Increased muscle proteolysis after local trauma mainly reflects macrophage-associated lysosomal proteolysis

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Farges, Marie-Chantal, Denis Balcerzak, Brian D. Fisher, Didier Attaix, Daniel Béchet, Marc Ferrara, and Vickie E. Baracos. Increased muscle proteolysis after local trauma mainly reflects macrophage-associated lysosomal proteolysis. Am J Physiol Endocrinol Metab 282:E326–E335, 2002; 10.1152/ajpendo.00345.2001.—Rat gastrocnemius showed increased protein degradation (+75–115%) at 48 h after traumatic injury. Injured muscle showed increased cathepsin B activity (+327%) and mRNA encoding cathepsin B (+670%), cathepsin L (+298%), cathepsin H (+159%), and cathepsin C (+268%). In situ hybridization, cathepsin B mRNA localized to the mononuclear cell infiltrate in injured muscle, and only background levels of hybridization were observed either over muscle cells in injured tissue or in uninjured muscle. Immunogold/electron microscopy showed specific staining for cathepsin B only in lysosome-like structures in cells of the mononuclear cell infiltrate in injured muscle. Muscle cells were uniformly negative in the immunocytochemistry. Matrix metalloproteinase-9 (granulocyte-macrophage gelatinase) mRNA and activity were not present in uninjured muscle but were expressed after trauma. There was no activation of the ATP-ubiquitin-proteasome-dependent proteolytic pathway in injured muscle, by contrast to diverse forms of muscle wasting where the activity of this system and the expression of genes encoding ubiquitin and proteasome elements rise. These results suggest that proteolytic systems of the muscle cells remain unstimulated after local injury and that lysosomal enzymes of the inflammatory infiltrated cells are likely to be the major participant in protein catabolism associated with local trauma.

IN A MODEL OF BLUNT TRAUMA TO MUSCLE, we demonstrated a period of degeneration lasting −3 days, characterized by gross disruption of muscle cells, hemorrhage, inflammation, invasion of the injured site by mononuclear cells, and a 26% loss of previously existing muscle protein (12). A large increase in the process of proteolytic activities on gelatin zymography and also expresses mRNA encoding MMP-1, -2, -9, -14, and-16 and TIMP-1, -2, and -3, as well as plasminogen activator and its receptor (2). Physiological regulation of this system in muscle has not been extensively characterized; however, the activity, expression, and localization of MMP-9 have recently been reported after experimental injury induced in normal muscle by cardiotoxin injection and denervation (20, 21). This proteinase is.

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mainly produced by inflammatory cells, including polymorphonuclear leukocytes, macrophages, eosinophils (36), and lymphocytes (29) and is involved in the migratory process of these cells in acute inflammation with remodeling and neovascularization (41).

The contribution of proteolytic systems to tissue catabolism after injury to nonmuscle tissue (i.e., systemic response to injury) has been studied. For example, muscle wasting after burn injury in rats has been attributed to the ATP-ubiquitin-proteasome-dependent system (11). Increased gene expression of elements of this system was also observed in peripheral muscle of head trauma victims (27). The relative roles of proteolytic systems after direct trauma to muscle are unknown, and we sought to clarify their nature through study of proteolytic activity, quantitation of mRNA encoding proteinases and their cofactors, and muscle incubation with specific proteinase inhibitors. Because initial studies (12, 37) suggested the participation of proteinases potentially derived from muscle cells and/or mononuclear cells of the inflammatory infiltrate, we also determined the localization of the most increased proteolytic activity, cathepsin B, by immunocytochemistry and of its mRNA by in situ hybridization.

METHODS

Experimental animals. Studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (200–300 g) from a colony maintained at the University of Alberta were used. Rats were housed in individual wire mesh cages in a temperature (24°C) and humidity (80%)-controlled room on a 12:12-h light-dark cycle. Rats were fed ground laboratory chow (Continental Grain, Chicago, IL) containing 24% crude protein. Rats were killed by CO2 asphyxiation. Animals were allocated by initial body weight to the two treatment groups (control and injured) such that the mean body weights and SE of the groups were similar. Injured rats were administered a single impact trauma to the medial aspect of the right hindlimb. The procedure produced a moderate contusion of the medial gastrocnemius and was conducted while the rats were under general anesthesia (12). Control uninjured rats were also anesthetized. In some experiments, the tissue receiving the direct impact of the device (right medial gastrocnemius) as well as uninjured muscle on the contralateral (left) limb of the same animal were studied. Experiments were carried out with 6–10 rats per treatment. All of the described experiments were repeated at least twice. The results of each treatment are presented as mean values ± SE. Statistical comparisons were made by ANOVA followed by Duncan’s test.

