Leupeptin, a Protease Inhibitor, Decreases Protein Degradation in Normal and Diseased Muscles

Abstract. *The protease inhibitor leupeptin decreases protein degradation in rat skeletal and cardiac muscle incubated in vitro, while protein synthesis remains unaltered. Leupeptin also lowers protein breakdown in denervated rat muscles and affected muscles from mice with hereditary muscular dystrophy. Leupeptin may thus be useful in retarding tissue atrophy. Since homogenates of leupeptin-treated muscles had decreased cathepsin B activity, this lysosomal protease may play a role in protein turnover in normal and diseased muscles.*

In muscle, as in other tissues, protein degradation as well as protein synthesis determines the concentration of cell protein (*1*). Therefore changes in the overall rate of protein catabolism can contribute to muscle growth or atrophy (*1, 2*). Although many proteases exist in muscle which might degrade muscle protein, it is not known which enzymes actually do so in various physiological and pathological states. In this study we have attempted to identify enzymes that participate in protein catabolism in intact muscles and to find agents that might retard this process. Both lysosomal (*3*) and nonlysosomal (*4, 5*) proteases can hydrolyze muscle protein in cell-free preparations, but such a finding does not prove that these enzymes serve this function in intact tissues. For instance, in the undisrupted cell, a particular protease may not have access to or may lack conditions necessary for degrading substrates it can hydrolyze in vitro. Also, proteases may serve a variety of cellular functions aside from turnover of cell protein, including limited proteolysis in metabolic regulation and protein maturation, and degradation of extracellular proteins and peptide hormones (*6*).

Studies with perfused liver (*7*), isolated hepatocytes (*8*) and cultured hepatoma cells (*9*), fibroblasts (*10*), and fetal mouse hearts (*11*) have shown decreased overall protein breakdown following treatment with various inhibitors of lysosomal proteases, although these studies did not establish protease inhibition as the mechanism of diminished proteolysis. In addition, these tissues are rich in lysosomes compared with normal adult muscle cells, which contain few structures clearly identifiable as lysosomes. It is even possible that in muscle, lysosomal enzymes occur largely in nonmyocyte cells (such as fibroblasts) or participate in protein degradation only in pathological conditions. Thus the possible role of lysosomes in protein turnover in muscle, even more than in other tissues, is a matter of considerable debate.

In an attempt to determine the physiological roles of muscle proteases, we studied the effect of a class of protease inhibitors produced by actinomycetes on protein degradation in intact muscles incubated in vitro. These inhibitors are aldehyde derivatives of oligopeptides isolated and characterized by Umezawa and co-workers (*12*). Unlike other protease inhibitors (such as diisopropyl fluorophosphate and the chloromethyl ketone derivatives), the compounds studied do not contain highly reactive groups that may interact with cell constituents other than proteases. Since these agents inhibit proteases maximally at micromolar concentrations, they should have fewer nonspecific effects than other inhibitors of lysosomal function, such as chloroquine (*10, 11*).

We determined the rate of protein degradation by measuring the release of free tyrosine from cell protein in paired contralateral hindlimb muscles or portions of rat atria incubated with or without the inhibitor under defined conditions (*13, 14*). Since tyrosine is neither degraded nor synthesized by muscle, its production reflects net protein breakdown (*13, 14*). In most experiments where protein degradation was measured as an isolated process, cycloheximide was used to block reutilization of tyrosine in protein synthesis.

During incubation in vitro the rat soleus, extensor digitorum longus (EDL), and atrial strip show linear rates of protein synthesis and degradation and are in nearly neutral nitrogen balance for the duration of these experiments (*13, 14*). As previously reported, rates of protein degradation and synthesis in these muscles vary from day to day, presumably as a consequence of fluctuations in such factors as food intake, endocrine status, and season. Consequently, all conclusions in this study were based on comparisons of paired contralateral muscles, or portions of the same atrium or diaphragm, from the same animal. In each experiment, five to seven inbred animals matched for age, weight, housing, and diet were studied.

