td>
<td>30.7 ± 1.4</td>
<td>4.9 ± 0.9</td>
</tr>
</tbody>
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|                     | Experiment 2 | Control | 26.5 ± 1.1 | 30.9 ± 1.0 | 8.1 ± 0.1 | 4.5 ± 0.1 |
|                     |              | aBBIC   | 27.3 ± 0.4 | 31.6 ± 0.7 | 8.1 ± 0.1 | 4.1 ± 0.1 |
|                     |              | Unloaded| 27.3 ± 0.4 | 28.6 ± 0.8* | 6.4 ± 0.2 | 4.3 ± 0.1 |
|                     |              | Unloaded + BBIC | 26.9 ± 1.0 | 28.5 ± 1.4* | 6.6 ± 0.2 | 5.0 ± 0.8 |

Values are means ± SE. BBIC, Bowman-Birk inhibitor concentrate. aBBIC, heat-inactivated (autoclaved) BBIC. *P < 0.05 vs. control.

RESULTS

Animal behavior and body weight. As in previous studies (28), hindlimb unloading had no obvious effects on the general behavior of our mice. Grooming, cage exploration, and responses to handling were indistinguishable between unloaded and control animals. Food and water intake were also unaltered by unloading or BBIC supplementation (Table 1). There were no differences in mean body weight among groups before the experiment (Table 1). After 12 days of hindlimb unloading, body weight of the unloaded group was less than that of freely ambulating control animals in both of our experimental series. Supplementation of the diet with BBIC, a concentrated BBI preparation, prevented the loss of body weight caused by unloading; supplementation with heat-inactivated BBIC did not.

Contractile function. Hindlimb unloading depressed the force developed by the soleus muscle across a broad range of stimulation frequencies (Fig. 1A). This reflected the combined effects of contractile dysfunction (Fig. 1B) and atrophy (Fig. 1C) in unloaded soleus muscle. Unloading also shifted the relative force-frequency relation leftward (Fig. 1D). BBIC supplementation increased force production in unloaded muscle (Fig. 1A) by preventing atrophy. In unloaded animals that consumed BBIC, soleus weight and cross-sectional area were not different from freely ambulating controls (Fig. 1C). BBIC did not prevent the contractile dysfunction (Fig. 1B) or force-frequency shift (Fig. 1D) caused by unloading.

In four separate groups of animals, an identical protocol was used to test for protective effects of heat-inactivated BBIC. The loss of soleus force with unloading was reproducible (Fig. 2). Unloading also decreased soleus weight, cross-sectional area, and force per area; heat-inactivated BBIC had no effect on any parameter tested (data not shown).

Fatigue characteristics. Repetitive 40-Hz stimulation induced fatigue of soleus muscles as reflected by a progressive fall in developed force over 300 s (Fig. 3). Differences among groups were evident during the 1st min, when unloaded muscles generated less force than control muscles. This effect was blunted by BBIC supplementation, which caused unloaded muscles to generate higher forces, and was unaffected by heat-inactivated BBIC (data not shown). After the first 60 s, developed force did not differ among groups. Later in the protocol, force was sustained between electrically stimulated contractions, a phenomenon that reflected incomplete relaxation of the fatiguing muscles. Incomplete relaxation was evident in control muscles after the 1st min of contractions and increased progressively thereafter. This phenomenon was less apparent in unloaded muscles and was unaffected by BBIC supplementation.

Muscle stability in vitro. We assessed stability by use of repetitive maximal force measurements. As shown in Fig. 4A, initial values were offset among groups according to primary effects of unloading and BBIC (Fig. 1A). Over time, maximal force fell modestly in muscles from ambulating animals but not in unloaded muscles. Among all groups, the change in force over time correlated negatively with muscle cross-sectional area (Fig. 4B) and force output (Fig. 4C). These are logical findings. Cross-sectional area determines the diffusion distance for oxygen in isolated muscles, a major determinant of stability (34), and force output reflects energetic demand. Both end points were diminished by unloading, which favored stability in vitro.

