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INHIBITION OF BASAL AND DEPRIVATION-INDUCED PROTEOLYSIS BY LEUPEPTIN AND PEPSTATIN IN PERFUSED RAT LIVER AND HEART

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SUMMARY

Isolated rat livers and hearts were perfused in the presence and absence of insulin and amino acids (or insulin alone) to generate the basal and 2x enhanced rates of intracellular protein degradation, respectively, that are normally demonstrable under these conditions. Additions of the thiol-proteinase inhibitor, leupeptin, to each tissue group inhibited both levels of degradation proportionately. In liver, administration of a pepstatin-liposome complex reduced deprivation-enhanced proteolysis by a small degree (20%), but when added with leupeptin, basal and deprivation rates were inhibited 55-60%. These findings are consistent with the notion that the lysosomal system is involved in both degradative components.

INTRODUCTION

It is generally agreed that lysosomes are responsible for the degradation of at least some intracellular proteins in mammalian cells (1-5), but a definitive role for this organelle in protein turnover has not been established. The increase in proteolysis that follows nutritional deprivation or the lack of insulin and serum growth factors (1,4,6,9-11) is clearly associated with enhanced cellular autophagy (11-15) and probably mediated by it (1-3,5,11). Other aspects of turnover, however, are less well understood. Additions of nutrients and growth factors markedly suppress the more overt, typical manifestations of autophagy (12,15), but fail to inhibit degradation by more than 50-60% (9,11,14,16). The notion that this basal component is carried out by a nonlysosomal mechanism has gained support from the failure of such protease and lysosomal inhibitors as leupeptin, chloraquine and ammonia

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to suppress proteolysis in cells cultured in growth media (3,11,17). On the other hand, we have found that lysosomes obtained from rat livers perfused with a medium enriched with insulin and amino acids contain degradation products of sequestered intracellular protein (14). The possibility that autophagy of some type is an ongoing phenomenon under basal conditions in liver and other tissues was examined by the combined use of two inhibitors of lysosomal endopeptidases, leupeptin and pepstatin.

MATERIALS AND METHODS

Measurement of Protein Degradation in Perfused Livers--Male Lewis rats (Microbiological Associates), weighing 125-140 g on the day of the experiment, were provided food and water ad libitum. Livers were cyclically perfused in situ as previously described (9,18) with a medium consisting of Krebs-Ringer bicarbonate buffer, 4% bovine albumin (Fraction V, Pentex) and 0.27 (v/v) washed bovine erythrocytes. Liver protein was previously labeled in vivo with L-[1-^{14}C]valine (18), and proteolytic rates were assessed from the release of [^{14}C]valine into a large pool (15 mM) of unlabeled valine, added at 60 min. Rates were calculated by dividing the subsequent accumulation of label by the specific radioactivity of perfusate valine determined prior to the addition of the 15 mM carrier (18). This procedure has been shown to provide a valid measurement of intracellular protein degradation with virtually no contribution from perfusate proteins (19).

Measurement of Protein Degradation in Perfused Hearts--Hearts were removed from 250-300 g, male Sprague-Dawley rats, maintained on Purina chow and water ad libitum, and perfused (10) with a medium consisting of Krebs-Henseleit bicarbonate buffer containing 3% bovine albumin, 15 mM glucose and normal plasma levels of amino acids, omitting phenylalanine (10). Total protein degradation was measured from the rate of accumulation of phenylalanine in intra- and extracellular water after the addition of 0.02 mM cycloheximide (10).

Preparation of Pepstatin-enriched Liposomes--The procedure used was slightly modified from that described by Dean (2). Liposome reagents were obtained in kit form (Lipoprep 1C Kit, Pabst Biochemicals, Inc.). Each sealed vial contained 63 μmol of egg lecithin, 18 μmol of stearylamine and 9 μmol of cholesterol in 5 ml of chloroform. The vial contents were placed in a glass tissue grinder (Dounce), dried on the vessel walls under a stream of N_2, and dessicated under vacuum. 10.4 ml of 5 mM KH_2PO_4 (pH 7.0) and 13 mg of pepstatin A were added and the mixture homogenized with 10 passes of the tight pestle. The homogenate then was sonicated (40 w, 30 s). 1.8 ml aliquots were added to the perfusate as described in Table I.

