Pathways of Protein Degradation in L6 Myotubes (44092)

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Abstract. **To assess the importance of different proteolytic pathways in anabolic and catabolic states, L6 myotubes were labeled with ^{3}H-phenylalanine for 24 hr, then exposed for another 24 hr to control medium, alone or with one of the following: insulin (1 μ/ml), insulin-like growth factor–I (IGF-I, 100 ng/ml), clenbuterol (100 μ*M*), or dexamethasone (1 μ*M*). Release of acid-soluble radioactivity and 3-methylhistidine were used to determine rates of total and myofibrillar protein degradation, respectively. Within each hormone treatment, cells were also exposed to none, one, or both of the following protease inhibitors: chloroquine (25 μ*M*); E-64d (100 μ*M*). Chloroquine inhibits lysosomal proteases, and E-64d is a cell-penetrating inhibitor of cysteine proteases (including the calpains). Total protein degradation was reduced by insulin (−33%) and IGF-I (−40%), increased by clenbuterol (+20%), and was unaffected by dexamethasone. Myofibrillar protein degradation was reduced by insulin (−35%), IGF-I (−55%), and clenbuterol (−22%), and was unaffected by dexamethasone. Therefore, total (mainly sarcoplasmic) and myofibrillar protein degradation were regulated independently of one another. The influence of the inhibitors was not consistent across hormone treatments. Chloroquine inhibited total but not myofibrillar proteolysis, indicating that lysosomes may be rate limiting in the former but not the latter. In contrast, E-64d had no effect on total protein degradation, but increased myofibrillar proteolysis. Interactions between anabolic and catabolic hormones and these two protease inhibitors indicate that both lysosomal and nonlysosomal proteolytic pathways are involved in regulation of total protein degradation in myotubes. In contrast, lysosomes are probably not involved in regulating the rate of myofibrillar protein degradation, the initial step likely being due to calpain activity.** [P.S.E.B.M. 1997, Vol 214]

Current understanding of the pathways of protein synthesis and its regulation is far more advanced than that of intracellular protein breakdown, in large part because of technical problems involved in studying degradative processes. Some of these problems, as well as the effects of various hormones on proteolysis, have been discussed (1). In many cell types, lysosomal enzymes are thought to catalyze the hydrolysis of polypeptides to constituent amino acids, although the manner in which specific proteins are targeted for proteolysis has not been fully elucidated (2). In skeletal muscle, lysosomes probably play an important role in the ultimate degradation of sarcoplasmic and myofibrillar proteins. Clearly, the myofibrillar apparatus cannot be engulfed by lysosomes; individual proteins must first be released and migrate to the extra-myofibrillar space before undergoing the final stages of proteolysis either by lysosomal (i.e., cathepsins) or nonlysosomal (i.e., proteasome) pathways. To date, the only proteases known to exist within the myofibril and capable of carrying out the initial step in myofibrillar proteolysis are calpains (2). Calpains are cysteine proteases that exist in the cytosol of muscle and many other cell types, and require calcium for their activity.

The objectives of the present study were to assess the relative importance of lysosomal and nonlysosomal proteolytic systems in the degradation of the myofibrillar and total protein pools in muscle cells. Rates of protein turnover were perturbed using several anabolic (insulin, insulin-like growth factor–I (IGF-I), or clenbuterol) and catabolic (dexamethasone) agents. In addition, specific pathways of protein degradation were

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Table I. Effects of Hormones and Protease Inhibitors on Total Protein Content in L6 Myotubes

Inhibitor	Control	Insulin	IGF-I	Clenbuterol	Dexamethasone
Control	$76.7^{a}{}_{w}$	102_{x}	$114^{a}{}_{y}$	65.9_{z}	$93.3^{a}{}_{x}$
Chloroquine (CQ)	66.3^{b}	112	88.7^{b}	72.1	63.4^{b}
E-64d	$73.5^{a,b}$	112	119^{a}	75.9	66.3^{b}
CQ + E-64d	63.7^{b}	107	121^{a}	72.1	70.3^{b}