Time course of atrophy and redox changes. To define unloading effects on muscle over time, we measured soleus weight and cytosolic oxidant activity after 1, 3, 6, and 12 days of hindlimb unloading. These data are shown in Fig. 5A. Soleus weight fell rapidly during the first 3 days and then declined more slowly, reaching a quasiplateau at ~60% of muscle weight in freely ambulating controls. In contrast, intracellular oxidant activity increased rapidly over the first 3 days and then remained elevated at 150–175% of control values. Figure 5B depicts the relation between atrophy and oxidant activity among individual soleus muscles. The data are negatively correlated.
correlated: muscle weight declines as oxidant activity increases (regression equation: $y = 0.00111 - 0.0000244x$, $P < 0.02$).

**BBIC as an antioxidant.** Data obtained in vivo and in vitro indicate that BBIC and its active ingredient BBI have antioxidant properties. Figure 6A depicts cytosolic oxidant activity in soleus muscles after 12 days of hindlimb unloading. As observed in the previous protocol, unloading increased oxidant activity within muscle fibers. This response was abolished by BBIC supplementation during unloading in mice that consumed BBIC during unloading. In freely ambulating animals, BBIC supplementation had no effect on oxidant activity in soleus muscles. Figure 6B shows that BBIC buffers reactive oxygen activity in vitro. The cytochrome $c$ reduction assay was used to measure reactive oxygen species generated using a hypoxanthine/xanthine oxidase system. In control studies, cytochrome $c$ redox state was unaffected by exposure to hypoxanthine, xanthine oxidase, or BBIC alone (data not shown). (BBIC supplementation in freely ambulating control mice had no effect on any point in A–D and, therefore, is not shown.)
shown). Addition of xanthine oxidase + hypoxanthine stimulated cytochrome c reduction, an indicator of superoxide anions (29, 30). BBIC diminished this signal by ~50%, suggesting that BBIC directly buffers reactive oxygen species, i.e., superoxide anions or their derivatives. Data in Fig. 6 indicate that BBIC does not quench NO. In the presence of an NO donor, the NO-sensitive probe DAF-FM is converted to its fluorescent derivative in a time-dependent reaction. Addition of hemoglobin, an NO quencher, largely abolishes this reaction. BBIC has the opposite action, augmenting the DAF-FM signal. This BBIC-associated increase appears to be NO dependent, because it is largely abolished by addition of hemoglobin or omission of the NO donor (data not shown).

Protease inhibition. Extracts of soleus muscles from unloaded and freely ambulating control animals were used to test effects of BBIC on serine protease activity (Fig. 7A) and chymotrypsin-like proteasome activity (Fig. 7B) in vitro. Unloading per se had no detectable effect on either end point. Addition of BBIC to the assay selectively inhibited serine protease activity in unloaded and control muscle; proteasome activity was unaltered. In contrast, the proteasome inhibitor MG-132 depressed proteasome, but not serine protease, activity. These data confirm that BBIC selectively inhibits the activity of serine proteases in muscle without altering proteasome function.

DISCUSSION

Overview of experimental findings. This study confirms the protective effects of BBIC against atrophy and weakness of unloaded muscle and expands our understanding of the underlying biology. New time course data show that mechanical unloading causes a rapid rise of oxidant activity within muscle fibers. This response peaks at 3 days, persists for up to 12 days,
BBIC is an antioxidant that protects unloaded muscle

and directly correlates with muscle atrophy. Furthermore, this report provides the first evidence that BBIC has direct antioxidant properties and that dietary supplementation with BBIC can mitigate oxidative stress and oxidant-mediated processes in vivo.

