Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 713-719

INHIBITIONS OF DEGRADATION OF RAT LIVER ALDOLASE AND LACTIC DEHYDROGENASE BY N-[N-(L-3-TRANS-CARBOXYOXIRANE-2-CARBONYL)-L-LEUCYL]AGMATINE OR LEUPEPTIN *IN VIVO*

Eiki KOMINAMI, Seiichi HASHIDA and Nobuhiko KATUNUMA

Department of Enzyme Chemistry, Institute for Enzyme Research, School of Medicine, Tokushima University, Tokushima 770, Japan

Received February 19,1980

SUMMARY: When injected into rats, leupeptin and E-64 (N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]agmatine), potent thiol protease inhibitors of microbial origin, inhibited cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.—) in the lysosomal fraction of liver. Both compounds strongly inhibited cathepsin B, but E-64 had more effect than leupeptin on cathepsin L. Neither compound inhibited cathepsin D (EC 3.4.23.5). E-64 reduced the apparent turnover rate of aldolase (EC 4.1.2.13) markedly and the turnover rates of lactic dehydrogenase (EC 1.1.1.27) and total soluble protein slightly. Leupeptin had apparently less effect on degradation of those enzymes, but significant effect on degradation of aldolase. These results indicate that proteinases, which are sensitive to inhibition by E-64 or leupeptin, especially cathepsin L and cathepsin B may be important in degradation of aldolase.

Little is known about the molecular mechanism of intracellular protein degradation or the intracellular proteases involved in the degradations of individual proteins. Recently, the roles of intracellular proteases in protein catabolism have been studied with help of protease inhibitors (1-6). These findings indicate that lysosome proteases are involved in breakdown of intracellular proteins. However, there are no studies on the roles of lysosomal proteases in degradations of individual enzymes. In this work we examined roles of lysosomal proteases in the degradation of fructose biphosphate aldolase and lactic dehydrogenase *in vivo* in rat liver cytosol, using the specific protease inhibitors leupeptin and E-64. E-64, which

Abbreviation: E-64, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]agmatine

0006-291X/80/070713-07$01.00/0

was isolated from *Aspergillus japonicus* (7,8) and leupeptin are powerful inhibitors of cathepsin B and cathepsin L (9-13). We choose for this study aldolase and lactic dehydrogenase, because the former is preferentially inactivated by cathepsin B, because the latter is resistant to cathepsin B (12,13). Present paper provides that E-64 is very effective for inhibition of degradation of aldolase *in vivo*.

MATERIALS AND METHODS

Chemicals: N-Benzoyl-DL-arginine-p-nitroanilide (BAPA) and azocasein were obtained from Sigma Chemical Co. Fructose biphosphate, NADH, triose monophosphate isomerase and glycero-3-phosphate dehydrogenase were from Boehringer Mannheim. [C^{14}]$NaHCO_3$ (60.3 mCi/mmol) was obtained from New England Corp. NCS solubilizer was from Amersham/Searle. Leupeptin and E-64 were gifts from Dr. Aoyagi (Institute of Microbial Chemistry) and Dr. Sawada (Taisho Pharmaceutical Co.), respectively. All other chemicals were of analytical grade and were obtained from Wako Chemical Industry (Tokyo) or Sigma Chemical Co.

Animals: Male Wistar-strain rats of 140-160 g body weight were maintained on regulatory chow and water *ad libitum* during the experimental period.

Injection of Protease Inhibitors and Preparation of Lysosome Fractions: Leupeptin was dissolved into 0.9% NaCl and injected intraperitoneally at the doses indicated in the legends to Figures and Tables; in turnover studies, it was injected once every 8 h. E-64 was administered subcutaneously as a 1:1 emulsion with complete adjuvant (DIFCO); in turnover studies, it was injected once every 8 h. Rats were killed by a blow on the head. All subsequent steps were carried out at 0-4°C. Three grams of liver was minced and homogenized in 10 w/v of 0.25 M cold sucrose in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 800 xg for 10 min, and the supernatant was recentrifuged at 12,000 xg for 20 min in a Sorval Superspeed RC 2-B. The resulting precipitate was washed twice with 0.25 M sucrose and suspended in 5.8 ml of 0.25 M sucrose, and then solubilized by addition of 0.2 ml of 10% Triton X-100. This extract was used for assays of cathepsin B and cathepsin L activities.