A time course study (6, 24, 48, and 72 h posttrauma) was done initially to determine the temporal sequence of induction of proteolysis. Because in vitro protein turnover measurement entails between-day variation, animals were killed at different times and then killed on the same day so that all incubations could be conducted at the same time. In all subsequent studies, control uninjured and injured rats were studied at 48 h after injury, when net protein mobilization and the process of protein catabolism occurred at the most rapid rate.

Lysoosomal enzyme inhibitors. The presence of cystolic inhibitors of cysteine proteinases (31) precludes direct assay of lysoosomal cathepsins in unfractioned muscle extracts. Preparation and purification of lysoosomal extracts were done as described previously (6, 30). Muscles were homogenized with a polytron in 10 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 50 mM KCl, and 1 mM EDTA. An aliquot of homogenate was brought to 0.25% Triton X-100 in acetate buffer, pH 5.0, and stored at −20°C until further analysis of N-acetyl-β-d-glucosaminidase activity and protein content. The homogenate was centrifuged 10 min at 1,000 g and then for 10 min at 2,500 g. The supernatant was centrifuged at 20,000 g for 20 min, and the pellet was resuspended in 30 mM sodium phosphate buffer, pH 5.8, and frozen overnight. After thawing, an aliquot was also made up to 0.2% in Triton X-100 and stored for determination of N-acetyl-β-d-glucosaminidase activity. The supernatant recovered after 20-min centrifugation at 60,000 g was designated the lysosomal extract and was used for determination of cathepsin B and B + L activity. N-acetyl-β-d-glucosaminidase activities were determined in lysozomal fractions to estimate the yield of lysosomes (7). Protein concentration was determined according to Bradford (9). Assays for Z-Arg-Arg-ammonomethylcoumarin (NMec; cathepsin B) and Z-Phe-Arg-NMec (cathepsins B and L) hydrolyses were carried out according to Barrett (5).

Gelatin zymography. To detect MMP-2 and MMP-9 activities present in control and injured muscles, samples were prepared and gelatin zymography conducted as described by Balcerzak et al. (2). Briefly, after extraction, soluble proteins (15 μg) were separated on a 15% SDS-PAGE gel containing gelatin (1 mg/ml). After migration, gels were washed in a Triton X-100 solution (2.5% in distilled water), incubated 20 h at 36°C in enzyme buffer (50 mM Tris·HCl, pH 7.5, 10 mM CaCl2, 0.05% Brij-35), and stained with naphthol blue-black solution.

Tissue RNA isolation, Northern hybridization analysis, and RT-PCR. Total RNA was extracted from frozen samples by the guanidinium isothiocyanate-CsCl method (34). Purity and quantitation of RNA were determined by measures of absorbance at 260 and 280 nm. A cDNA insert encoding rat cathepsin B was subcloned into the EcoRI site of pGEM-blue (Promega, Madison, WI). Antisense riboprobes suited to Northern hybridization and in situ hybridization were generated from this plasmid pGEM rat cathepsin after HindIII digestion and synthesis with T7 RNA polymerase. The riboprobe is complementary to 289 bases of rat cathepsin B mRNA. Northern hybridization with cathepsin B [32P]cRNA was performed as previously described (6, 42).

Dot blot hybridization was performed as described previously (38) using probes encoding rat cathepsins C and H, mouse cathepsins B, L, and D, rat calpain I, chicken ubiquitin, rat 20S proteasome subunits C2, C3, C5, C6, and C9, and human subunit S5α of the 19S complex. A probe encoding rat mitochondrial rRNA 12S (F17) was used as a control. Radioactivity in dot blots was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RT-PCR was performed using the Superscript One-Step RT-PCR system (Life Technologies, Burlington, ON, Canada). The MMP-9 product (497 bp) was amplified using 2 μg of total RNA and 10 μM of the following primers: sense GCCAAGGATGTTCTACTGGC; antisense GACGCACATC-3' and human subunit S5α of the 19S complex. A probe encoding rat cathepsin B mRNA was performed as previously described (6, 42).