Note. Values are least square means for L6 myotube protein content (μg/well); pooled SEM = 7.29 (n = 4/group). L6 myoblasts were grown in DMEM on 24-well plastic plates and induced to fuse by reducing fetal calf serum from 5% to 1%. Myotubes were exposed to the various hormones over a 24-hr period, then harvested using 10% trichloroacetic acid.
^{a,b,c} Means in the same column lacking a common superscript differ ($P < 0.05$).
^{w,x,y,z} Means in the control row lacking a common subscript differ ($P < 0.05$).

blocked using inhibitors of lysosomal (chloroquine) and cysteine (including calpains; E-64d) proteases, alone or in combination.

Materials and Methods

Cell Cultures. L6 muscle cells (derived from neonatal rat muscle) (3) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 2% penicillin/streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were grown in 75-cm^2 tissue culture flasks (Corning Inc., Corning, NY), then plated (before confluence) onto multiwell plates (24 wells/plate). Once cells achieved confluence, the concentration of FBS was reduced to 1% in order to induce differentiation and formation of myotubes. Experiments were conducted using only confluent myotubes.

Treatments. Anabolic treatments consisted of insulin (1 μg/ml), insulin-like growth factor–I (IGF-I, 100 ng/ml), and clenbuterol (100 μM), whereas dexamethasone (1 μM) was intended to be catabolic. These were compared with control wells incubated with no added hormones. Each of these treatments was applied in the presence of chase medium containing no inhibitors or one or both of the following: chloroquine (CQ, 25 μM) or E-64d (E, 100 μM).

Measurement of Protein Degradation. After exposure to DMEM containing 1% FBS for 24 hr, the medium was replaced with labeling medium (DMEM + 1% FBS + 1 mM phenylalanine [Phe] + 1 μCi/ml ^{3}H-Phe). After a 24-hr labeling period, the medium was removed, and monolayers were washed four times with unlabeled medium (DMEM + 1 mM Phe + 1% FBS), then replaced with 1 ml/well of chase medium (DMEM + 1% FBS + 20 mM Phe) containing one or more of the hormones and inhibitors (see Table I). A high concentration of Phe was used in the chase medium to minimize re-utilization of ^{3}H-Phe released following degradation of cellular proteins. Treatment combinations were applied for a total of 24 hr, with a medium change and cell washing (twice) after the first 3 hr, followed by 21 hr with no further medium changes. At the end of the 24-hr chase period, medium was harvested for measurement of ^{3}H-Phe and 3-methylhistidine (3MH) release, and 0.75 ml trichloroacetic acid (TCA, 10%) was added to lyse cells and precipitate cell proteins. After aspirating and discarding the TCA solution, the remaining precipitate was dissolved overnight at 37°C using 0.5 ml NaOH solution (0.5 N NaOH plus 0.1% Triton X-100). The solution was transferred to a microfuge tube and the plate washed with 0.25 ml NaOH, which was added to the tube. Finally, the plate was washed with 0.75 ml of ice-cold TCA (30%), which was then added to the microfuge tube. This precipitate (i.e., cell proteins) was used to determine total cell protein content and ^{3}H-Phe incorporation, as described below.

Analytical. Tubes containing TCA-precipitable cell protein were centrifuged at 15,000g for 10 min at 4°C; the pellets were dissolved in 1 ml of NaOH solution and placed in a shaking water bath (37°C) for 60 min. Glacial acetic acid (100 μl, 1.74 N) was used to neutralize NaOH in the protein samples only. Aliquots (250 μl) of dissolved protein solutions and medium (for ^{3}H-Phe incorporation and release, respectively) were combined with 10 ml of scintillation cocktail (Aquassure, New England Nuclear, Boston, MA) and counted in a liquid scintillation counter (LKB Wallace 1209 Rackbeta). The appropriate quench controls were used to calculate radioactivity (dpm/ml). Protein contents of the dissolved cell protein samples were determined using a commercial kit (Bio-Rad Laboratories, Richmond, CA) and expressed as μg protein/ml. The concentration of 3MH in the medium was determined by HPLC using the Pico-Tag system (Waters, Millipore Corp., Concord, MA), after deproteinization with acetonitrile and addition of norleucine as an internal standard (4).

