Proteasome blockers inhibit protein breakdown in skeletal muscle after burn injury in rats

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1. Burn injury stimulates ubiquitin-dependent protein breakdown in skeletal muscle. The 20S proteasome is the proteolytic core of the 26S proteasome that degrades ubiquitin conjugates. We examined the effects of the proteasome inhibitors N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL), lactacystin and β-lactone on protein breakdown in muscles from burned rats.

2. A full-thickness burn of 30% total body surface area was inflicted on the back of rats. Control rats underwent a sham procedure. After 24 h, extensor digitorum longus muscles were incubated in the absence or presence of 20S proteasome blocker and protein turnover rates and ubiquitin mRNA levels were determined.

3. LLnL resulted in a dose- and time-dependent inhibition of total protein breakdown in incubated muscles from burned rats. Lactacystin and β-lactone blocked both total and myofibrillar muscle protein breakdown. In addition to inhibiting protein breakdown, LLnL increased ubiquitin mRNA levels, possibly reflecting inhibited proteasome-associated RNase activity.

4. Inhibited muscle protein breakdown caused by LLnL, lactacystin and β-lactone supports the concept that the ubiquitin–proteasome pathway plays a central role in burn-induced muscle proteolysis. Because the proteasome has multiple important functions in the cell, in addition to regulating general protein breakdown, further studies are needed to test the role of proteasome blockers in the treatment or prevention of muscle catabolism.

INTRODUCTION

Muscle catabolism is a characteristic metabolic response to burn injury, both in patients [1] and experimental animals [2–4]. Although the catabolic response is characterized by both reduced protein synthesis and increased protein degradation, the stimulated protein breakdown, in particular myofibrillar protein breakdown, is the most important component of muscle catabolism in this condition [2,3].

Intracellular protein degradation is regulated by lysosomal and non-lysosomal proteolytic pathways. Among the non-lysosomal pathways, the energy–ubiquitin-dependent mechanism is involved in the regulation of muscle protein breakdown in a number of catabolic conditions, including sepsis [5–7], cancer [8], fasting [9] and renal failure [10]. In recent studies, we found evidence that this mechanism is important for the catabolic response in skeletal muscle after burn injury as well [4]. Thus, energy-dependent total and myofibrillar protein breakdown rates were substantially elevated in muscles of burned rats and tissue levels of ubiquitin mRNA were increased. Lysosomal and calcium-dependent protein breakdown was also increased after burn injury but...
because myofibrillar protein degradation is not regulated by these mechanisms, ubiquitin-dependent proteolysis is probably the most important component of burn-induced muscle proteolysis [4].

In the energy–ubiquitin-dependent proteolytic pathway, proteins that are to be degraded are first conjugated to ubiquitin, a 76-amino-acid peptide, whereafter they are recognized and broken down by the 26S proteasome, which is a large 26 kDa catalytic complex [11,12]. The 20S proteasome, a cylinder-formed particle composed of four stacked rings, is the catalytic core of the 26S proteasome [12,13]. Each ring of the 20S proteasome consists of seven subunits, the outer of which are called the α-subunits and the inner of which are called the β-subunits. The functions of the α-subunits include interaction between the 20S proteasome and various regulators whereas the hydrolytic sites are located on the β-subunits. The structure and function of the 20S proteasome have been reviewed elsewhere [12–15].

Although previous reports of a concomitant increase in energy-dependent protein breakdown and expression of ubiquitin mRNA suggest that the ubiquitin–proteasome pathway is upregulated in skeletal muscle after burn injury [4], the results do not prove that this mechanism is responsible for burn-induced muscle catabolism. Another way to further examine the role of the ubiquitin–proteasome mechanism in burn-induced muscle proteolysis would be to test the effects of specific blockers of this proteolytic pathway. The recent description of 20S proteasome inhibitors has made such experiments possible [16–18]. In recent studies, 20S proteasome inhibitors blocked the increase in muscle protein breakdown associated with chronic renal failure [19], diabetes [20] and sepsis [21,22]. The influence of these substances on muscle protein breakdown after thermal injury is not known.