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Light microscopy and in situ hybridization analysis. For light and electron microscopy, control (uninjured) and injured animals were killed and immediately perfused in a single pass with phosphate-buffered physiological saline (PBS) and then with 0.5% glutaraldehyde-4% paraformaldehyde in 0.1 M cacodylate HCl buffer, pH 7.2. Samples of medial gastrocnemius (3 mm² (n = 5/muscle) were dissected from the injured area and from the same anatomical location on the contralateral (uninjured) limb. Muscle pieces were postfixed for 2 h at 4°C in a solution containing 4% paraformaldehyde and 4% sucrose in 0.1 M cacodylate HCl buffer, pH 7.2. Finally, tissue pieces were washed in the same buffer containing 7.5% sucrose. This fixation protocol was developed and employed for immunocytochemistry with the use of affinity-purified anti-cathepsin B (14, 24, 40).

Samples for light microscopy collected after fixation by perfusion were frozen in isopentane, cooled in liquid nitrogen, and mounted in OCT compound (Ames, Elkhart, IN) before sectioning (5 μm) in a cryostat (Reichert-Jung, Nussloch, Germany). To determine the general tissue architecture, tissue sections were stained with hematoxylin and eosin, and mounted for light microscopy by standard procedures. In situ hybridization was carried out as described previously (15). Briefly, tissue sections were prepared by treatment with proteinase K (20 μg/ml; 8 min), blocking [10 mM dithiothreitol (DTT), 1.85 mg/ml iodoacetamide, 1.25 mg/ml N-ethylmaleimide for 25 min at 45°C] and acetylation (0.25% acetic anhydride in triethanolamine HCl, pH 8.0). Sections were incubated in a prehybridization medium containing 50% formamide, 5 × PIPES, pH 6.8, 5 × Denhardt's solution, 2% SDS, 0.25 mg/ml salmon sperm DNA, 0.25 mg/ml tRNA, and 0.1 mM DTT at 43°C for 2 h. The hybridization solution was of the same composition as that used for prehybridization, except that it contained no DTT and included 0.1 g/ml dextran sulfate and an antisense [35S]CTP-cathepsin B riboprobe (120,000 dpm/μl). Hybridization was at 43°C for 4–16 h. After hybridization and rinsing with 4 × standard saline citrate (SSC; 0.15 M NaCl, 0.015 M Na citrate)-0.1% mercaptoethanol, sections were digested with ribonuclease A (40 μg/ml) and T1 (800 U/ml) for 30 min at 37°C. Finally, sections were counterstained with hematoxylin and eosin, and liquid emulsion microautoradiography was carried (15). Photomicrography was performed using light field optics. To check the specificity of in situ hybridization reactions, some sections were hybridized with an unrelated antisense riboprobe, [35S]CTP-glucagon, or with a labeled sense transcript of the cathepsin B cDNA.

Immunocytochemistry. For electron microscopy, tissue blocks were washed three times with PBS, pH 7.4, and then transferred to PBS containing 0.5% OsO4 for 10 min at 25°C. Tissues were dehydrated in a graded series of ethanol and then transferred to 100% propylene oxide. Samples were embedded in araldite CY212. Thin sections were cut on a Reichert Om U2 ultramicrotome and mounted on nickel grids. All of the following steps were conducted at 25°C. Grids bearing sections were floated face down on 1% NaIO4 for 1 h and then rinsed with distilled water three times for 10 min each. To block nonspecific binding, sections were incubated for 10 min in a solution of PBS that contained 1% bovine serum albumin (BSA), 1% gelatin, and 0.05% Tween-20 and transferred to a solution of 0.02 M glycine-PBS for 5 min. Affinity-purified IgG anti-cathepsin B was a generous gift of Dr. E. Kominami (Juntendo University, Tokyo, Japan). Antibodies against cathepsin B were raised in rabbits and purified by affinity chromatography (22). These antibodies have been characterized by immunoblotting and immunohistochemistry (19, 40). Affinity-purified IgG anti-cathepsin B was diluted to concentrations of 2.5, 5, or 10 μg/ml in PBS containing 1% BSA, 1% gelatin, and 0.05% Tween-20. The muscle sections were incubated with the primary antibody for 1 h at 25°C on a slow shaker and then washed with PBS containing 1% BSA and 1% gelatin. Protein A-gold (G-3766, Sigma, Oakville, ON, Canada) was used for immunocytochemical staining. Protein A was coupled to colloidal gold particles of the nominal size 10–15 nm. The final solution of protein A-gold was prepared in 0.5% BSA in 0.01 M PBS, pH 7.4, containing 0.05% Tween-20. The grids were placed on drops of protein A-gold at a 20-fold dilution of stock solution and incubated at room temperature for 1 h. The grids were rinsed with PBS and finally rinsed with distilled water. The sections were subsequently stained with 2% uranyl acetate for 30 min and with lead citrate for 5 min. The specimens were examined in a Hitachi H-7000 electron microscope at 75 kV. The specificity of the affinity-purified antibody was determined by immunoblotting on whole skeletal muscle obtained from injured and uninjured control animals. For a negative control for immunocytochemistry, the primary antibody was omitted.