Statistical Analysis. Rates of protein degradation were expressed as dpm/μg protein/hr (total) or as picomoles of 3MH/μg protein/hr (myofibrillar). Data were analyzed by the GLM procedure of SAS (SAS Institute, Cary, NC). The model fitted was

$$y_{ijk} = \mu + \text{Hormone}_i + \text{Inhibitor}_j + \text{Hormone} \times \text{Inhibitor}_{ij} + e_{ijk},$$

where y_{ijk} is the response of the k-th cell in the j-th inhibitor group given the i-th hormone. Differences be-

Table II. Effects of Hormones and Protease Inhibitors on Total Protein Degradation in L6 Myotubes

Inhibitor	Control	Insulin	IGF-I	Clenbuterol	Dexamethasone
Control	6.06^{a}_{x}	4.06^{a}_{y}	3.61^{b}_{y}	7.25^{a}_{z}	5.47^{b}_{x}
Chloroquine (CQ)	5.32^{b}	2.74^{b}	4.63^{a}	5.29^{b}	5.33^{b}
E-64d	6.65^{a}	3.88^{a}	3.06^{b}	5.94^{b}	7.61^{a}
CQ + E-64d	4.53^{c}	2.80^{b}	2.33^{c}	3.61^{c}	4.97^{b}

Note. Values are least-square means for ^{3}H-phenylalanine release from L6 myotubes (dpm/μg protein/hr); pooled SEM = 0.251 (n = 4/group). L6 myotubes were pre-labeled for 24 hr with ^{3}H-phenylalanine, then exposed to the various hormones for another 24 hr. Total protein degradation was estimated from release of radioactivity into the medium during the second 24-hr period.
^{a,b,c} Means in the same column lacking a common superscript differ ($P < 0.05$).
^{x,y,z} Means in the control row lacking a common subscript differ ($P < 0.05$).

tween means were determined by Tukey's procedure (5). For simplicity, due to the large number of interactions among hormone and inhibitor treatments the only hormone effects examined were those obtained in control wells with no added inhibitors.

Results and Discussion

This experiment was designed to assess the responses to anabolic and catabolic factors (according to their effect on total cell protein, Table I) in L6 myotubes. In particular, the relative importance of lysosomal (e.g., cathepsins) and nonlysosomal (e.g., calpains) proteolytic systems in the degradation of the total and myofibrillar protein pools were evaluated. Specific pathways of protein degradation were blocked using inhibitors of lysosomal (i.e., cathepsins) and cysteine (including calpains) proteases (chloroquine and E-64d, respectively), alone or in combination. The degradation data shown in Tables II and III were interpreted as follows: (i) when an inhibitor reduced or abolished the effect of a hormone that increased proteolysis, this was considered to be evidence for a role of the inhibited pathway in the hormone-induced increase in degradation. (ii) Similarly, when an inhibitor failed to reduce proteolysis beyond that produced by a hormone, this was also considered to indicate a role of that pathway in the hormone-induced decrease in degradation. (iii) On the other hand, if an inhibitor reduced proteolysis below that produced by a hormone, this was considered to argue against a primary role of the inhibited pathway in the action of that hormone. Other combinations, of course, were possible and in fact were observed. (iv) For example, when a hormone reduces proteolysis in the absence of an inhibitor but not in its presence, interpretation is difficult. In such cases, one must bear in mind that these inhibitors may produce a variety of changes within cells, perhaps including alerations in receptors or postreceptor signaling events.

