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INDUCTION OF HEMOGLOBIN-HYDROLASE ACTIVITY BY THE THIOL-PROTEASE INHIBITORS LEUPEPTIN AND ANTIPAIN IN ADULT RAT LIVER CELLS IN PRIMARY CULTURE[1]

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SUMMARY: Addition of the inhibitors of thiol-protease leupeptin and antipain to adult rat hepatocytes in primary culture caused rapid inhibition of α-N-benzoylarginine-β-naphthylamide (BANA)[2]-hydrolase and induction of the acid-protease, hemoglobin (Hb)-hydrolase. This induction was inhibited completely by cycloheximide and puromycin, and partially by actinomycin D. Addition of leupeptin did not affect the activity of acid phosphatase, cytochrome c oxidase, or NADPH cytochrome c reductase or protein synthesis. Pepstatin, an inhibitor of acid-protease, did not induce BANA-hydrolase activity. These findings suggest that inhibitors of thiol-protease specifically induced acid-protease in lysosomes by some unknown mechanism.

We reported previously that primary cultures of hepatocytes from adult rats maintain various liver functions and sensitivities to various hormones (1). Their rate of protein turnover is also affected by various hormones (2). Therefore, these cells seem to be a good experimental system to use in studies on lysosomal functions in protein turnover in the liver. There are two main types of endopeptidase in liver lysosomes, acid-protease, such as cathepsin D [EC 3,4,23,5] and thiol-protease, such as cathepsin B [EC 3,4,22,1](3). Leupeptin and antipain specifically inhibit cathepsin B, while pepstatin specifically inhibits cathepsin D (4).

In the present work we examined the effects of these inhibitors on the activities of various lysosomal enzymes related to protein turnover in adult rat hepatocytes in primary culture.

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2. Abbreviations: BANA, α-N-benzoyl-DL-arginine-β-naphthylamide; Hb, hemoglobin.

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Materials and Methods

Materials --- All protease inhibitors were obtained from the Peptide Institute Inc.,Minō; BANA was from Nakarai Chemicals,Ltd., Kyoto; bovine Hb was from Wako Pure Chemical Industries, Osaka. Other materials were as reported previously (1,2).

Culture of Hepatocytes --- Hepatocytes of adult Wistar strain rats were obtained by perfusing liver with collagenase. The cells were cultured as monolayers under 5% CO_2 and 30% O_2 in air in Williams medium E containing 10% calf serum, 10^{-7}M insulin and 10^{-5}M dexamethasone for 3 days. The presence of both hormones was necessary for maintaining various liver functions (1).

Enzyme Assays --- Cultured cells at a density of 5 x 10^6 cells /50 cm^2 were incubated with inhibitors under otherwise standard culture conditions. Then the cells were washed with Hanks salt solution and harvested with a rubber policeman. The cells were suspended with 1 ml of 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and disrupted by freeze-thawing using dry ice-acetone. The disrupted cells were centrifuged at 20,000 x g for 30 min and the supernatant was used for measurement of various enzyme activities. The activities measured were those of Hb-hydrolase (5), BANA-hydrolase (6), acid phosphatase (7), cytochrome c oxidase (8), NADPH cytochrome c reductase (9) and protein synthesis (1). Protein was measured by the method of Lowry et al.(10).

Results and Discussion

Acid- and thiol-protease activities were measured with Hb and BANA, respectively, as substrate. These activities probably mainly represent the activities of cathepsin D and B, respectively (3). Cathepsin B has been subdivided into cathepsin B-1, H and L (11), while BANA hydrolase has been classified as cathepsin H, although this classification has not yet been clearly established.

When leupeptin was added to hepatocytes, the activity of BANA-hydrolase was markedly inhibited within 10 hr, while the activity of Hb-hydrolase started to increase gradually after 2-3 hr, reaching 6 times the control value after 30 hr (Fig.1). The inhibition of BANA-hydrolase and increase of Hb-hydrolase were proportional to the concentration of inhibitor (Fig.2). Addition of leupeptin did not change the rate of protein synthesis,or the activities of acid phosphatase, cytochrome c oxidase or NADPH cytochrome c reductase (data not shown). These results suggest that leupeptin specifically induces the lysosomal Hb-hydrolase, but that it does not induce other enzymes of mitochondrial or microsomal fractions.