Muscle incubations. Incubations are routinely done with muscles that are small and thin enough to permit the diffusion of oxygen and substrates into the tissue. The use of surgically prepared longitudinal strips of larger muscles has been validated for studies of muscle protein turnover (43) as well as glucose transport (13). Because it would not have been possible to traumatize some of the more commonly incubated muscles, we dissected four thin longitudinal strips from control and injured medial gastrocnemius. Muscle strips weighed ~20 mg and were ~12 mm in length (i.e., dissected from origin to insertion of the gastrocnemius muscle) and not thicker than 1.2 mm. The strips were not mounted on a physical support because of the lack of long tendons on this muscle. Muscle strips were incubated in vitro as described extensively in other work from our laboratories (3, 4, 42). Muscles were preincubated in individual flasks containing 3 ml of a modified Krebs-Ringer bicarbonate medium (KRB) composed as previously described (3). In all studies, tissues were incubated at 35°C in medium with 95% O2-5% CO2. KRB buffer contained 1.0 mM CaCl2, 8 mM bovine insulin, 0.5 mM cycloheximide, and 5 mM glucose. A control strip from each injured and uninjured muscle was incubated in this medium, and in a second strip dissected from the same muscles, lysosomal proteinases were inhibited by adding methylamine HCl (4). A separate experiment was conducted to study the possible involvement of proteasome-dependent proteolysis. Media were formulated as indicated above; however, Ca2+-dependent proteases were inhibited by deleting Ca2+ from the medium and adding 10 μg/ml Na-dantrolene to prevent release of intracellular Ca2+ (3); methylamine HCl was added as indicated to inhibit lysosomal protease activity. In this approach, the contribution of the non-proteasome-dependent systems is first eliminated. We used a proteasome inhibitor MG 132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Calbiochem, La Jolla, CA) (10 μM, final concentration) dissolved in dimethyl sulfoxide (35, 39). Control muscles were incubated in the same medium containing an equivalent amount of dimethyl sulfoxide but no inhibitor.

All muscles were preincubated for 1 h and then incubated for 2 h in fresh medium of identical composition. At the end of incubation, muscles were blotted and frozen in liquid nitrogen and stored at −20°C until analysis. Muscle protein mass was determined using the bichinchonic acid procedure (BCA Protein Assay; Pierce Chemical, Rockford, IL) after tissue solubilization in 1.0 N NaOH at 25°C.
Protein degradation was determined as the amount of tyrosine released by the tissue into the medium during incubation in the presence of cycloheximide to prevent amino acid reincorporation into proteins (3). Preliminary studies established that changes in intracellular pools of tyrosine during incubation were small and could be ignored for such measurements. Tyrosine release was linear for up to 120 min of incubation.

RESULTS

Gastrocnemius muscle preparation and effects of trauma on proteolysis. Rates of protein catabolism in control uninjured gastrocnemius strips fell within a range from 2.09 to 2.55 nmol tyrosine·mg protein\(^{-1}\)·h\(^{-1}\), and these values were similar to those reported for incubated epiprocthepiras (3). In preliminary experiments, we determined that the four strips from each muscle had highly similar rates of catabolism (coefficient of variation, 3%). Tissue levels of free tyrosine were 0.625 ± 0.031 nmol/mg protein in control muscles and 0.685 ± 0.043 nmol/mg protein in injured muscles, and these values were not significantly different from each other before or after tissue incubation. Injury resulted in increased release of tyrosine into the incubation medium (Fig. 1). Because tissue levels of tyrosine did not change over the course of incubation and protein synthesis was inhibited, tyrosine appearing in the medium originated from proteolysis. At 6 h after injury, protein degradation tended to rise (+25%, \(P = 0.149\) vs. control) and was significantly elevated at 24, 48, and 72 h. Day 2 posttrauma was the most catabolic day (+115% vs. control, \(P < 0.0001\)). In different experiments, the magnitude of the proteolytic response on day 2 showed some variation, from +75 to +115%.