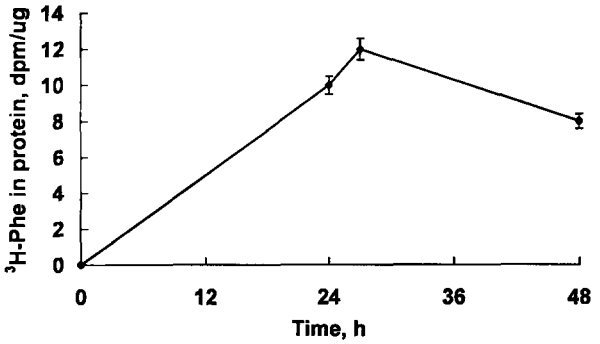

Figure 1. Time course of incorporation of ^{3}H-phenylalanine into L6 myotube cell proteins, and its release after label removal. Myotubes were pulsed with ^{3}H-phenylalanine (1 μCi/ml, 1 m*M*) for 24 hr, then chased using 20 m*M* phenylalanine in Dulbecco's modified Eagle's medium.

The methods used here to estimate rates of protein degradation depend upon some basic assumptions. Total protein degradation was assessed by the release of TCA-soluble radioactivity from cultures pre-labeled with ^{3}H-Phe. The time course of cell labeling and release is shown in Figure 1. This approach depends upon the muscle cell's inability to metabolize Phe. Due to the far greater rate of turnover of sarcoplasmic proteins relative

Table III. Effects of Hormones and Protease Inhibitors on Myofibrillar Protein Degradation in L6 Myotubes

Inhibitor	Control	Insulin	IGF-I	Clenbuterol	Dexamethasone
Control	$8.48^{a,b}_{x}$	5.48^{a}_{y}	3.85^{a}_{z}	6.62^{a}_{y}	$6.97^{a}_{x,y}$
Chloroquine (CQ)	8.09^{b}	7.68^{c}	$4.02^{a,c}$	$7.35^{a,c}$	13.8^{c}
E-64d	10.4^{c}	3.54^{b}	$5.04^{a,c}$	5.93^{a}	6.54^{a}
CQ + E-64d	$10.0^{a,c}$	5.76^{a}	5.79^{c}	8.84^{c}	5.89^{a}

Note. Values are least square means for 3-methylhistidine release from L6 myotubes (pmol 3MH/μg protein/hr); pooled SEM = 0.513 (n = 4/group). L6 myotubes were exposed to the various hormones for 24 hr, and myofibrillar protein degradation was estimated from release of 3-methylhistidine into the medium during the 24-hr period.
^{a,b,c} Means in the same column lacking a common superscript differ ($P < 0.05$).
^{x,y,z} Means in the control row lacking a common subscript differ ($P < 0.05$).

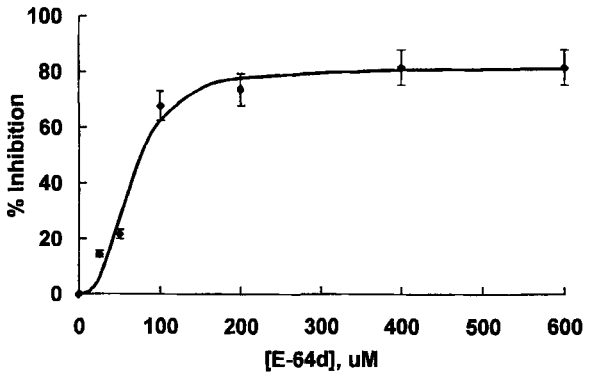

Figure 2. Dose-response curve for inhibition of m-calpain by E-64d *in vitro* (K_i = 63.9 μM). m-Calpain was partially purified from ovine muscle using a DEAE-Sephacel column and a NaCl gradient, and assayed against casein in the presence of varying amounts of E-64d.

to myofibrillar proteins, total release of label measures predominantly sarcoplasmic protein degradation. Myofibrillar protein breakdown was evaluated in terms of the release of 3-methylhistidine into the medium. The excretion of 3MH has been used as an indicator of myofibrillar protein degradation *in vivo,* because (i) skeletal muscle is the major source of 3MH, and (ii) 3MH released during protein breakdown is neither reutilized for protein synthesis nor metabolized (6). The use of 3MH release as an index of myofibrillar protein degradation *in vitro* is less common but is probably more defensible, since there is no interference from smooth muscle (e.g., from the digestive tract) proteolysis (1).