In the present experiments we tested the effects of the 20S proteasome blockers N-acetyl-l-leucinyl-l-leucinal- l-norleucinal [LNNL] and lactacystin on protein breakdown in muscles from burned rats. Because recent studies indicated that lactacystin in solution undergoes rapid spontaneous hydrolysis to β-lactone which rapidly enters the cell and is the active proteasome blocking substance [18], the effect of β-lactone was also tested. We found that the proteasome blockers inhibited most of the burn-induced increase in muscle proteolysis, supporting the concept that muscle protein breakdown after burn injury at least in part reflects stimulated ubiquitin–proteasome-dependent proteolysis.

**MATERIALS AND METHODS**

**Animals and burn protocol**

Male Sprague–Dawley rats (Harlan Company, Indianapolis, IN) weighing 40–60 g were housed at an ambient temperature of 25 °C with a 12-h light and dark cycle. Rats of this size were used because they possess lower extremity muscles which are thin enough to allow for the measurement of protein turnover rates during incubation *in vitro* [23,24]. After 3 days of acclimatization, the rats were anaesthetized with pentobarbital (Nembutal, 35 mg/kg intraperitoneally; Abbot Laboratories, North Chicago, IL., U.S.A.), the dorsal fur was closely clipped and a full-thickness burn of 30% total body surface area was inflicted on the back by burning two layers of kerosene-soaked gauze for 15 s. The size of the burn wound was determined using the formula of Horst et al. [25]. The rats were resuscitated with 0.9% saline solution (10 ml/100 g body weight) administered intraperitoneally immediately before the burn. Rats in the control group were anaesthetized, clipped and resuscitated with saline solution, but not burned. After the burn or sham procedure, rats were housed individually with free access to water and muscles were harvested after 24 h. Rats in the control group were fed an equal amount of chow (10 g/100 g body weight) to that consumed by the rats during the first 24 h after burn in the present experimental model [3,4]. This provided a ‘pair-fed’ group of control rats although each individual rat was not pair-fed with a corresponding burned rat. All rats were cared for in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

**Muscle incubations**

Twenty-four hours after the burn or sham procedure, total and myofibrillar protein breakdown rates were determined by measuring net release of tyrosine and 3-methylhistidine (3-MH) respectively from incubated extensor digitorum longus (EDL) muscles as described in detail previously [3,14,24]. In other muscles, protein synthesis rates were determined by measuring the incorporation of [14C]phenylalanine into protein as described previously [3]. Unless stated otherwise, the incubation medium consisted of calcium-free oxygenated (O2:CO2 = 95:5) Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose, 1 m-unit/ml insulin, 10 mM methylamine and five times normal rat plasma concentrations of the branched-chain amino acids leucine, isoleucine and valine [5]. Muscles were preincubated for 30 min and were then transferred to fresh medium and incubated for 2 h unless stated otherwise. When protein breakdown rates were determined, the medium contained cycloheximide (0.5 mM) to prevent reincorporation of amino acids released by proteolysis. All incubations were performed in a shaking water bath at 37 °C. Groups of muscles were preincubated and incubated in the absence or presence of different concentrations of LNNL (Sigma Co., St. Louis, MO, U.S.A.),
Muscle proteolysis after burn injury

227

lactacystin (Dr E. J. Corey, Cambridge, MA, U.S.A.), or β-lactone (Boston Biochem Inc. and ProScript Inc., Cambridge, MA, U.S.A.). The incubation medium was calcium-free to avoid the influence of calcium-dependent protein breakdown and contained insulin, methylamine and branched-chain amino acids to minimize the influence of lysosomal protein breakdown [26]. Tyrosine and 3-MH were determined by HPLC as described previously [24]. Tissue levels of ATP were determined spectrophotometrically [5].