This time point, which reflected the peak rate of net protein mobilization (12) and the peak rate of protein catabolism, was selected for all further studies.

Proteinase activity. We tested for modifications of lysosomal proteinase activity. Similar lysosomal yields were obtained from control and injured muscles as demonstrated by glucosaminidase activity (Table 1). Both cathepsin B and B + L activities in the medial gastrocnemius increased after trauma. The large increase in proteolysis observed on day 2 posttrauma was associated with a maximal activity of both cathepsin B and B + L in muscle homogenates (+327% and +123% vs. control, respectively). On day 7 posttrauma, cathepsin activities in lysosomal extracts returned to normal levels compared with control rats; however, these remained elevated in injured muscle homogenates (+96 and +53% for cathepsin B and B + L, respectively, vs. control).

Proteinase gene expression. Injury was associated with increased expression of cathepsin B mRNA compared with a control gene, glyceraldehyde phosphate dehydrogenase (Fig. 2). Day 2, the most catabolic day, corresponded to the maximal expression of cathepsin B. Results of quantitative dot blot for proteinase gene expression are shown in Table 2. Lysosomal proteinase mRNAs increased (cathepsins B, H, L) or tended to increase (cathepsins C and D) in injured muscle. Of these, cathepsin B showed the largest increase, 6.72-fold, compared with control muscles. No significant

### Table 1. Lysosomal enzyme activities increase in injured gastrocnemius

<table>
<thead>
<tr>
<th>Activity</th>
<th>Control</th>
<th>Injured</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Phe-Arg-NMec hydrolysis (cathepsin B + L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In homogenate, µU/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days posttrauma</td>
<td>0.83 ± 0.04*</td>
<td>2.17 ± 0.23†</td>
<td>+161%</td>
</tr>
<tr>
<td>7 days posttrauma</td>
<td>0.27 ± 0.02*</td>
<td>0.53 ± 0.09†</td>
<td>+96%</td>
</tr>
<tr>
<td>In lysosomal fraction, µU/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days posttrauma</td>
<td>27.2 ± 4*</td>
<td>60.8 ± 9†</td>
<td>+123%</td>
</tr>
<tr>
<td>7 days posttrauma</td>
<td>10.7 ± 2*</td>
<td>10.3 ± 1*</td>
<td>NS</td>
</tr>
<tr>
<td>Z-Arg-Arg-NMec hydrolysis (cathepsin B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In homogenate, µU/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days posttrauma</td>
<td>0.26 ± 0.01*</td>
<td>1.11 ± 0.13†</td>
<td>+327%</td>
</tr>
<tr>
<td>7 days posttrauma</td>
<td>0.36 ± 0.32*</td>
<td>0.55 ± 0.09†</td>
<td>+53%</td>
</tr>
<tr>
<td>In lysosomal fraction, µU/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days posttrauma</td>
<td>6.2 ± 1.3*</td>
<td>26.2 ± 3.6†</td>
<td>+323%</td>
</tr>
<tr>
<td>7 days posttrauma</td>
<td>20.4 ± 3.1*</td>
<td>21.8 ± 2.6*</td>
<td>NS</td>
</tr>
<tr>
<td>β-d-glucosaminase, U/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days posttrauma</td>
<td>38 ± 2*</td>
<td>48 ± 4†</td>
<td>+26%</td>
</tr>
<tr>
<td>7 days posttrauma</td>
<td>47 ± 4*</td>
<td>42 ± 2*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cathepsin activities were measured in muscle homogenates and lysosomal extract by use of specific substrates. Cathepsin activities were expressed relative to the protein content of the muscle homogenate. One unit (U) of activity is the release of 1 µmol aminomethylcoumarin (Mec/min). Means with different superscript symbols in the same line are significantly different (\(P < 0.05; n = 6\)). NS, not significant.

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*Fig. 1. Time course of protein breakdown after injury.* Proteolysis was estimated by the release of tyrosine into the incubation medium in the presence of cycloheximide. Medial gastrocnemius from control and injured rats (4 thin strips/muscle) were preincubated for 1 h in Krebs-Ringer bicarbonate buffer containing cycloheximide (0.5 mM), glucose (5 mM), insulin (8 nM), and CaCl\(_2\) (1.0 mM). Muscles were transferred to fresh media of the same composition and incubated for a further 3 h. The average value from each of the 4 strips was calculated for each animal. The 4 strips from each muscle had highly similar rates of catabolism (coefficient of variation, 3%). Values not sharing the same letters are significantly different (\(P < 0.01, n = 4\)).
changes were seen in expression of calpain, ubiquitin, or proteasome subunit mRNAs.