Administration of Isotope: Rats were injected with inhibitors as described above and given a single intraperitoneal injection of 0.1 mCi of [C^{14}]$NaHCO_3$ in 1 ml of 0.9% NaCl 1 h before sacrifice. Three rats each were killed 1, 25, 49 and 73 h after the first injection of inhibitor. Their livers were combined and homogenized in 0.25 M sucrose containing 10 mM Tris-HCl buffer, pH 7.5. Protein in the supernatant obtained by centrifugation at 105,000 xg, for 60 min was precipitated with 10% trichloroacetic acid and washed as described by Siekevitz (14). The protein was then dissolved in 0.1 ml of NCS solubilizer and neutralized with acetate and its radioactivity was counted in

10 ml of Triton-toluene. Radioactivity in purified enzyme solutions was counted directly without precipitating the protein with trichloroacetic acid. The rates of decay of enzymes and protein fractions are shown as decreases in total counts in these fractions.

Enzyme Purification: Rat liver aldolase and lactic dehydrogenase was purified by a modification of the methods of Rutter et al. (15) and Scopes (16), respectively, permitting isolation of the two enzymes from the same extract of liver. Obtained enzymes appeared homogeneous on sodium dodecyl sulphate polyacrylamide gel electrophoresis. The recoveries of both enzymes were 60-80%.

Enzyme Assays: Fructose biphosphate aldolase was measured at 25°C by the method of Rutter et al. (15). Lactic dehydrogenase was assayed by measuring the decrease in absorbance at 340 nm in a final volume of 1 ml at 25°C. The assay mixture contained 50 mM Tris-HCl buffer, pH 7.5, 1 mM pyruvate and 0.2 mM NADH. Activities in cathepsin B was measured by the method of Otto (17). The p-nitroaniline liberated was converted to an azodye by diazotization and coupling, and its concentration was measured at 560 nm (18). Cathepsin L was assayed as described by Kirschke et al. (9). One unit is expressed as the amount of enzyme cause a change of A_{366}/min per ml.

RESULTS AND DISCUSSION

Effects of Administration of Protease Inhibitors on Cathepsin B and Cathepsin L Activities: Fig. 1 shows the time courses of changes in activity of cathepsin B after a single intraperitoneal injection of 0.8 mg/rat of leupeptin as a solution in saline and subcutaneous injection of 1.5 mg/rat of E-64 as an emulsion in oil. Cathepsin B activity was inhibited appreciably within 1.5 h after injection of leupeptin or E-64 and the inhibition continues for at least 6 h, but disappeared completely within 24 h. When E-64 was administered intraperitoneally, inhibition of cathepsin B started to decrease within 3 h and disappeared completely within 6 h. Thus, E-64 was administered subcutaneously as an oil emulsion in subsequent studies. The changes in cathepsin B and L activities 6 h after administration of various concentrations of the inhibitors are shown in Fig. 2. Both inhibitors showed dose-dependent inhibition of cathepsin B causing at least 80% inhibition at high concentrations. However,