Northern blot analysis

Ubiquitin mRNA levels were determined by Northern blot analysis as described in detail previously [4,5]. In brief, total RNA was extracted by the guanidinium thiocyanate–phenol–chloroform method [27] using an RNA STAT-60 kit (Tel-Test ‘B’ Inc., Friendswood, TX, U.S.A.). RNA was denatured and separated by electrophoresis on 1% agarose gel containing formaldehyde. The RNA was transferred from the gel to nylon membranes (Micron Separations Inc., Westboro, MA, U.S.A.) by capillary action in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) overnight. RNA was immobilized by ultraviolet crosslinking. The blots were hybridized at 42 °C for 4 h in 50% formaldehyde and 6 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM NaH2PO4, 1 M EDTA), 5 × Denhardt’s solution, 0.5% SDS and 100 µg/ml salmon sperm DNA. A cDNA probe for ubiquitin [5] was labelled by random priming with [32P]dATP (Stratagene, LaJolla, CA, U.S.A.). The blots were hybridized with the 32P-labelled ubiquitin cDNA probe at 42 °C overnight. The blots were then washed twice in 1 × SSC and 0.1% SDS and once in 0.1 × SSC and 0.1% SDS at room temperature and autoradiographed at −70 °C. An 18S rat ribosomal oligonucleotide probe [5] was used to control for equal loading of RNA. Blots were quantified on a phosphoimager using the Image Quant Program (Molecular Dynamics, Inc., Sunnyvale, CA, U.S.A.) and the relative mRNA abundance was expressed as the ratio between ubiquitin mRNA and 18S.

Ubiquitin mRNA stability

Ubiquitin mRNA stability was assessed by determining the decline in mRNA levels in muscles from burned rats incubated in the absence or presence of 100 µM LLnL. One muscle from each rat was immediately frozen in liquid nitrogen. Ubiquitin mRNA in this muscle served as the time zero level. The contralateral muscle was incubated for 4 h at 37 °C in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 5 µg/ml actinomycin D to block RNA synthesis. In control experiments, this concentration of actinomycin D reduced [3H]uridine incorporation into RNA to background levels in incubated muscles, confirming that RNA synthesis was effectively blocked (results not shown). After incubation, the muscle was frozen in liquid nitrogen. Northern blots were generated using 10 µg of total RNA and the ubiquitin probe as described above. Ubiquitin mRNA levels were normalized to the 18S band and compared between the muscle excised and immediately frozen (time zero) and the muscle incubated in the presence of actinomycin D. Blots were quantified by phosphoimager analysis and the amount of ubiquitin mRNA remaining in the incubated muscle was expressed as a percentage of the amount of ubiquitin mRNA present in the muscle at time zero.

Statistical analysis

Results are presented as means ± S.E.M. Student’s t-test or analysis of variance (ANOVA) followed by Tukey’s test was used for statistical analysis.

RESULTS

Total muscle protein breakdown, measured as net release of tyrosine, was increased by approximately 45% after burn injury (Figure 1), similar to previous reports from our laboratory [3,4]. Because muscles were incubated in calcium-free medium containing insulin, methylamine and a high concentration of the branched-chain amino acids, the burn-induced increase in muscle proteolysis mainly reflected calcium-independent, non-lysosomal protein breakdown. In the presence of 100 µM LLnL, protein breakdown was inhibited in muscles from both sham-burned and burned rats. Protein breakdown rates

Figure 1 Total protein breakdown (tyrosine release) in incubated EDL muscles from sham-burned and burned rats

Muscles were incubated in the absence (open bars) or presence (filled bars) of 100 µM LLnL. Results are means ± S.E.M. with n = 7 in each group. *P < 0.05 versus muscles incubated in the absence of LLnL; †P < 0.05 versus all other groups by ANOVA.