Reagents--In addition to sources of materials specifically mentioned, L-[1-^{14}C]valine was obtained from New England Nuclear Corp.; leupeptin and pepstatin A from Protein Research Foundation, Osaka; trypsin-treated, crystalline zinc pork insulin (Lot #615-D63-10, 25 U/mg) from Lilly Research Laboratories. All other reagents were of the highest commercial grade obtainable.

RESULTS AND DISCUSSION

Data in Table I show that additions of pepstatin-enriched liposomes significantly inhibit protein degradation in rat livers perfused under un-

Table I. Effect of pepstatin on protein degradation in rat livers perfused in the absence of added amino acids and insulin.

Additions	Protein Degradation (nmol valine released · min^{-1}/liver 100 g rat)	%
None (14)	280 ± 18	100
DMSO (6)	287 ± 24	103
DMSO + pepstatin (5)	217 ± 19[a]	78
Pepstatin-liposomes (→)	225 ± 8[b]	80

a, $p < .05$; b, $p < .02$ Experimental procedures were generally the same as those described for liver in Figure 1. Pepstatin was added at 60 min either as a solution with dimethyl sulfoxide (DMSO) or incorporated in liposomes (see MATERIALS AND METHODS). In the first procedure, the initial perfusate concentrations were: pepstatin, 50 μg/ml; DMSO, 0.3%. Results are expressed as means ± S.E.; the numbers of observations are in parentheses.

supplemented conditions, results which confirm an earlier observation by Dean (2). A similar inhibitory response was obtained using 0.3% dimethyl sulfoxide (DMSO) as a pepstatin carrier. It should be noted that our average suppression (21%) was appreciably smaller than Dean's and more in line with that of Hopgood et al. (4) who employed DMSO. The reason for the difference is not altogether clear. However, it does seem likely that the nature of the carrier is not critically important providing it effectively facilitates the cellular entry of pepstatin. That a carrier is necessary has been shown by the fact that pepstatin has no activity when added alone to the perfused rat liver (2). Experiments with pepstatin-DMSO were also tried in perfused rat hearts, but the results were equivocal owing in part to a suppressive effect of DMSO on proteolysis. Other workers have failed to elicit pepstatin responses in muscle (20).

As depicted in Fig. 1, leupeptin exhibited moderately strong inhibitory activity both in liver and heart. The effects were seen by 15 min and they persisted throughout the course of perfusion. In each group, responses obtained under supplemented and deprived conditions were proportional, a

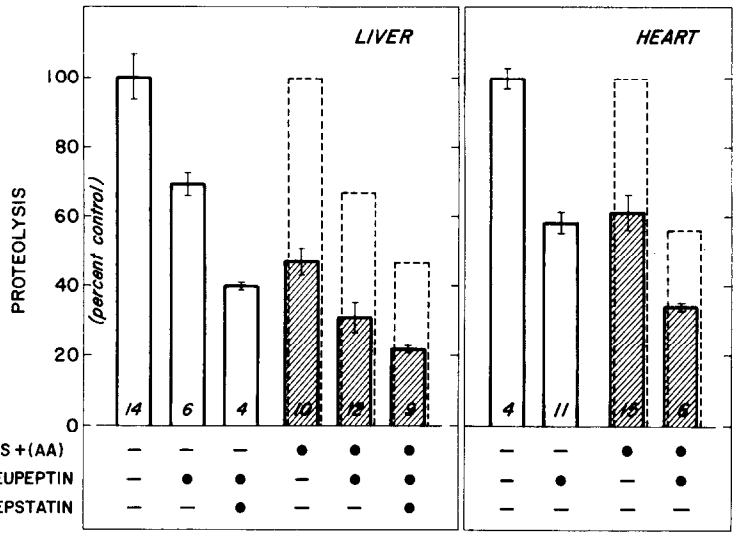

Figure 1. Effects of leupeptin and pepstatin on protein degradation in rat livers and hearts perfused in the presence and absence of added amino acids and insulin.