The inhibitors of proteolysis used in this study have been shown to be effective in reducing activities of lysosomal proteases and calpains (chloroquine and E-64d, respectively). Chloroquine is rapidly and selectively concentrated in lysosomes, where it increases the lysosomal pH, and specifically inhibits cathepsins. In control (i.e., no added hormone) cells, chloroquine reduced total and myofibrillar protein degradation by 12% and 5%, respectively. In previous work with L8 myotubes, chloroquine produced similar reductions in total (12%–14%) (7, 8) and myofibrillar (no change) (7) protein degradation. On the other hand, E-64d is a cell-permeable oxirane inhibitor of cysteine proteases, notably the calpains (9). Effects of E-64d on other nonlysosomal proteases (i.e., the proteasome) have not been reported. In this study, E-64d increased total (+10%, $P > 0.05$) and myofibrillar (+23%, $P < 0.05$) protein degradation despite the well-documented inhibition of calpains (Fig. 2), as well as cathepsins B and L (9). These results conflict with those of Tsujinaka *et al.* (10), who found that the half-life of long-lived proteins in C2C12 myotubes was increased by E-64. It must be noted that, due to the limited permeability of the plasma membrane to E-64 (9), one would not expect dramatic effects of the parent compound on intracellular protein degradation. Therefore, differences between that study and this one remain unexplained.

Insulin increased total protein content and reduced total protein degradation (−33%) in L6 myotubes (Tables I and II). This is in agreement with many previous studies using L6 (11–13), L8 (14), and primary cell cultures (15, 16), as well as in whole diaphragm muscle (17). In the case of total protein degradation, the effect of insulin was even greater in the presence of chloroquine, whereas E-64d did not alter the decrease in degradation due to insulin. These results suggest that E-64d, but not chloroquine, affected the activity of the proteolytic pathways that were decreased by insulin. In addition, myofibrillar protein degradation was also decreased by insulin (−35%; Table III). In contrast to the effects of insulin on total protein degradation, the reduction in myofibrillar protein degradation was eliminated by chloroquine, indicating that impairment of lysosomal function somehow interfered with the inhibitory effect of insulin. On the other hand, the greater inhibition in the presence of E-64d suggests that calpains were not involved in the insulin-mediated reduction of myofibrillar protein degradation.

IGF-I was even more anabolic in L6 myotubes than insulin (Table I), a response that was at least partly due to large decreases in the rates of total (−40%), Table II) and myofibrillar (−55%, Table III) protein degradation. Large reductions in total protein degradation by IGF-I have been demonstrated in L6 (11, 12), L8 (7, 8, 14), and primary (12, 15, 16) myotube cultures. In contrast with the effects of insulin, the reduction in total protein degradation by IGF-I was significantly diminished by chloroquine. On the other hand, E-64d (alone or in combination with chloroquine) decreased total protein degradation even further. These results indicate that the reduction in total protein degradation by IGF-I somehow involved normal lysosomal function, and that calpains were probably not involved. The recent detection of proteasome degradation within lysosomes (18) raises the possibility that IGF-I decreases total protein degradation by increasing lysosomal breakdown of proteasomes, and that chloroquine interferes with this process. This would be analogous to the IGF-I–mediated increase in breakdown of cathepsins in myotubes (10). In contrast to its effects on total protein degradation, the large reduction in myofibrillar protein degradation by IGF-I was unaffected by both chloroquine and E-64d, alone or in combination. The reduction in myofibrillar protein degradation by IGF-I observed in this study has not been previously reported. To our knowledge, the only study to date showed no effect of IGF-I on myofibrillar protein degradation in L8 myotubes (7). That study also showed no effect on CQ on myofibrillar protein degradation, so that the lack of agreement between those previous data and the present results extend beyond the effect of IGF-I and may be due to differences in cells, methodology, or some other factor. The present results suggest that both cytoplasmic and lysosomal

pathways may be involved in the suppression of myofibrillar protein degradation by IGF-I.

