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The selective role of cathepsins B and D in the lysosomal degradation of endogenous and exogenous proteins

Eiki Kominami[1], Takashi Ueno[1], Daisaku Muno[1] and Nobuhiko Katunuma[2]

[1] *Department of Biochemistry, School of Medicine, Juntendo University, Hongo 2-1-1, Bunkyo-ku, Tokyo 113, Japan* and [2] *Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Kuramoto-cho 3-18-15, Tokushima-shi, Tokushima 770, Japan*

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A selective inhibitor of cathepsin B, a derivative of E-64 (compound CA-074), and pepstatin-asialofetuin, a potent inhibitor of cathepsin D, were used for an in vivo study of the selective role of these proteinases in lysosomal proteolysis. Administration of compound CA-074 or pepstatin-asialofetuin to rats caused only a slight shift of the lysosomal density and no increase in sequestered enzymes in the autolysosomal fraction, although cathepsin B or D activity in the liver was markedly inhibited. These treatments also had little effect on the inhibition of the degradation of endocytosed FITC-labeled asialofetuin. In contrast, leupeptin treatment caused marked inhibition of lysosomal degradation of endogenous and exogenous proteins. These results suggest a small contribution of cathepsins B and D to the initiation of lysosomal proteolysis.

Cysteine proteinase inhibitory E-64 derivative; Pepstatin; Leupeptin; Cathepsin B; Cathepsin L; Cathepsin D

1. INTRODUCTION

Lysosomal cathepsins B, H, L and D are major endopeptidases that are important in intracellular protein degradation [1,2], antigen processing [3,4], and accelerated protein turnover in various pathological conditions [5,6]. However, it is not certain which proteinase is most important in lysosomal proteolysis. Leupeptin and E-64, powerful inhibitors of cysteine proteinases, effectively inhibit the degradation of endogenous proteins in tissues [7] as well as in cultured cells [8], however, they do not show selective inhibition of cathepsins B and L in vitro or in vivo. Murata et al. [9] recently synthesized derivatives of E-64 in order to develop a specific inhibitor of cathepsin B, and Towatari et al. [10] found that one of these compounds, CA-074, is a potent selective inhibitor of cathepsin B in vivo. Furuno et al. [11] reported that pepstatin-asialofetuin given to rats in vivo inhibits markedly the activity of cathepsin D in liver. We used these two specific inhibitors in vivo in order to study the selective role of cathepsins B and D in lysosomal proteolysis. Our results show that cathepsins B and D do not play a major role in the lysosomal degradation of endogenous or exogenous proteins.

Abbreviations: z, benzoyloxycarbonyl; MCA, methylcoumarylamide; BCA, bicinchoninic acid; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; FITC, fluorescein isothiocyanate.

Enzymes: cathepsin B (EC 3.4.22.1), cathepsin H (EC 3.4.22.16), cathepsin L (EC 3.4.22.15), cathepsin D (EC 3.4.23.5).

Correspondence address: E. Kominami, Department of Biochemistry, School of Medicine, Juntendo University, Hongo 2-1-1, Bunkyo-ku, Tokyo 113, Japan.

2. MATERIALS AND METHODS

2.1. *Materials*

Arg-MCA, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, pepstatin and leupeptin were purchased from the Peptide Institute Inc., Osaka, Japan. Fetuin (type 3) and neuraminidase were obtained from Sigma Chemical Co., and Nakalai Tesque Inc. (Kyoto, Japan), respectively. All other chemicals were of analytical grade. Compound CA-074, (*N*-(L-3-*trans*-propyl-carbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline) prepared by M. Murata et al. [9] was donated by Dr. Hanada, Taisho Pharmaceutical Co. (Saitama, Japan).