Table 1. Effect of leupeptin on protein degradation and synthesis in rat muscle. (A) Paired leg muscles or atrial strips were incubated at 37°C in vitro in Krebs-Ringer bicarbonate solution equilibrated with 95 percent O_2 and 5 percent CO_2, and supplemented with glucose (10 m*M*) and insulin (0.1 U/ml); +AA indicates that amino acids were included at five times the normal concentrations in rat plasma (*13*). In the presence of cycloheximide (0.5 m*M*), the net production of tyrosine (that released into the medium plus any lost from the tissue pools) reflects protein degradation. Skeletal muscles were dissected and incubated as described previously (*13*). Atrial strips were obtained by bisecting left atria of freshly excised rat hearts (*14*). A 90-minute incubation in identical media (with or without leupeptin) preceded the measurement of protein degradation. In the experiments on atrial strips, the tissue pools of tyrosine were too small to measure accurately, and only tyrosine release into the medium is reported. The concentration of leupeptin was 25 μM in the skeletal muscle experiments and 50 μM in the experiment on cardiac muscle. Here and in Tables 2 and 3, results are given as the mean ± standard error of the mean with $N \geq 5$. The P values were determined by the Student t-test for paired observations. (B) Tissues were incubated as described in (A), except that cycloheximide was omitted. The rate of protein synthesis was determined by measuring incorporation of [^{14}C]tyrosine into acid-insoluble material and correcting for the specific activity of the intracellular pool of tyrosine (*12*). The leupeptin concentration was 25 μM. The results of two experiments performed on different days are shown. (The differences in the control values illustrate the day-to-day variations mentioned in the text.)

Tissue	Medium enrichment	Tyrosine [nmole mg^{-1} (2 hours)$^{-1}$]		Change (%)
		Control	Leupeptin	
		A. Protein degradation		
Soleus		0.420 ± 0.038	0.264 ± 0.028*	36
	+AA	0.389 ± 0.029	0.242 ± 0.018*	36
EDL		0.320 ± 0.009	0.148 ± 0.015*	54
	+AA	0.304 ± 0.015	0.121 ± 0.007*	60
Atrium		0.330 ± 0.015	0.271 ± 0.010*	18
		B. Protein synthesis		
Soleus	+AA	0.477 ± 0.038	0.467 ± 0.032†	
	+AA	0.552 ± 0.027	0.565 ± 0.037†	
EDL	+AA	0.398 ± 0.020	0.333 ± 0.023†	
	+AA	0.517 ± 0.025	0.471 ± 0.011†	

*Significantly different from control at $P < .01$. †Not significantly different from control.

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Preliminary experiments showed that neither pepstatin (which inhibits cathepsin D), antipain (which inhibits cathepsins A and B), nor elastinal (which inhibits elastase) affected protein degradation in rat diaphragm. These agents also had no effect in the presence of dimethyl sulfoxide (2 percent by volume), which may facilitate penetration of the inhibitors into the cells.

However, leupeptin consistently decreased protein degradation in rat muscles (Table 1A). Leupeptin (a mixture of propionyl- and acetyl-leucyl-leucyl-arginal) inhibits trypsin, plasmin, papain, and cathepsin B (*12*). It decreased protein breakdown in the soleus (a red muscle) and the EDL (a pale muscle) as well as in cardiac muscle. Similar results were obtained when muscles were incubated in the presence or absence of amino acids (Table 1A). In general, leupeptin seemed to reduce proteolysis more in the EDL than in the soleus or atrium, although it is not known why. Leupeptin also decreased net protein degradation (the balance between protein synthesis and breakdown) in other experiments (data not shown) performed in the absence of cycloheximide.

To investigate whether leupeptin decreased protein degradation by a nonspecific toxic effect, we measured the rate of protein synthesis in leupeptin-treated muscles, since this process is a sensitive index of energy supply and viability. We observed no effect of leupeptin on protein synthesis in rat hindlimb muscles at concentrations of the inhibitor which consistently decreased protein breakdown (Table 1B). Since leupeptin does not appear to nonspecifically poison the cell, this decrease in protein catabolism is probably due to a direct effect on one or more cellular proteases.

Cathepsin B is a leupeptin-sensitive protease found in muscle. This sulfhydryl endoprotease has an acid *p*H optimum, occurs in lysosomes, and has been partially purified from skeletal muscle (*3*). To determine whether this enzyme is inhibited when leupeptin decreases muscle protein degradation, we measured cathepsin B activity and protein breakdown in muscles previously treated with leupeptin, after washing to exclude leupeptin from the extracellular space (Table 2).