Clenbuterol failed to increase protein content of L6 myotubes in this experiment. Moreover, total protein degradation was increased (+20%) in response to clenbuterol, although myofibrillar protein degradation was decreased (−22%; Tables II and III). Previous studies have shown varying effects of β-agonists on total protein degradation, ranging from no effect in L6 (19) or L8 (20) myotubes, to decreased proteolysis in myotubes from primary cultures (19, 21). In this study, both chloroquine and E-64d blocked the effect of clenbuterol on total protein degradation, being most effective in combination. Therefore, the effect of clenbuterol on total protein degradation in myotubes appears to be mediated through both lysosomal and nonlysosomal (i.e., calpains) processes. The effects of clenbuterol on myofibrillar protein degradation were more straightforward: the reduction by clenbuterol was abolished by chloroquine, indicating that (as seen for insulin) impairment of lysosomal function interfered with the effects of clenbuterol. In conrast, the clenbuterol-mediated reduction in myofibrillar protein degradation was unaffected by E-64d, indicating that calpains were probably involved in this response. These results are consistent with a wide body of evidence for reduced calpain (or increased calpastatin) activities in muscles of animals treated with β-adrenergic agonists (22).

Dexamethasone failed to produce the expected catabolic effects, with increased protein content and unchanged total and myofibrillar protein degradation (Table II and III). These results conflict with those of Yeh *et al.* (23), who found that 3-methylhistidine excretion was reduced by up to 48% in dexamethasone-treated rabbits. Those authors also found no change in calpain and calpastatin activities, although since they used frozen muscles samples for those analyses the results must be viewed with extreme caution. On the other hand, reports of no change in muscle protein degradation due to corticosterone or dexamethasone also exist (1). Several other studies have demonstrated increased 3MH excretion when glucocorticoids were administered *in vivo* to rats (24, 25) or broilers (26). Similarly, total protein degradation was increased by dexamethasone in L8 (8) and primary chicken (16) myotubes. In one of those studies (8), the dexamethasone-stimulated proteolysis was accompanied by increased expression of cathepsin D and m-calpain. In the present work, dexamethasone had no effect on total and myofibrillar protein degradation in the absence of any inhibitors but increased total protein degradation when E-64d was present. This suggests that calpains may somehow be involved in the response of total protein degradation to dexamethasone. In contrast, dexamethasone increased myofibrillar protein degradation in the presence of chloroquine. Although this observation raises serious questions about the interactions between chloroquine and dexamethasone (and other hormones), it also demonstrates that lysosomes are not required for stimulation of myofibrillar protein degradation. Clearly, much remains to be known about the effects of glucocorticoids on muscle protein metabolism and the mechanisms of myofibril breakdown.

In conclusion, total (mainly sarcoplasmic) and myofibrillar protein degradation were regulated independently of one another. Moreover, the effects of anabolic and catabolic agents on the processes not only differed but were also mediated *via* different pathways. Chloroquine inhibited total but not myofibrillar proteolysis, indicating that lysosomes may be rate limiting in the former but not the latter. In contrast, E-64d had no effect on total protein degradation, but increased myofibrillar proteolysis. Since this compound is known to inhibit activities of calpains (as well as cathepsins B and L), the stimulation of myofibrillar protein degradation by E-64d may be a result of interference with a receptor or postreceptor signaling event. The observed interactions between anabolic and catabolic hormones and these two protease inhibitors are consistent with the general concept that both lysosomal and nonlysosomal proteolytic pathways are involved in regulation of total protein degradation in myotubes. In contrast, whereas lysosomes probably are not involved, calpains may play a role in regulating the rate of myofibrillar protein degradation.

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