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in muscles from sham-burned and burned rats incubated in the presence of LLnL were almost identical, suggesting that the burn-induced increase in protein breakdown was proteasome-dependent. Because LLnL interfered with the HPLC method used to determine 3-MH release (C.-H. Fang and P.-O. Hasselgren, unpublished work), the effect of LLnL on myofibrillar proteolysis could not be determined in the present experiments.

The inhibition by LLnL of total protein breakdown in muscles from sham-burned rats (Figure 1) may reflect inhibition of proteasome-dependent proteolysis that was increased due to the sham procedure itself and the ensuing 24 h pair-feeding. Alternatively, the results may reflect a proteasome-dependent component of basal protein breakdown. To further test that possibility, we next incubated muscles from untreated, freely fed rats in the absence or presence of 100 µM LLnL. In these muscles, protein breakdown was also inhibited by LLnL (from 272 ± 15 to 158 ± 9 nmol tyrosine/g wet weight per 2 h; \( P < 0.05 \), \( n = 7 \) in each group), suggesting that basal muscle protein breakdown is at least in part regulated by the 20S proteasome.

A potentially important clinical aspect of the results shown in Figure 1 is that the burn-induced increase in muscle proteolysis was completely blocked by LLnL. In subsequent experiments we further characterized the effects of LLnL on protein turnover in muscles from burned rats. First we established a dose–response curve for different concentrations of LLnL. A significant inhibition of tyrosine release was noted at a concentration of 50 µM and a maximal effect was seen at 100 µM of LLnL (Figure 2). In subsequent experiments, LLnL was used at a concentration of 100 µM.

When muscles were incubated for various periods of time in the presence of LLnL, a significant inhibition of tyrosine release was noted after 30 min; after 90 min no further tyrosine release occurred, suggesting that protein breakdown was completely blocked (Figure 3). The early effect of LLnL suggests that it is rapidly taken up by the muscle cell and readily reaches the 20S proteasome. Other studies demonstrated that LLnL readily penetrated cell membranes of cultured lymphoblasts as well [16]. It should be noted that in most of our experiments, muscles were incubated for 2 h and proteolytic rates were expressed as net amount of amino acid released per 2 h. This experimental design was based on previous studies in which net release of tyrosine and 3-MH was linear during incubation for up to 2 h [24]. Also, in the present study, tyrosine release was linear during 2 h when muscles were incubated in medium without proteasome blocker (see Figure 3). Because proteolysis was completely blocked during the last 30 min of incubation in the presence of LLnL, the effect of LLnL was underestimated when tyrosine was measured after 2 h incubation. This may in part explain why protein breakdown was apparently not completely blocked (as expected) when muscles were incubated under conditions inhibiting lysosomal, calcium-dependent and proteasome-mediated protein degradation (see Figure 1).

Because protein breakdown in the ubiquitin–proteasome pathway is energy dependent [11,12], it is possible that the effect of LLnL on muscle protein degradation was related to reduced tissue energy levels. To test that possibility, we measured ATP levels in muscles after incubation for 2 h in the absence or presence of LLnL. ATP concentrations were 3.16 ± 0.48 and 3.98 ± 0.34 µmol/g in muscles incubated in the absence and presence of LLnL respectively (\( n = 5 \) paired muscles; \( P \) not significant). Thus, the inhibition of proteolysis by LLnL was not caused by energy depletion.

**Figure 2** Effects of different concentrations of LLnL on protein breakdown in muscles from burned rats

\( n \geq 7 \) in each group. *\( P < 0.05 \) versus no LLnL; **\( P < 0.05 \) versus 0, 10 and 50 µM LLnL.