2.2. *Methods*

2.2.1. *Analytical procedures.* Cathepsin activities were assayed with Z-Phe-Arg-MCA at pH 5.5 for cathepsin B and L and Arg-MCA at pH 6.8 for cathepsin H as substrates by the method of Barrett and Kirschke [2]. Cathepsins L and B were measured by assay in the presence and absence of 2 μg/ml of compound CA-074 and cathepsin B was calculated as the compound CA-074-sensitive Z-Phe-Arg-MCA hydrolyzing activity; the rest of the activity was regarded as cathepsin L activity. Assay of cathepsin D was performed with denatured hemoglobin as described previously [12]. Lactic dehydrogenase and β-hexosaminidase activities were determined as described previously [13,14]. Protein was measured by the BCA method (Pierce Chemical Co.) with bovine serum albumin as a standard. FITC-labeled asialofetuin in the samples was measured fluorometrically as described previously [15] with FITC-asialofetuin from the same batch as that injected into rats as a standard.

2.2.2. *Preparation of FITC-labeled asialofetuin and pepstatin-asialofetuin.* Asialofetuin was prepared by neuraminidase treatment of fetuin and labeled with FITC as described previously [15]. Coupling of pepstatin to asialofetuin was performed as described by Furuno

et al. [11]. About 40 μg of pepstatin was conjugated to 1 mg of asialofetuin in the preparation.

2.2.3. *Treatment of animals.* Male Wistar rats weighing 200–250 g were maintained in an environmentally controlled room (lights on 06.00–20.00 h) for at least 2 weeks before the start of experiments. All rats were fed a standard laboratory pelleted diet. Compound CA-074 or leupeptin was injected intraperitoneally in saline solution at doses of 5 mg or 2 mg/100 g body weight, respectively. Pepstatin-asialofetuin (3 mg protein/100 g body weight) and FITC-asialofetuin (3 mg protein/100 g body weight) were injected intravenously as a solution in saline. The rats were sacrificed one hour after injection, the livers were perfused with cold saline, and 4 g of each liver was homogenized as described by Furuno et al. [16]. The resultant homogenates were centrifuged at $800 \times g$ for 5 min and the supernatants were centrifuged at $11\,000 \times g$ for 20 min. The precipitates (crude mitochondrial–lysosomal fraction) were suspended in 4 ml of 0.05 M acetate buffer, pH 5.0, and freeze-thawed for measurements of cathepsins B, H, L and D activities. In some experiments, the mitochondrial–lysosomal fraction was suspended in 25 ml of isosmotic Percoll at a density of 1.10 g/ml, and centrifuged at $63\,600 \times g$ for 40 min. Fractions of 1.15 ml were subjected to assays for the marker enzymes.

3. RESULTS AND DISCUSSION

3.1. *Inactivation of cathepsin activities by compound CA-074, leupeptin, and pepstatin-asialofetuin in vivo*

The data in Table I show that leupeptin inactivated cathepsins B and L markedly and cathepsin H moderately, whereas CA-074 inactivated cathepsin B selectively. Pepstatin-asialofetuin also inactivated cathepsin D selectively. To test the possibility that inactivation of cathepsin B is caused not by the presence of compound CA-074 in lysosomes but by adsorption of the inhibitor to the lysosomal membrane, we subjected the mitochondrial–lysosomal fraction to two cycles of freeze–thawing and then measured cathepsin B activities in the supernatant obtained by centrifugation at $105\,000 \times g$, 60 min. Results showed that similar decrease of cathepsin B to that shown in Table I was observed in CA-074-treated rats (not shown). This finding suggests that compound CA-074 is present inside of lysosomes.

3.2. *The effect of proteinase inhibitors on the degradation of endogenous proteins*

The administration of leupeptin to rats markedly increases the density of liver lysosomes due to protein accumulation [16,17]. We previously demonstrated that active or undegraded proteins such as cytosolic enzymes, cytoskeletal proteins (myosin), and mitochondrial enzymes, are sequestered in autolysosomes [17,18]. Compound CA-074, leupeptin, and pepstatin-asialofetuin were injected into rats and the animals were killed 1 h later. The mitochondrial–lysosomal fraction prepared from the liver was subjected to Percoll density gradient analysis. The profiles of liver lysosomes on the Percoll density gradient determined by measuring β-glucuronidase activity are shown in Fig. 1. The lysosomes of untreated rats show a typical broad density distribution. Leupeptin treatment caused a marked shift in lysosomal density as reported previously [16,17], but injection of compound CA-074 or pepstatin-asialofetuin resulted in a much smaller shift. Lactic dehydrogenase, which is a cytosolic enzyme, was detected in the autolysosomal fraction of leupeptin-treated rats, but only faintly in that of compound CA-074- or pepstatin-asialofetuin-treated rats, indicating that the inhibition of cathepsin B or cathepsin D by administration of compound CA-074 or pepstatin-asialofetuin, respectively, does not lead to a blockage of the lysosomal digestion of sequestered proteins.