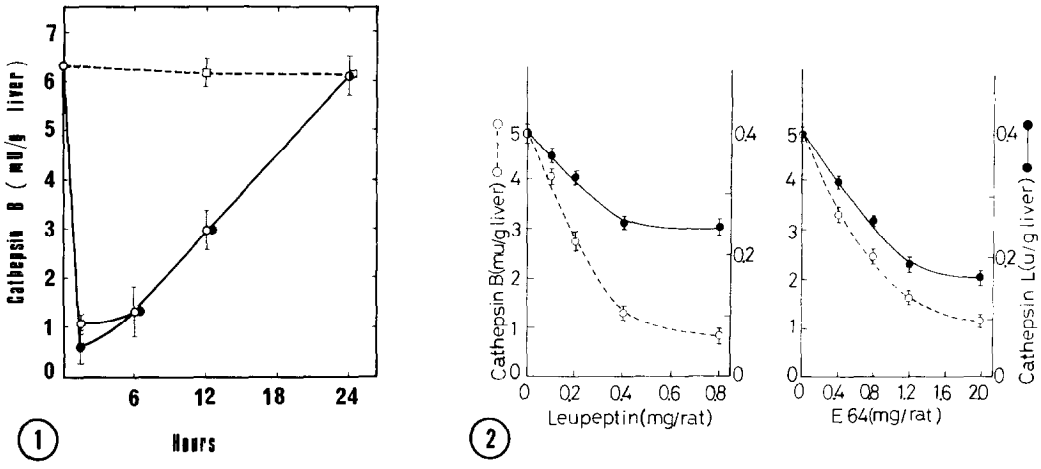

Fig. 1. Time course of change in cathepsin B activity in isolated lysosomal fractions from rat liver treated with protease inhibitors. Leupeptin in 0.9% saline was injected intraperitoneally at 0 time at a dose of 0.8 mg/rat, and 1.5 mg/rat of E-64 was injected subcutaneously as an emulsion with oil. Control rats received only 0.9% saline or oil. At the indicated times, rats were killed and the lysosomal fraction was isolated as described in the "Materials and Methods" and the cathepsin B activity was determined. □---□ , control activities; ○——○ , cathepsin B activity after treatment with leupeptin; ●——● , cathepsin B activity after treatment with E-64.

Fig. 2. Effect of the dose of leupeptin (1) or E-64 (2) on cathepsin B and L activities in the lysosomal fraction of 6 h after treatment. Leupeptin was injected intraperitoneally in 0.9% saline and E-64 was injected subcutanelusly as an emulsion with oil. 6 h after injections, rats were killed, the lysosomal fraction of the liver was isolated as described in the "Materials and Methods", and cathepsin B and L activities were determined; Cathepsin B activity (○----○), Cathepsin L activity (●——●).

their effects on cathepsin L activity were rather different; leupeptin was less inhibitory than E-64, causing less than 40% inhibition even at high concentrations, whereas E-64 caused more than 60% inhibition at a dose of 2.0 mg/rat. Actual inhibition of cathepsin L activity by E-64 or leupeptin could be higher, since the observed activity includes that of other protease(s) besides cathepsin L, because of use of non-specific substrate, azocasein (11). Neither inhibitor had any effect on cathepsin D or acid phosphatase activity, when assayed in the same preparations. Their action, however, may not be limited to those

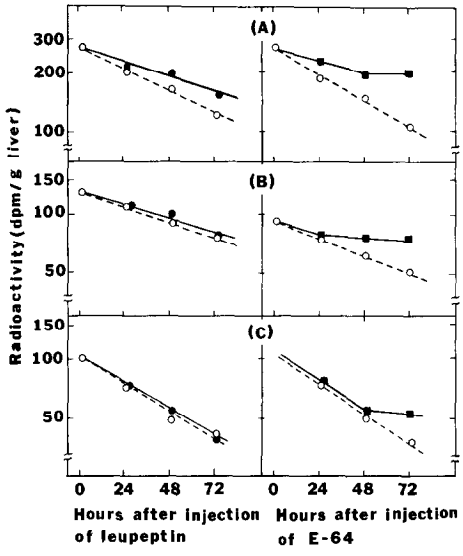

Fig. 3. Effects of protease inhibitors on the rates of degradation of fructose biphosphate aldolase (A), lactic dehydrogenase (B) and total soluble proteins (C). Rats weighing 140 to 160 g were given a single injection of 100 μCi of [^{14}C]$NaHCO_3$ and then injected with an inhibitor and every 8 h starting one h later. Dose of 0.8 mg/rat of leupeptin and 2.0 mg/rat of E-64 were given. Leupeptin was injected intraperitoneally in 0.9% saline and E-64 was injected subctaneously as an emulsion with oil. Rats were killed at the indicated times after the first injection of inhibitor, and aldolase and lactic dehydrogenase were isolated from the pooled livers from 3 rats. Results are shown as total counts of radioactivity in enzyme protein or protein fractions/g liver. O----O , specific radioactivities of control; ●——● , specific radioactivities after treatment with leupeptin; ■——■ , specific radioactivities after treatment with E-64.

proteases, since potentially inhibitable endoproteinases have been demonstrated in liver (19-21).

Turnover Rates of Aldolase and Lactic Dehydrogenase in Control and Protease Inhibitors-Treated Rats: Rats were fed on normal laboratory chow *ad libitum* and injected with leupeptin or E-64 once every 8 h. Injection of the inhibitors was started 1 h after injection of [C^{14}]$NaHCO_3$. The rates of decay of enzymes and protein fractions were measured as the rates of decrease in total counts in these fractions.