**Figure 3** Tyrosine release from muscles of burned rats incubated in the absence (○) or presence (●) of 100 µM LLnL

\( n \geq 6 \) for each data point. *\( P < 0.05 \) versus no LLnL at corresponding time point.
Figure 4 Effects of LLnL on ubiquitin mRNA levels
Upper panel: Ubiquitin mRNA levels determined by Northern blotting. Paired muscles from burned rats were incubated without (−) or with (+) 100 µM LLnL. The blots were hybridized with a cDNA probe for ubiquitin, stripped and rehybridized with an 18S oligonucleotide probe to control for loading. Lower panel: Quantification of ubiquitin mRNA in muscles from burned rats incubated in the absence (control) or presence of 100 µM LLnL. n = 7 in each group. *P < 0.05 versus control.

Because previous studies suggest that the proteasome is associated with RNase activity [28], we hypothesized that treatment of muscles with LLnL may increase ubiquitin mRNA stability. After incubation of muscles from burned rats in medium with LLnL, ubiquitin mRNA levels were approximately three times higher than in muscles incubated in medium without LLnL (Figure 4). To test whether the elevated ubiquitin mRNA levels were caused by increased stability of the messenger, as would be expected if the results reflected inhibited RNase activity after treatment with LLnL, we examined the breakdown of ubiquitin mRNA in muscles incubated with or without the proteasome blocker. In this experiment, all muscles were incubated in the presence of actinomycin D. Thus, any decline in ubiquitin mRNA levels reflected breakdown of mRNA. The amount of ubiquitin mRNA remaining in the muscles after incubation with LLnL was significantly higher than after incubation without LLnL, suggesting that inhibition of the 20S proteasome increased the stability of ubiquitin mRNA (Figure 5). The different sizes of ubiquitin mRNA noted in Figures 4 and 5 are consistent with previous reports of a ubiquitin multigene family [29,30]. The predominant ubiquitin mRNA sizes observed in the present study were 2.8 and 3.2 kb which correspond to the two sizes of ubiquitin C mRNA reported in rats [30]. LLnL is not a completely specific proteasome blocker but may inhibit calpains and lysosomal cysteine proteases as well [31]. Because muscles were incubated in calcium-free medium containing insulin, methylamine and branched-chain amino acids, the results from the experiments described above in which LLnL blocked protein breakdown most probably reflected inhibited proteasome-dependent proteolysis. To further test the specificity with respect to the 20S proteasome mechanism, we next examined the effect of lactacystin on muscle protein breakdown. Lactacystin, a more selective proteasome blocker than LLnL [17], inhibited total protein breakdown in muscles from sham-burned and burned rats (Figure 6A). The inhibitory effect on protein breakdown by lactacystin was less pronounced than the effect of LLnL (compare with Figure 1), possibly reflecting the higher specificity of lactacystin as a proteasome blocker. Because lactacystin did not interfere with the HPLC
method used to measure 3-MH, the effect of the drug on myofibrillar protein breakdown could be determined. Lactacystin blocked the burn-induced increase in myofibrillar protein breakdown (Figure 6B).

A recent study provided evidence that lactacystin in solution is rapidly hydrolysed to \(\beta\)-lactone [18]. \(\beta\)-Lactone is the active substance blocking 20S proteasome activity and is rapidly taken up by the cell. Treatment of incubated muscles with 100 \(\mu\)M \(\beta\)-lactone inhibited total and myofibrillar protein breakdown in muscles from sham-burned and burned rats, similar to the effects of lactacystin (Figure 7).

Previous reports suggest that proteasomes not only regulate intracellular protein degradation but protein synthesis as well. For example, proteasomes interfered with biosynthetic activity in cultured HeLa cells [32] and prevented the formation of 80S initiation complexes in a reticulocyte cell-free system [33]. To examine whether proteasomes may be involved in the regulation of protein synthesis in muscle tissue as well, we next measured protein synthesis rates in muscles incubated in the absence or presence of LLnL. The proteasome blocker significantly reduced protein synthesis in muscles from both sham-burned and burned rats (Figure 8). The lower protein synthesis rate in muscles from burned rats compared with muscles from sham-burned rats is similar to previous reports of burn-induced inhibition of muscle protein synthesis [3].