Perfused Livers--Livers were cyclically perfused in situ as described under MATERIALS AND METHODS. An amino acid mixture simulating 10x plasma amino acids, omitting tyrosine (7,14), was added at 60, 90 and 120 min of perfusion. Insulin was infused continuously from 60 min on at a rate of 2.4 μg/h after the addition of a priming dose of 0.6 μg. Leupeptin, dissolved in 0.85% NaCl, was introduced at 60 min in an amount calculated to give an initial concentration of 100 μg/ml. Pepstatin-enriched liposomes, prepared as described under MATERIALS AND METHODS, were added at 60 min. Rates of proteolysis were determined from 90 to 150 min of perfusion. The values shown are means ± S.E., given as the percentage of the mean control degradation rate which was 280 ± 18 nmol valine · min^{-1} per liver of 100 g rat. This rate is equivalent to 3.4% of total liver protein per h. Numbers of observations are indicated by the figures at the bottom of the bars. The open bars depict deprived and the shaded bars, basal conditions; the various rates are expressed as percentages of the deprived control. For purposes of comparison, basal rates were also normalized (dashed bars) by setting the basal control equal to 100%. All effects of inhibitors were significant to the 95% confidence level or greater.

Perfused Hearts--Following 10 min of preliminary perfusion, hearts were perfused for 120 min as described under MATERIALS AND METHODS. Where indicated, the following additions were made at the start of perfusion: leupeptin, 100 μg/ml; insulin, 1 μg (25 mU)/ml; cycloheximide, 0.02 mM. Rates of protein degradation were measured in the 120-min period after the additions. As in the above liver experiments, the values are means ± S.E., given as the percentage of the mean control degradation rate which was 299 ± 9 nmol phenylalanine/g · h. All inhibitory effects were significant to the 95% confidence level or greater.

pattern that is particularly evident because of the wide difference in degradation rates between the two conditions. In liver, pepstatin-enriched liposomes significantly enhanced the inhibition by leupeptin, and the

augmentation was similar in both proteolytic states (Fig. 1). The combined additions of leupeptin and pepstatin reduced basal degradation rates from 1.6% to a low of 0.7% of total protein per h, an inhibition of 56%.

Leupeptin rapidly enters the cell and is a potent inhibitor of the lysosomal thiol-proteinases, cathepsins B_1 and L (20,22,23). Its action, however, may not be limited to this organelle since potentially inhibitable, soluble endopeptidases have been demonstrated in muscle and liver (24-26). The function of these neutral proteolytic enzymes remains to be clarified. Should they eventually prove to be quantitatively important in total protein degradation, the processing of protein fragments by lysosomes might still represent an obligatory, final step in the overall proteolytic scheme. This would be so since endopeptidases catalyze only the limited hydrolysis of proteins. On the other hand, the action of pepstatin appears to be restricted to a single intracellular locus. It binds strongly to carboxyl-proteinases (21) of which cathepsin D is the only known intracellular representative. Inasmuch as this enzyme is intralysosomal, one may presume that the latter is the primary site of pepstatin action (2).

Based on the above considerations, we have concluded from the pepstatin effects in Fig. 1 that the lysosome is implicated in basal as well as deprivation-induced proteolysis in liver. Although we recognize the obvious limitations of any attempt to interpret the leupeptin inhibition similarly, nevertheless, our data are not inconsistent with the belief that analogous proteolytic mechanisms exist in heart. We can offer no explanation for the reported failure of protease inhibitors to affect basal proteolysis in cultured cells (3,11,17), but our findings, as well as those of Libby and Goldberg in rat skeletal muscle (20) clearly show that this unresponsiveness is not a general phenomenon.

The possibility that some fractions of cellular protein are continuously internalized and degraded by lysosomes in the absence of overt autophagy has received some recognition (27,28), but little experimental support. Perhaps

the strongest evidence for the existence of such a process under steady state conditions is the presence of glycogen within lysosomes of normal, fed rat livers (29) and of livers perfused with insulin and amino acids (14,15). Since smooth endoplasmic reticulum and associated proteins are undoubtedly sequestered along with glycogen (14,30), this uptake, if continuous, could account for the degradation of some intracellular proteins and membrane-bound components in the basal state.

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