Muscle unloading and BBIC supplementation. Mechanical unloading causes muscle weakness due to two parallel processes, overt atrophy and contractile dysfunction. Atrophy reflects a mismatch between synthesis and breakdown of muscle protein; mechanical unloading affects both, slowing protein synthesis (26) and accelerating breakdown (7). Contractile dysfunction has been observed in human studies (1, 11) and animal experiments (16, 37) but is a more enigmatic process. Putative mechanisms include loss of thin filaments (12) and oxidant effects on excitation-contraction coupling (4, 28).

Morris et al. (31) were the first to demonstrate that dietary supplementation with BBIC can inhibit unloading-induced weakness. These investigators studied mice subjected to 14 days of hindlimb unloading. They showed that a diet containing 1% BBIC partially protected soleus and gastrocnemius muscles against atrophy and lessened the fall of tetanic force in soleus muscles. We designed our present study to mimic that of Morris et al., and our primary findings are identical. BBIC supplementation clearly mitigates atrophy and weakness in unloaded muscle, a robust finding in this animal model.

Morris et al. (31) did not observe contractile dysfunction in unloaded soleus muscle. The decrement in force per area measured during tetanic (100-Hz) contractions was small (≈11%) and not statistically significant. In contrast, we found that force per area was diminished by ≈30% in unloaded muscle, evidence of contractile dysfunction across a broad range of stimulus frequencies. Despite the differences, the two studies agree that BBIC supplementation does not alter force per area in skeletal muscle: BBIC had no effect on any group in either study. Failure of BBIC supplementation to prevent contractile dysfunction does not rule out a redox mechanism. BBQ tissue levels may have been inadequate to fully abolish oxidant effects; BBIC’s antioxidant chemistry (currently undefined) may not buffer the specific reactions that affect contraction, or BBQ distribution within muscle cells may be compartmentalized, limiting its biological availability. Existing data cannot discriminate among these possibilities.

Bioavailability and mechanisms of action. The bioavailability of BBIC has been studied extensively. Briefly, BBIC survives the digestive tract and reaches the colon in an active form (5, 41). BBIC is an 8-kDa molecule. It is taken up by intestinal epithelial cells and enters the bloodstream, where it is distributed intact throughout the body (5). Studies using anti-BBI antibodies and 125I-labeled BBIC have defined the distribution after ingestion; BBIC is taken up by most organs but crosses the blood-brain barrier weakly, if at all (5, 19, 32, 41).

BBIC is widely recognized as a serine protease inhibitor (19) that opposes the activity of various enzymes in this class, including elastase, chymotrypsin, trypsin, and cathepsin G (6, 22, 38, 40). In muscle extracts, Morris et al. (31) showed that BBIC dietary supplementation leads to inhibition of matrix metalloproteinase (MMP) activities in muscle extracts but does not affect proteasome activity. Our data corroborate these findings. Although BBIC is not expected to directly inhibit the catalytic activity of MMPs, it can prevent their activation; this could lead to lower levels of MMP activity, as observed by Morris et al. The serine protease response to unloading may differ among limb muscles. Morris et al. found that gastrocnemius muscle atrophies more slowly than soleus muscle and that serine protease activity remains elevated in gastrocnemius muscle for up to 14 days. In contrast, we found that soleus muscle atrophies most rapidly in the first 6 days of unloading; by 12 days, atrophy is almost undetectable, and serine protease activity is not elevated.

The antioxidant action of BBIC is less well recognized. Frenkel et al. (14) originally showed that BBIC inhibits hydrogen peroxide production by activated human polymorphonuclear leukocytes, an effect attributed to chymotrypsin inhibition. Ware et al. (39) later demonstrated that high concentrations of BBIC inhibit superoxide anion production by differentiated HL-60 cells; failure of BBIC to inhibit superoxide production by cell extracts led to the conclusion that BBIC does not act as a simple free radical scavenger. Most recently, Guan...
et al. (15) showed that BBIC supplementation could protect the total antioxidant status of mice subjected to whole body proton or highly ionizing (HZE) irradiation. Our results indicate that BBIC has direct antioxidant properties. The data suggest that BBIC scavenges superoxide anion radicals, spontaneously formed superoxide derivatives (e.g., hydrogen peroxide and hydroxyl radicals), or some combination thereof. This is consistent with the cysteine-rich composition of BBIC, which yields cysteine, cystin, N-acetylcysteine, and cystamine on thermal decomposition (24). Cysteine and N-acetylcysteine are nonspecific antioxidants that support cellular resynthesis of glutathione (33). Cystamine increases glutathione levels in mice (25) and increases L-cysteine levels in rodents and cell culture (13). Our data indicate BBIC does not quench NO. On the contrary, BBIC appeared to increase the DAF-FM signal via an NO-dependent mechanism. The underlying chemistry is not clear; BBIC may have stimulated NO...