The inhibition of protein synthesis by LLnL noted...
here contrasts with previous studies in which LLnL did not affect protein synthesis in incubated rat diaphragm [21] and in which MG132, another proteasome inhibitor, did not reduce protein synthesis in incubated rat epitrochlearis muscles [19]. To test the possibility that the effect of LLnL on protein synthesis was specific for EDL muscles, we next measured protein synthesis in diaphragm and soleus muscles incubated in the absence or presence of 100 μM LLnL. Protein synthesis was inhibited by LLnL from 189±6 to 121±2 nmol phenylalanine/μg×h in diaphragm (P<0.001; n=7 paired observations), and from 296±9 to 176±5 nmol phenylalanine/μg×h in soleus muscles (P<0.001; n=7 paired observations). Thus, the effect of LLnL on protein synthesis noted here (see Figure 8) did not seem to reflect a specific effect in EDL muscles.

Another potential explanation as to why LLnL inhibited protein synthesis may be that muscles were incubated in medium containing high concentrations of insulin and branched-chain amino acids which by themselves may stimulate protein synthesis independently of the proteasome. To test that possibility, we next incubated EDL muscles from sham-burned rats in unsupplemented medium (without the addition of branched-chain amino acids and insulin) in the absence or presence of 100 μM LLnL. Under these experimental conditions, LLnL still inhibited protein synthesis (from 181±3 to 151±4 nmol phenylalanine/μg×h; P<0.001, n=7 paired observations).

Finally, it is possible that the effect of LLnL on protein synthesis reflected the fact that LLnL is not a completely specific proteasome blocker [31]. We therefore examined the effect of β-lactone on protein synthesis in incubated EDL muscles from sham-burned and burned rats. β-Lactone, at a concentration of 100 μM, did not influence protein synthesis in muscles from sham-burned or burned rats; protein synthesis rates were 198±7 and 190±7 nmol phenylalanine/μg×h in muscles from sham-burned rats incubated in the absence and presence of β-lactone respectively (P not significant, n=7 paired observations). The corresponding protein synthesis rates in muscles from burned rats were 177±4 and 180±5 nmol phenylalanine/μg×h (P not significant, n=7 paired observations). Thus, the effect of LLnL on protein synthesis may not be proteasome-related.

**DISCUSSION**

In the present study, treatment of incubated muscles with one of the proteasome blockers LLnL, lactacystin or β-lactone inhibited the burn-induced increase in protein breakdown, suggesting that burn injury stimulates proteasome-dependent muscle proteolysis. The results support a recent study from our laboratory of upregulated energy–ubiquitin-dependent muscle protein breakdown after burn injury [4]. The influence of proteasome blockers on muscle protein breakdown after burn injury has not been previously reported. In other studies, however, proteasome blockers inhibited muscle proteolysis associated with chronic renal failure [19], diabetes [20] and sepsis [21,22], supporting the concept that the ubiquitin–proteasome pathway plays an important role in the regulation of muscle protein breakdown in different catabolic conditions.

LLnL is not a completely specific proteasome inhibitor but may block calpains and lysosomal cysteine proteases as well [31]. We recently found evidence that burn injury is associated with increased energy–ubiquitin-dependent proteolysis as well as increased lysosomal and calcium-dependent total protein breakdown [4]. In the present study, muscles were incubated in calcium-free medium containing high concentrations of the branched-chain amino acids, insulin, and methylamine, so the influence of calcium-dependent and lysosomal protein breakdown was minimized. Therefore, the effects of LLnL noted here mainly reflected inhibited non-lysosomal, calcium-independent proteolysis. The inhibition of protein breakdown by lactacystin and β-lactone, the most specific proteasome blockers presently known [17,18], supports the interpretation that a substantial portion of the protein breakdown in incubated muscles from burned rats was proteasome-mediated.