3.3. *The effect of proteinase inhibitors on the degradation of FITC-asialofetuin*

FITC-asialofetuin was injected into rats administered compound CA-074, leupeptin, or pepstatin-asialofetuin 10 min before, and the effect of proteinase inhibitors on the digestion of this exogenous protein in liver lysosomes was examined. When 3 mg of FITC-asialofetuin was administered to rats, the recovery of FITC in control liver 30 min after injection was about 70% of the injected dose and 25% after 2 h. As shown in Table II, compound CA-074 and pepstatin-asialofetuin caused little delay in the degradation of endocytosed FITC-asialofetuin in the liver. In contrast, leupeptin strongly inhibited the digestion of FITC-asialofetuin and almost all the injected dose remained undigested even 2 h after the injection.

Cathepsin B from mammalian liver displays both endopeptidase and exopeptidase activities, depending on the substrate [1,2], although higher concentrations of cathepsin B are required to show endopeptidase activity

Table I

Lysosomal cathepsin activities in the livers of proteinase inhibitor-treated rats

	Cathepsin B (mu/mg–%Activity)	Cathepsin L (mu/mg–%Activity)	Cathepsin H (mu/mg–%Activity)	Cathepsin D (mu/mg–%Activity)
Control	420 ± 30 (100)	170 ± 20 (100)	103 ± 18 (100)	69.8 ± 6.8 (100)
CA-074	38 ± 7 (9)	178 ± 18 (105)	106 ± 21 (103)	68.8 ± 7.2 (98)
Leupeptin	56 ± 11 (13)	22 ± 4 (13)	37 ± 6 (36)	69.0 ± 6.2 (98)
Pepstatin-asialofetuin	410 ± 42 (98)	168 ± 26 (99)	99 ± 17 (96)	15.2 ± 1.8 (22)

Rats were treated i.p. with compound CA-074 (5 mg/100 g body weight) or leupeptin (2 mg/100 g body weight) or i.v. with pepstatin-asialofetuin (3 mg/100 g body weight). One hour later, mitochondrial–lysosomal fractions of the livers were prepared and the activities of cathepsins B, L, H and D were measured. Values are means for 4 rats.