The results in Fig. 3 show that leupeptin was less inhibitory than E-64 on the rates of degradation of the marker proteins,

aldolase and lactic dehydrogenase in the cytosol. But the apparent turnover rate of aldolase was reduced significantly by leupeptin. In E-64-treated animals the half lives of the proteins, and especially aldolase, were longer. The half lives of aldolase, lactic dehydrogenase and total soluble proteins in control animals were estimated as 2.4 days, 3.5 days and 2.1 days, respectively. Stronger effect of E-64 to reduce turnover rates of marker proteins seems to partly be due to the difference in inhibitory effects of the two inhibitors on cathepsin B and L (especially cathepsin L). It is not, however, ruled out that the unknown mechanism of proteinase inhibitors by their administration *in vivo* may operate for occurence of this difference.

Injection of these inhibitors did not cause loss of body weight or decrease in food intake or have any effect on protein synthesis. The rate of aldolase and lactic dehydrogenase syntheses before and after injection of protease inhibitors was measured by pulse-labeling and isolation of the enzymes. No significant effect on the rates of synthesis of the two enzymes by treatment of protease inhibitors was observed (not shown). Leupeptin was also found not to influence protein synthesis in cell cultures by Hopgood et al. (22) and Seglen et al. (23). Therefore, these protease inhibitors appear to decrease degradation of enzymes by a specific action, not by a non-specific toxic effect.

Injection of the protease inhibitors *in vivo* resulted in less than 30% decrease in aldolase activity in the cytosol and had no effect on lactic dehydrogenase activity in the cytosol. Then, the rates of decay of enzymes are expressed as decreases in total counts in these fractions. Preliminary results showed that after injection of inhibitors rat liver contains a

enzymatically less active form of aldolase with lower specific activity. Studies on the mechanisms of less active form of aldolase are now in progress.

ACKNOWLEDGMENT: This work was supported by Grant-in-Aid for Scientific Research (No. 348379) from the Ministry of Education of Japan.

REFERENCES

1. Dean, R.T. (1975) Nature 257, 414-416
2. Wibo, M. & Pool, B. (1974) J. Cell. Biol. 63, 430-440
3. Libby, P. & Goldberg, A.L. (1978) Science 199, 534-536
4. Seglen, P.O., Grinde, B. & Solheim, A.E. (1979) Eur. J. Biochem. 95, 215-225
5. Ward, W.F., Chua, B.L., Li, J.B., Morgan, H.E. & Mortimore, G.E. (1979) Biochem. Biophys. Res. Commun. 87, 92-98
6. Dean, R.T. (1979) Biochem. J. 180, 339-345
7. Hanada, K., Tamai, M., Yamagishi, M., Ohmura, S., Sawada, J. & Tanaka, I. (1978) Agr. Biol. Chem. 42, 523-528
8. Hanada, K., Tamai, M., Ohmura, S., Sawada, J., Seki, T. & Tanaka, I. (1978) Agr. Biol. Chem. 42, 529-536
9. Aoyagi, T., Miyata, S., Nanba, M., Kojima, F., Ishizuka, M., Takeuchi, T. & Umezawa, H. (1969) J. Antibiol. 22, 558-568
10. Ikezawa, H., Aoyagi, T., Takeuchi, T. & Umezawa, H. (1971) J. Antibiol. 24, 488-490
11. Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. & Bohley, P. (1977) Eur. J. Biochem. 74, 293-301
12. Towatari, T., Kawabata, Y. & Katunuma, N. (1979) Eur. J. Biochem. 102, 289-299
13. Towatari, T., Tanaka, K., Yoshikawa, D. & Katunuma, N. (1978) J. Biochem. 84, 659-671
14. Siekevitz, P. (1952) J. Biol. Chem. 195, 549-565
15. Rutter, W.J., Hunsley, J.R., Groves, W.E., Calder, J., Rajkumar, T.V. & Woodfin, B.M. (1966) Method Enzymol. 9, 479-498
16. Scopes, R.T. (1977) Biochem. J. 161, 253-263
17. Otto, K. & Bhakdi, S. (1969) Hoope-Seyler's Z. Physiol. Chem. 348, 482-490
18. Thompson, J.F. (1970) Method Enzymol. 17, 894-900
19. Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Broghammer, U. (1976) Acta Biol. med. Germ. 35, 285-299
20. Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Hanson, H. (1977) Acta Biol. Med. Germ. 36, 185-199
21. Takai, Y., Yamamoto, M., Inoue, M., Kishimoto, A. & Nishizuka, Y. (1977) Biochem. Biophys. Res. Commun. 77, 542-550
22. Hopgood, M.F., Clark, M.G. & Ballard, F.J. (1977) Biochem. J. 164, 399-407
23. Seglen, P.P. (1978) Biochem. J. 174, 469-474