The 20S proteasome contains multiple proteolytic activities [13,14]. By using specific fluorogenic peptide substrates, a recent study provided evidence that lactacystin inhibits the trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide hydrolysing activities of the proteasome and that some of the effect is caused by covalent binding of lactacystin to and modification of the amino-terminal threonine of the proteasome subunit MBI [17]. In contrast to LLnL, lactacystin did not block calpain and cathepsin B or any other protease tested.

In previous studies, we and others have found evidence that burn injury mainly stimulates the breakdown of myofibrillar proteins in skeletal muscle and that most of the increase in total protein breakdown reflects increased myofibrillar breakdown [2–4]. Because LLnL interfered with the HPLC method used to measure 3MH, the influence of LLnL on myofibrillar protein breakdown could not be determined. The inhibition of myofibrillar protein breakdown in muscles treated with lactacystin or β-lactone, however, supports the concept that burn-induced myofibrillar protein breakdown in skeletal muscle is proteasome-dependent. This is in line with previous reports that myofibrillar proteins are degraded by an energy-dependent, non-lysosomal mechanism, most probably the ubiquitin–proteasome pathway [34].

Among the different proteolytic activities of the 20S proteasome, the branched-chain-amino-acid-prefering activity is particularly important for the regulation of myofibrillar protein breakdown [35]. It is therefore
possible that the effects of the proteasome blockers tested here at least in part reflected inhibition of branched-chain-amino-acid-prefering peptidase activity. Further studies using specific substrates are needed to determine which proteolytic activity of the 20S proteasome is preferentially inhibited by LLnL and lactacystin/β-lactone in skeletal muscle.

Although previous studies suggest that proteasomes may influence protein synthesis (in addition to regulating protein breakdown) [32,33], the present experiments in which protein synthesis was measured in the presence of β-lactone suggest that muscle protein synthesis is not regulated by the proteasome. The mechanism of the inhibited protein synthesis noted in the presence of LLnL is not known from the present study but a toxic effect of LLnL is less likely considering the maintained ATP levels in the muscles incubated in the presence of LLnL. The effect of LLnL on protein synthesis does not seem to be muscle specific since an inhibitory effect on protein synthesis was noted in EDL (a white, fast-twitch muscle), soleus (a red, slow-twitch muscle) and diaphragm (a mixed-fibre type muscle). Although the inhibited protein synthesis in muscles incubated in the presence of LLnL noted here is in line with a recent study from our laboratory in which the regulation of muscle protein turnover during sepsis was examined [22], it should be noted that conflicting results have been reported. Thus, Tawa et al. [21] found that protein synthesis in incubated rat diaphragm was not affected by LLnL. The reason for this apparent discrepancy is not known at present.

Although the present result of increased steady-state levels of ubiquitin mRNA in muscles incubated in the presence of LLnL may be consistent with inhibited proteasome-associated RNase activity [28], the results need to be interpreted with caution for several reasons. First, because mRNA levels were determined at only two time-points, no definitive statement can be made regarding mRNA half-life. Second, it is possible that the results reflected effects of LLnL unrelated to the proteasome. Third, an alternative explanation for the result may be that inhibition of the proteasome blocks the degradation of a protein that stabilizes ubiquitin mRNA. Actual measurements of RNAase activity will be needed in future studies to determine the mechanism of increased ubiquitin mRNA levels caused by treatment with LLnL.

The results in the present study do not necessarily mean that proteasome blockers will be useful in the treatment of patients with burn injury or other catabolic conditions. In fact, the multiple functions of the proteasome, in addition to regulating general protein breakdown, may make such an approach less likely to be successful. Nevertheless, the present results are important because they provide further evidence that burn-induced muscle proteolysis is regulated by the ubiquitin–proteasome pathway. It will be an important area for future research to determine whether this proteolytic pathway can be inhibited in catabolic muscle without a non-specific blockade of the proteasome.

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