Light microscopy and in situ hybridization. In uninjured muscle, only scattered silver grains in a random pattern were observed (Fig. 3B), and this was not different from muscle sections that were treated with an unrelated antisense riboprobe, [35S]CTP-glucagon, or with a sense transcript of the cathepsin B cDNA. In injured gastrocnemius, cathepsin B mRNA localized to the area of tissue damage (Fig. 3A). Dense clusters of silver grains were seen over and around mononuclear cell infiltrates localized in the widened interstitial spaces and around the damaged myofibers.

Immunoelectron microscopy. Because of the energy level of the isotope used in in situ hybridization, the silver grains are scattered about the source of radioactivity, and we chose the immunogold technique to more precisely localize cathepsin B at a higher level of resolution. At 2 days posttrauma, muscle damage was characterized by the presence of widened interstitial

Table 2. Proteinase gene expression in injured and control muscles at 48 h posttrauma

<table>
<thead>
<tr>
<th>Factor</th>
<th>mRNA Level, Injured/Control</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial rRNA 12S</td>
<td>1.03</td>
<td>0.22</td>
<td>NS</td>
</tr>
<tr>
<td>Lysosomal enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>6.72</td>
<td>0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>2.98</td>
<td>0.70</td>
<td>0.04</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>1.59</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>2.68</td>
<td>0.44</td>
<td>0.05</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>1.60</td>
<td>0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>Calpain I</td>
<td>2.23</td>
<td>0.67</td>
<td>NS</td>
</tr>
<tr>
<td>Proteasome-dependent system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>0.94</td>
<td>0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Proteasome subunits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>1.24</td>
<td>0.13</td>
<td>NS</td>
</tr>
<tr>
<td>C3</td>
<td>1.22</td>
<td>0.10</td>
<td>NS</td>
</tr>
<tr>
<td>C5</td>
<td>1.15</td>
<td>0.10</td>
<td>NS</td>
</tr>
<tr>
<td>C8</td>
<td>1.47</td>
<td>0.19</td>
<td>NS</td>
</tr>
<tr>
<td>C9</td>
<td>1.21</td>
<td>0.16</td>
<td>NS</td>
</tr>
<tr>
<td>S5a</td>
<td>1.21</td>
<td>0.10</td>
<td>NS</td>
</tr>
</tbody>
</table>

mRNA levels were determined by quantitative dot blot hybridization in total RNA from control and injured muscle. C2–C9, 20S proteasome subunits; S5a, human subunit of 19S complex; n = 6.

Fig. 3. In situ hybridization analysis of cathepsin B mRNA in injured and control gastrocnemius. A: injured muscle at a site of focal damage, showing widened interstitial spaces and mononuclear cell infiltrate. Dense clusters of silver grains can be seen in cells comprising the mononuclear cell infiltrate. B: uninjured muscle. Bar, 50 μm.
strips from the same muscle incubated with inhibitor. Methylamine (20 mM), an inhibitor of lysosomal acidification, had no significant effect on protein degradation in uninjured muscles but suppressed the increase due to trauma by 67% (Table 3). An inhibitor of proteasome activity substantially inhibited total proteolysis in uninjured muscles but had no effect on the activation of proteolysis induced by injury (Table 4).

DISCUSSION

**Contribution of proteolytic systems to trauma-induced proteolysis.** After the experimental injury used here, a degenerative phase of ~3 days duration is characterized by acute inflammation and muscle protein loss (12). The present study clearly demonstrates that a sustained increase in protein breakdown is one determinant of protein loss previously reported in acute muscle trauma (12). The specific aim of this study was to determine which proteolytic system(s) contribute to muscle degeneration after trauma. Using different approaches, we have shown that it is mainly the lysosomal proteolytic system that is activated during the degenerative phase. Lysosomal proteolysis accounted substantially (~67%) for the overall increase in protein breakdown based on an in vitro approach with the use of an inhibitor. Concordant results were found with several different approaches. Increased activity of cathepsin B and B + L was found in the atrophying muscles. On day 2 after trauma, these enzyme activities increased both in muscle homogenates and in lysosome fractions in parallel with the rise in the lysosomal process in incubated muscles. Moreover, elevated mRNA levels coding for lysosomal cathepsins were markedly expressed in injured muscle, particularly on day 2 posttrauma.