Fig. 6. Antioxidant action of BBIC. A: dietary supplementation with BBIC depresses cytosolic oxidant activity in unloaded muscle. Data depict oxidant activity in soleus muscles from freely ambulating control animals, mice unloaded for 3 days, and hindlimb-unloaded mice fed a diet supplemented with 1% BBIC. Oxidant activity in muscles from freely ambulating animals fed 1% BBIC (40.0 ± 8.5) was not different from control. DCF, 2′,7′-dichlorofluorescein; AU, arbitrary units. Values are means ± SE. *P < 0.05 vs. control. B: BBIC directly buffers reactive oxygen species activity in vitro. Superoxide anions generated by a xanthine oxidase/hypoxanthine system were detected using cytochrome c reduction assay. This signal was blunted by addition of 1% BBIC. *P < 0.01 vs. buffer control. C: BBIC does not buffer nitric oxide (NO) activity. In vitro reaction of 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) with NO generated by N-ethyl-2(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethamine (NOC) is augmented by addition of 1% BBIC (NOC + BBIC). Hemoglobin (50 μM), an NO quencher, inhibits DAF-FM reaction with NO (NOC + hemoglobin). Values are means ± SE; n = 2/group at each time point.

Fig. 7. Serine protease inhibition by BBI, shown as enzyme activities in homogenates of soleus muscles from freely ambulating control mice (solid bars) and animals subjected to unloading (shaded bars). A: in both groups, serine protease activity was diminished by BBI, but not by the selective proteasome inhibitor MG-132. MCA, methylcoumarin. B: proteasome activity was unaffected by BBI and depressed by MG-132. AMC, amidomethylcoumarin. Values are means ± SE. *P < 0.05 vs. basal.

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release by NOC-12 and/or facilitated NO reaction with DAFFM. The biological relevance is also not obvious. To our knowledge, BBIC effects on NO signaling have not been tested in cellular systems or animals.

Dietary supplementation with BBIC opposes the effects of mechanical unloading on muscle, a condition in which weakness is strongly linked to oxidative stress (4, 23, 28, 35). We find that atrophy of unloaded muscles correlates with increased oxidant activity. BBIC supplementation prevents both changes, abolishing redox changes and partially protecting muscle function. These findings are consistent with antioxidant protection by BBIC but do not define the relative importance of antioxidant effects vs. protease inhibition. This issue has yet to be resolved.

In conclusion, the present study provides new information on the role of oxidative stress in unloaded muscle. Time-course data show that elevated oxidant activity is a ubiquitous influence on muscle fibers, inasmuch as they adapt to mechanical unloading, not just a transient effect or late-phase outcome. The biological significance of this response is reinforced by the fact that oxidant activity correlates with atrophy. The present data also provide new insights into the antioxidant properties of BBIC, showing its biological activity in vivo and the capacity of BBIC to directly scavenge reactive oxygen species. The most important finding of this study is that BBIC supplementation protects skeletal muscle against the atrophy and weakness caused by mechanical unloading. Our data closely agree with those of Morris et al. (31), establishing the reproducibility of BBIC protection in rodents. This highlights the potential value of BBIC supplementation as a countermeasure for unloading-induced weakness and strengthens the justification for testing BBIC efficacy in humans.

GRANTS

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