Cathepsin B activity in the individual muscle homogenates was measured fluorimetrically, using the specific substrate carbobenzoxy-alanyl-arginyl-arginyl-4-methoxy-2-naphthylamine (Enzyme Systems Products, Livermore, California) (*15*). The enzyme activity measured in muscle exhibited various properties characteristic of cathepsin B from other tissues (such as *p*H optimum and sensitivity to sulfhydryl group inactivators), and leupeptin inhibited proteolysis in muscle homogenates at acid *p*H but not at *p*H 7.8 (*16*). Pretreatment with leupeptin lowered protein breakdown and concomitantly decreased cathepsin B activity (Table 2). In analogous experiments, pretreatment with antipain, also an inhibitor of cathepsin B, had no effect on protein degradation in muscles or on the cathepsin B activity of their homogenates. Thus, the washing procedure employed was sufficient to free the extracellular space of an inhibitor similar in size to leupeptin. These data provide evidence that leupeptin, but not antipain, actually entered cells (*17*) and inhibited a lysosomal protease. They are consistent with a physiological role for cathepsin B in protein breakdown in muscles from normal rats.

In some pathological conditions, such as certain forms of muscular dystrophy and denervation atrophy, muscle wasting or failure of normal growth may result from increased protein catabolism rather than decreased protein synthesis (*18, 19*). Enzymatic and morphological studies suggest that lysosomal protease activities increase in the affected muscles (*20*). Since these enzymes may cause this increased protein breakdown, we determined the effect of leupeptin on protein degradation in denervated rat muscles and dystrophic muscles from C57B16J mice homozygous for the dy^2 gene. Leupeptin decreased protein degradation in these abnormal muscles as in the muscles from healthy animals (Table 3).

The results of this study by no means imply that cathepsin B is the only enzyme responsible for catabolizing muscle proteins. Muscle contains cathepsin D activity, and the failure to decrease protein breakdown with pepstatin may reflect the inability of this agent to

Table 2. Effect of pretreatment with leupeptin on protein degradation and cathepsin B activity in rat hindlimb muscles. The muscles were preincubated as described in Table 1 for 110 minutes with or without leupeptin (50 μM). They were then washed in 3 ml of fresh medium lacking leupeptin for 10 minutes with shaking and transferred to another 3 ml of fresh medium without leupeptin for a 2-hour incubation. Tyrosine release into this medium is an index of protein degradation (*13*). Subsequently, the individual muscles were homogenized in phosphate-citrate buffer (50 mM), *p*H 6.0, containing 2-mercaptoethanol (2 mM) and EDTA (1 mM). Cathepsin B activity was determined during a 2-hour incubation with substrate (final concentration, 50 μM). The abbreviation MNA denotes 4-methoxy-2-naphthylamine, the fluorescent product of substrate hydrolysis.

Muscle	Treatment	Protein degradation [nmole tyrosine mg^{-1} (2 hours)$^{-1}$]	Cathepsin B activity [nmole MNA mg^{-1} (2 hours)$^{-1}$]
Soleus	Control	0.279 ± 0.018	0.438 ± 0.015
	Leupeptin	0.225 ± 0.010*	0.325 ± 0.024*
EDL	Control	0.267 ± 0.015	0.366 ± 0.014
	Leupeptin	0.217 ± 0.008†	0.243 ± 0.010*

*Significantly different from control at $P < .01$. †Significantly different from control at $P < .05$.

Table 3. Effect of leupeptin on protein breakdown in diseased muscles. (A) Dystrophic muscles: muscles from 5- to 7-week-old mice with hereditary muscular dystrophy (C57B16J/dy^2) were studied by the techniques described for rat muscles. Tyrosine release into the medium was taken as a measure of protein degradation. No correction was made for possible changes in the intracellular pools of tyrosine since these pools were too small to measure. In one experiment [EDL (net proteolysis)] net protein degradation (without cycloheximide) was measured in the EDL. The leupeptin concentration was 25 μM. (B) Denervated muscles: 24 hours before removal of the muscles, the rats were anesthetized with ether, and at least 5 mm of sciatic nerve was removed just distal to the hip joint bilaterally (*19*). The leupeptin concentration was 50 μM.