**Cellular source of lysosomal enzymes.** Various cellular components of the muscle tissue were altered after injury (12). At 1–2 days after injury, inflammation appeared to be fully established in the muscle, and this period was characterized by large numbers of mononuclear cells that had not previously been present. Mononuclear cells were seen both in the endomysial connective tissue and within some damaged muscle fibers (12). Mononuclear cells were seen beneath the basement membrane of the muscle cells in focal aggregates. These mononuclear cells had the distinctive morphology of phagocytes and may include tissue macrophages that were previously present, monocytes that were attracted to the site of injury and crossed the vascular wall to become macrophages, B lymphocytes, and cytotoxic T lymphocytes. Mononuclear phagocytes, when stimulated, synthesize and secrete >80 defined molecules, which serve to mediate the inflammatory, antibacterial, and antitumor activities of these cells. Hydrolytic enzymes, and particularly proteinases, figure prominently in the enzymes synthesized by phagocytes. Thus two potential sources for increased lysosomal enzyme levels in injured muscle may be considered: 1) infiltration of mononuclear cells into the muscle tissue and 2) activation of the lysosomal system.
endogenous to the muscle cells. The cellular components released from mechanically disrupted muscle cells and vascular walls provide strong stimuli for the influx of inflammatory cells into the injured site, and these cells have a large capacity for phagocytosis and lysosomal degradation of proteins. We localized cathepsin B and cathepsin B mRNA to determine their potential cellular source(s). Cathepsin B mRNA, studied at the resolution of light microscopy, localized to cells of the mononuclear cell infiltrate and not in muscle cells. At the higher resolution of transmission electron microscopy, immunoreactive cathepsin B was detected only in lysosome-like structures within mononuclear cells.

The presence of inflammatory cell infiltrate and its possible contribution to proteolytic events after injury is additionally suggested by the appearance of MMP-9 activity and mRNA in the injured muscles. MMP-9 activity and mRNA were present only in the traumatized area dissected from the injured medial gastrocnemius. The infiltration of inflammatory cells is linked to the presence of the MMP-9 activity observed, as macrophages and lymphocytes are an important source of this particular gelatinase (29, 36). MMP-9 may be involved in the migration of the inflammatory cells into the traumatized area. By contrast, MMP-2, an MMP constitutively expressed in skeletal muscle and connec-

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Fig. 5. Immunocytochemical localization of cathepsin B in mononuclear cell infiltrate of injured gastrocnemius. Muscles were treated with affinity-purified anti-cathepsin B. A: low-magnification view of mononuclear cell. B and C: gold particles are localized in lysosome-like structures of uniform electron density. Solid bar, 5 μm.

Fig. 6. Gelatinase activities and matrix metalloproteinase (MMP)-9 gene expression in injured and uninjured gastrocnemius. A: MMP-2 and MMP-9 activities detected using the gelatin zymography technique in uninjured and injured muscles. Proteins (15 μg) were separated on a 15% SDS-PAGE gel containing gelatin (1 mg/ml). After migration, the gel was washed in a Triton X-100 solution (2.5% in distilled water), incubated 20 h at 36°C in enzyme buffer, and stained with naphthol blue-black solution. B: determination of MMP-9 expression by RT-PCR. Total RNA (2 μg) isolated from a positive control tissue rat lung (L), uninjured muscle (U), and injured muscle (I) was used. The 100-bp DNA ladder is presented in the lanes denoted M.
and CaCl₂ (1.0 mM). Muscle strips were transferred to fresh medium Ringer bicarbonate (KRB) containing glucose (5 mM), insulin (8 nM), before and after injury.