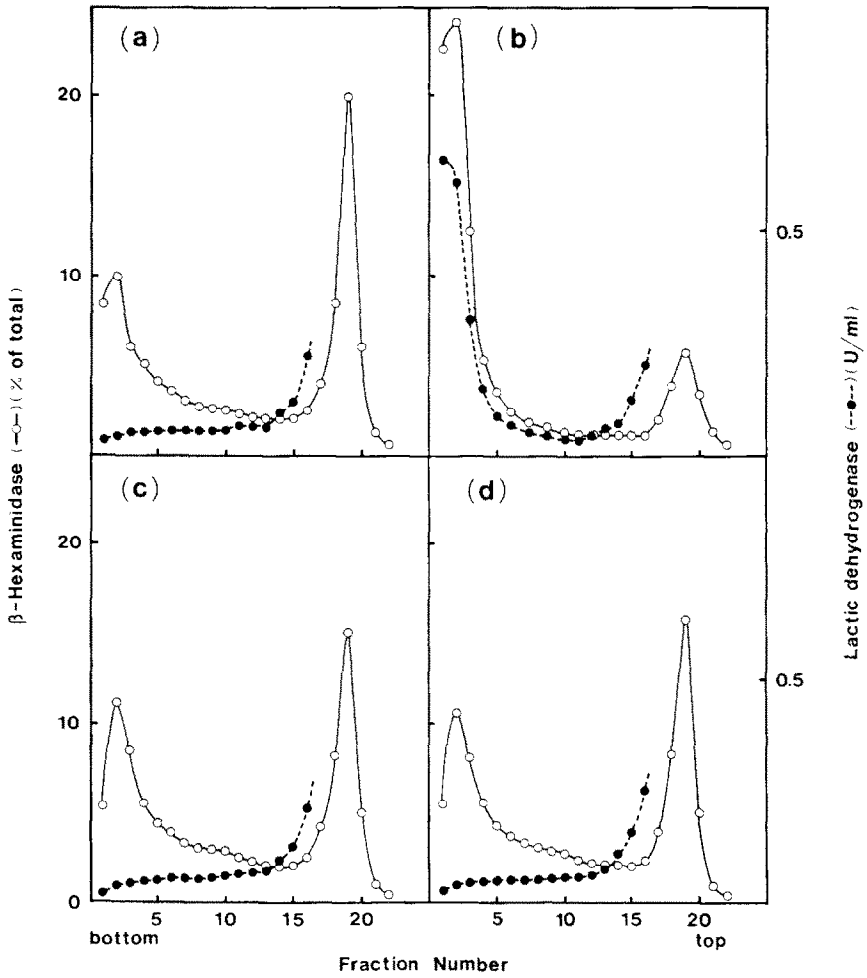

Fig. 1. Percoll density gradient distributions of β-hexosaminidase and lactic dehydrogenase in the lysosomal fractions of livers from control (a), leupeptin (b), compound CA-074 (c), and pepstatin-asialofetuin (d) treated rats. Rats were injected i.p. with leupeptin (2 mg/100 g body weight) or compound CA-074 (5 mg/100 g body weight) or i.v. with pepstatin-asialofetuin (3 mg/100 g body weight) and killed 1 h later. Mitochondrial-lysosomal fractions were prepared and subjected to Percoll density gradient centrifugation (1.10 g/ml). Gradients were collected in 22 fractions of 1.15 ml each and their β-hexosaminidase and lactic dehydrogenase activities were measured.

than are necessary for dipeptidyl carboxypeptidase activity. Also, the fact that cathepsin B exists in lysosomes at very high concentrations [19] suggests that it might be expected to show potent endopeptidase activity in that location. But, the present investigation using compound CA-074, a selective cathepsin B inhibitor, revealed that cathepsin B is not much involved in the degradation of endogenous or exogenous proteins in lysosomes. Cathepsin D has limited action against native proteins, but considerable activity against denatured proteins, at pH 3.5–5. Our results indicating that the marked inhibition of cathepsin D in liver lysosomes by injection of pepstatin-asialofetuin had no significant effect on lysosomal protein degradation suggest that like cathepsin B, cathepsin D by itself cannot initiate protein breakdown in lysosomes.

Table II

Effect of proteinase inhibitors on the degradation of FITC-asialofetuin in rat liver

	FITC concentration in liver (ng/mg protein)		
Treatment	30 min	60 min	120 min
Control	2.8 ± 0.4	2.2 ± 0.3	1.0 ± 0.2
Leupeptin	3.0 ± 0.3	3.4 ± 0.4	3.1 ± 0.2
CA-074	2.8 ± 0.3	2.6 ± 0.2	1.5 ± 0.3
Pepstatin-asialofetuin	2.9 ± 0.4	2.3 ± 0.3	1.1 ± 0.2

Rats were treated with various proteinase inhibitors as described in the legend to Table I and Fig. 1, ten min before intravenous injection of FITC-asialofetuin (3 mg as protein base). Values are means for 4 rats.

Administration of leupeptin, a potent cysteine proteinase inhibitor, causes an accumulation of autolysosomes in liver [11] due to retarded digestion of sequestered proteins (Fig. 1) and endocytosed proteins (Table II). Since leupeptin is known to inhibit cathepsins B and L strongly and cathepsin H much less ([1] and Table I), the results suggest that cathepsin H is less important than cathepsins L and B in lysosomal pro-

teolysis. Cathepsin L is one of the most powerful lysosomal cysteine proteinases when assayed using protein substrates [1,2], and it is thus a strong candidate as the initiator of protein breakdown in lysosomes. Since marked inhibition of lysosomal proteolysis by injection of leupeptin occurs as a result of blocking of not only cathepsin L but also other cysteine proteinases, the identification of cathepsin L as the most important protein in lysosomal protein breakdown must await the development of a selective inhibitor for cathepsin L in vivo.

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