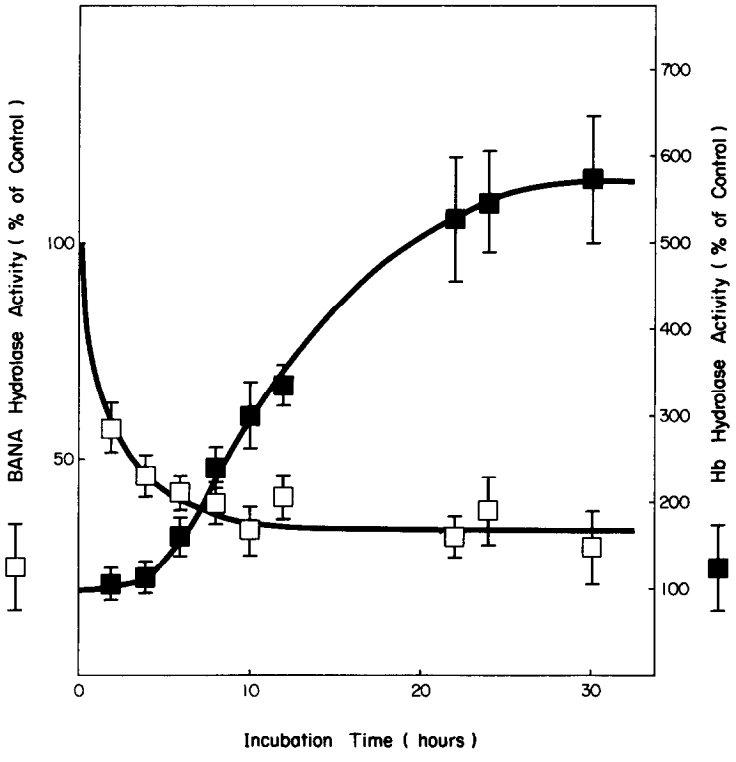

Fig.1. Inhibition of BANA hydrolase activity and increase of Hb-hydrolase activity by leupeptin. Cells from 3-day cultures were cultured further with or without leupeptin (50 μg/ml) for 30 hr. At the indicated times protease activities were measured. Each value represents the mean ± s.d. of 3 experiments.

Induction of Hb-hydrolase was inhibited completely by cycloheximide (5 μg/ml) and puromycin (0.05 μg/ml), and was inhibited 50% by actinomycin D (1 μg/ml). Antipain, another inhibitor of thiol-protease, had similar effects to those of leupeptin (data not shown), but pepstatin, an inhibitor of acid-protease, did not induce BANA-hydrolase, and inhibited the activity of Hb-hydrolase (Table I). The induction of Hb-hydrolase by leupeptin was not seen in a cell-free system (data not shown).

The present results show that thiol-protease inhibitors induce acid-protease activity, but that inhibitors of acid-protease do not induce thiol-protease. The mechanism and physiological significance of the induction of acid-protease activity are still un-

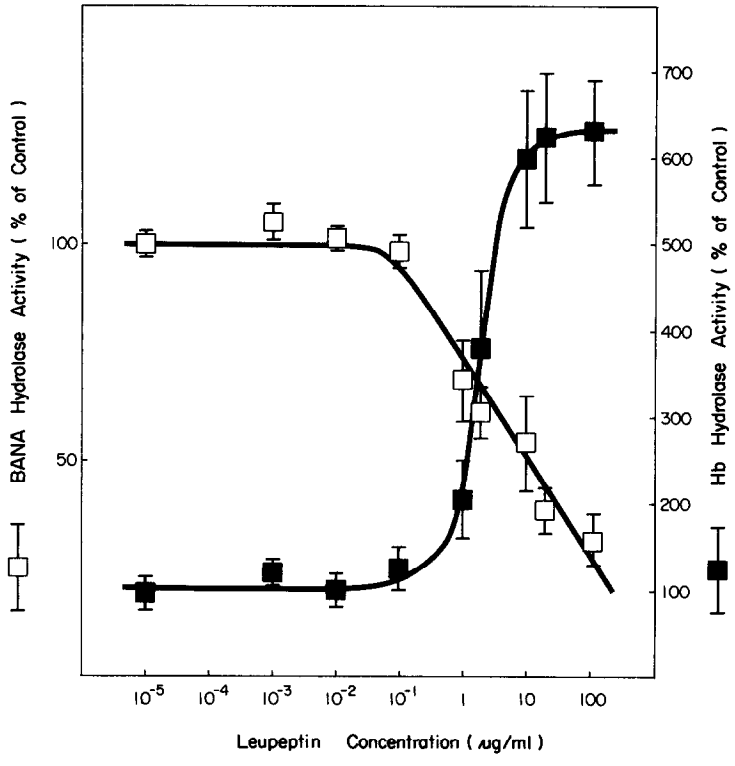

Fig.2. Effect of leupeptin concentration on the activities of BANA- and Hb-hydrolase. Cells were treated with different concentrations of leupeptin for 24 hr. Other conditions were as described in Fig.1.

known. It is unlikely that the number of lysosomes is increased by inhibitors of thiol-protease, because the activity of an other lysosomal enzyme, acid phosphatase, was not increased by these

Table I. Effect of pepstatin on the activities of Hb- and BANA-hydrolase. Pepstatin dissolved in 1% dimethylsulfoxide was added to the medium (50 μg/ml) and cells were cultured for 24 hr. Addition of dimethylsulfoxide alone showed no effect on the activities of these enzymes. Results are means ± s.d. of 4 experiments.

Addition	Hb-hydrolase (μg tyrosine formed /min/mg protein)	BANA-hydrolase (nmol β-naphthylamine formed/min/mg protein)
None	2.09 ± 0.08	22.2 ± 4.5
Pepstatin	0.65 ± 0.17	20.6 ± 3.4

inhibitors. The reciprocal relation of the activities of Hb- and BANA-hydrolases suggests an interconversion of these enzymes. Our preliminary results have shown that the Hb-hydrolase induced by leupeptin was pepstatin-insensitive, but otherwise very similar to cathepsin D, suggesting that it may be due to a modified form of cathepsin D. These possibilities must be studied bearing in mind that the induction must be accompanied by de novo synthesis of enzyme and that the induction did not occur in a cell-free system. Another possibility is that the induction may represent formation of a new unknown protease. In any case, comparative studies on non-induced and induced Hb-hydrolase should be useful in clarifing the mechanism of enzyme induction.

We showed previously that cathepsin D activity was induced when mouse L cells grew to the confluency, but that cathepsin B activity of the cells was not affected by their growth state (12). Therefore, it is possible that cathepsin D may function mainly, if not entirely, in regulation of protein turnover in cells. The possibility that decrease of one protease activity may cause compensatory induction of another protease to maintain the homeostasis of cellular proteins requires study.

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