Table 3. Lysosomal protein catabolism is activated at 48 h posttrauma

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>Control</th>
<th>Injured</th>
<th>Increase After Trauma</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.06 ± 0.08</td>
<td>3.64 ± 0.34</td>
<td>1.58 ± 0.33</td>
</tr>
<tr>
<td>Methylamine</td>
<td>2.09 ± 0.10</td>
<td>2.61 ± 0.28</td>
<td>0.52 ± 0.24*</td>
</tr>
<tr>
<td>Lysosomal process, % total proteolysis</td>
<td>0%</td>
<td>28%</td>
<td>67%</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol tyrosine-mg protein⁻¹*2 h⁻¹. Strips of medial gastrocnemius muscles dissected from control (n = 6) and injured rats (n = 6) were preincubated for 1 h in Krebs-Ringer bicarbonate (KRB) containing glucose (5 mM), insulin (8 nM), and CaCl₂ (1.0 mM). Muscle strips were transferred to fresh medium of the same composition and incubated for a further 2 h. Proteolysis was estimated by the release of tyrosine into the incubation medium in the presence of cycloheximide (0.5 mM). One muscle strip/animal was incubated under control conditions. Lysosomal proteolysis was determined in 1 muscle strip/animal in the presence of methylamine HCl (20 mM). *Significant effect of methylamine (P < 0.05).

Table 4. Proteasome inhibitor MG132 does not inhibit the activation of protein catabolism at 48 h posttrauma

<table>
<thead>
<tr>
<th>Additions to Medium</th>
<th>Control</th>
<th>Injured</th>
<th>Increase After Trauma</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.40 ± 0.08</td>
<td>4.57 ± 0.33</td>
<td>2.17 ± 0.31</td>
</tr>
<tr>
<td>Methylamine, Na dantrolene in Ca²⁺-free medium</td>
<td>1.63 ± 0.09</td>
<td>2.61 ± 0.21</td>
<td>0.98 ± 0.33</td>
</tr>
<tr>
<td>MG132, methylamine, dantrolene in Ca²⁺-free medium</td>
<td>0.93 ± 0.04*</td>
<td>2.03 ± 0.24*</td>
<td>1.10 ± 0.25</td>
</tr>
<tr>
<td>Proteasome-dependent process, % total proteolysis</td>
<td>29.2%</td>
<td>12.7%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol tyrosine-mg protein⁻¹*2 h⁻¹. Strips of medial gastrocnemius muscles dissected from control (n = 7) and injured (n = 7) rats were preincubated for 1 h in KRB containing glucose (5 mM), insulin (8 nM), and CaCl₂ (1.0 mM). Muscle strips were transferred to fresh medium of the same composition and incubated for a further 2 h. Proteolysis was estimated by the release of tyrosine into the incubation medium in the presence of cycloheximide (0.5 mM). One muscle strip/animal was incubated under control conditions. One muscle strip/animal was incubated with inhibitors of lysosomal and Ca²⁺-dependent proteolytic systems. One muscle strip/animal was incubated with inhibitors of lysosomal, Ca²⁺-dependent, and proteasome-dependent pathways (MG132). *Significant effect of MG132 (P < 0.05).
Only a few results are available regarding the participation of proteolytic systems after trauma. In one of the few studies done to date in humans (27), mRNAs encoding multiple elements of the ATP-ubiquitin-proteasome system were increased in peripheral skeletal muscles of head trauma victims. Superficially, these results would seem to contradict those presented here, which suggest that proteolysis in traumatized muscle is largely lysosomal and is associated with mononuclear phagocytes. However, it seems that there is more likely to be both a systemic and a local proteolytic response to injury. A severe injury to the head would induce a systemic response by alterations in hormones such as glucocorticoids, which are known to activate ATP-ubiquitin-proteasome-dependent catabolism in peripheral muscle (1). Because the extent of the injury in our model was limited to a small part of the medial gastrocnemius, it is possible that a systemic response was small or not present and that, within the local environs of injury to muscle, proteolytic activity associated mainly with inflammatory cells prevailed. Although we did not obtain data on glucocorticoid levels in the injured animals, the absence of any reduction in food intake after injury (12) suggests that the overall level of stress was minimal.

A full appreciation of the role of locally and systemically produced inflammatory mediators in muscle injury and regeneration will permit therapeutic strategies to limit excess muscle catabolism and enhance regeneration. The results presented here clarify the role of proteinases in injury-induced local muscle catabolism. Lysosomal enzymes accounted in large part for the increased protein catabolism associated with muscle trauma, and these enzymes and their mRNA localized to cells of the inflammatory infiltrate, not to muscle cells, in the injured tissue. The lack of increase in mRNA of elements of the ATP-ubiquitin-proteasome-dependent proteolytic pathway, considered to be a key participant in diverse forms of muscle wasting, further supports a limited role of intramuscular proteolytic pathways in the remodeling of muscle after local injury.

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REFERENCES


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