Muscle	Protein degradation [nmole tyrosine mg^{-1} (2 hours)$^{-1}$]		Change (%)
	Control	Leupeptin	
	A. Dystrophic muscles		
EDL	0.414 ± 0.050	0.234 ± 0.021*	34
Plantaris	0.397 ± 0.028	0.289 ± 0.030†	24
EDL (net proteolysis)	0.401 ± 0.013	0.229 ± 0.016‡	43
	B. Denervated muscles		
Soleus	0.475 ± 0.021	0.215 ± 0.014†	54
EDL	0.454 ± 0.024	0.283 ± 0.021‡	37

*Significantly different from control at $P < .02$. †Significantly different from control at $P < .01$. ‡Significantly different from control at $P < .001$.

enter cells. Furthermore, a leupeptin-insensitive alkaline protease also seems to participate in protein turnover in skeletal muscle (*16*).

These results also do not establish that cathepsin B is the only protease inhibited by leupeptin in this tissue. Cathepsin L, an enzyme very similar to cathepsin B and also inhibited by leupeptin, has been found in liver lysosomes (*21*). In addition, muscle contains a soluble alkaline protease, activated by calcium (*5*), which seems sensitive to leupeptin (*16*). These enzymes or perhaps unknown proteases may also contribute to the effects of leupeptin reported here.

These experiments illustrate the value of inhibitors of this class as probes for investigating the functions of proteases in vivo and for elucidating the pathway of protein catabolism. In addition, the results with atrophying muscles provide an experimental rationale for attempting to treat muscle atrophy with leupeptin or other protease inhibitors. Stracher and co-workers (*22*) reported delayed degeneration of cultured cells from normal and dystrophic chick embryo muscles exposed to leupeptin, antipain, and pepstatin. They hypothesized that this was a consequence of decreased protein breakdown. Our results with intact muscles show that leupeptin could indeed act as these workers suggest. Leupeptin may have potential therapeutic uses, since it is apparently nontoxic and is absorbed orally (*23*). However, it may not be an ideal compound for therapeutic use since it is rapidly excreted in the urine and in some species may inhibit other important proteolytic enzymes (such as proteases involved in hemostasis, fibrinolysis, or maturation of secreted proteins) (*23*). Nonetheless, further pharmacological studies with leupeptin and related compounds seem warranted by the results reported here and elsewhere (*22*).

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Paramecium Fusion Rosettes: Possible Function as Ca^{2+} Gates

Abstract. *The function of a specific intramembrane particle array, "the fusion rosette," an essential requirement for exocytosis of trichocysts in* Paramecium, *was probed with a temperature sensitive secretory mutant (nd9). The cells were grown at 27°C, the nonpermissive, nonreleasing temperature at which fusion rosettes do not assemble. Exocytosis could be triggered, nonetheless, by addition of 40 μM ionophore A23187 and 15 mM Ca^{2+} but not Mg^{2+}. Rosette function is bypassed by this procedure, suggesting that during normal release, the rosette acts as a Ca^{2+} channel that allows development of a site-specific increase in Ca^{2+}, which in turn induces fusion and release.*

New information about molecular events involved in the process of vesicular secretion has been obtained with the freeze fracture technique (*1, 2*). The secretory process has been carefully studied in two ciliated protozoa, *Tetrahymena* and *Paramecium*, where the presence of an intramembrane particle array, the fusion rosette, marks the sites toward which the secretory organelles of these cells (mucocysts and trichocysts, respectively) have migrated and docked prior to membrane fusion and release (*2, 3*). The rosette consists of 11 P and E face particles, 15 nm in diameter with one central particle. In *Paramecium*, but not in *Tetrahymena*, the rosette is surrounded by one or two rings of particles (about 7.5 nm in diameter), which play no role in secretion (*4, 5*). An intimate connection between the rosette and secretion was clearly demonstrated with a series of secretory mutants of *Paramecium*. Beisson *et al.* (*4*) showed that a temperature-sensitive mutant, nd9, when grown at the nonpermissive temperature (27°C) neither assembles rosettes nor secretes mature attached trichocysts; however, the same cell grown at the permissive temperature (18°C) both assembles rosettes and regains the normal capacity for secretion.

In many systems, control of secretion appears to be dependent on the presence of Ca^{2+} (*6*). Although the exact role that this ion plays at a molecular level is still unclear, it is postulated that a rise in cytoplasmic free Ca^{2+} is usually necessary for normal stimulus secretion coupling. Secretion can be induced in wild-type *Paramecium* in the presence of extracellular Ca^{2+} by exposure to ionophores such as X-537A or A23187, compounds that facilitate transport of diva-

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