Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 440-447

IN VITRO AND IN VIVO INHIBITION OF RAT LIVER CATHEPSIN L BY EPIDERMAL PROTEINASE INHIBITOR

Toshihiko Hibino, Kimie Fukuyama, and William L. Epstein

Department of Dermatology, University of California
San Francisco, California 94143

Received February 6,1980

SUMMARY: An SH-proteinase inhibitor (EPI) purified from newborn rat epidermis was shown to inhibit casein and BAPNA hydrolytic activities of rat liver extract in vitro. The greatest inhibition was seen in the cathepsin L fraction purified by CM Sephadex C-50. Fifty percent inhibition of 1 U cathepsin L and papain were seen with 325 μg and 50 μg EPI, respectively. Intradermal injection of cathepsin L in newborn rats caused inflammatory reactions and a reduction of EPI from living epidermal cells of the skin site.

The findings suggest that EPI is excreted from the epidermal cells into the dermis where SH-proteinase activity is involved in the inflammatory process

INTRODUCTION

An SH-proteinase inhibitor which localizes in the superficial layers (granular cells) of newborn rat epidermis was reported by Järvinen et al (1), who thought that the inhibitor might act as a defense mechanism against microbial invasion. Although general chemical properties of this inhibitor were similar to those of an SH-proteinase inhibitor found in the healing phase of Arthus reaction (2), the authors suggested that the epidermal SH-proteinase inhibitor does not participate in dermal inflammation, because the epidermal inhibitor was not found in dermis or serum. Early reports by others also seemed to support this view: Hayashi (3) observed large numbers of mononuclear cells in the healing Arthus reaction and postulated they secrete the inhibitor in the lesions as shown in vitro (4). Furthermore, Tokaji (5) isolated another inhibitor that might relate to healing of dermal injury from sera of rabbit, guinea pig, and cow.

Recently we have isolated and purified a protein with a molecular weight of about 12,000 from newborn rat epidermis (6). This protein (EPI) demonstrated in vitro inhibitor activity to papain and ficin, but not to trypsin,

0006-291X/80/060440-08$01.00/0

cathepsin D or pepsin. General chemical properties appeared closely related to the protein isolated by Järvinen et al (1) except for its localization in the epidermis. EPI was found by both immunohistological and immunochemical techniques to be present throughout the epidermis, including the deeper layers (spinous and basal cells). In this study, we examined the biological function of EPI using papain and cathepsin L. Immunofluorescence technique was used for in vivo detection of EPI and its behavior following dermal injury.

MATERIALS AND METHODS

Isolation and Purification of EPI: Cornified cells from the epidermis of newborn rats (Sprague-Dawley strain) were homogenized in 0.1 M Tris-HCl (pH 8.0) containing 0.15 M NaCl and 10 μg/ml of phenylmethylsulfonylfluoride and incubated at 4°C for 1 hr. Supernatant was separated by centrifugation at 30,000 x g, dialyzed against H_2O, lyophilized, and the proteins were extracted with 0.12 M $HClO_4$. The pH was adjusted to 4.5 and the soluble fraction was subjected to Sephadex G-50 column chromatography. A protein peak with the highest inhibitor activity was applied on a rabbit anti-EPI IgG affinity column as described previously (6). EPI absorbed in the column and dissociated by glycine-HCl buffer, pH 2.3, was neutralized, dialyzed, and lyophilized.

Purification of Rat Liver Cathepsin L: Subcellular fractions from liver of adult male rats (Sprague-Dawley strain) were prepared by the procedure of de Duve (7). Cathepsin L was then purified from the light mitochondrial (crude lysosome) fraction according to the method of Kirschke et al (8). Each fraction from Sephadex G-75 column was tested for 1) BAPNA and casein hydrolyses, and 2) EPI inhibition of enzyme activity. The fraction containing EPI-inhibited casein hydrolysis was applied on a CM Sephadex C-50 column and eluted with a stepwise KCl gradient.

Enzyme Assay: Buffers used were 0.2 M acetate buffer (pH 5.0) for the liver enzymes and 0.2 M Tris-HCl buffer (pH 7.5) for papain; both contained 8 mM EDTA and 4 mM DTT. 1) Casein hydrolysis (9): Assay mixture consisted of 0.1 ml buffer, 0.05 ml H_2O, and 0.1 ml enzyme solution. The mixture was incubated for 30 min at 37°C after addition of 0.25 ml of 1% casein in H_2O. The reaction was stopped by the addition of 0.75 ml of 5% TCA and the sample kept at 4°C for 1 hr. After removing the precipitate by centrifugation, the reaction product was read at 280 nm. Enzyme activity was expressed as a unit causing an increase of absorbance at 280 nm by 1.0 per 10 min. 2) α-*N*-benzoyl-DL-arginine-p-nitroanilide (BAPNA) hydrolysis (10): The assay mixture consisted of 0.1 ml buffer, 0.1 ml H_2O and 0.1 ml enzyme solution. After addition of 0.1 ml, 0.01 M BAPNA (dissolved in DMSO) in buffer, the mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 0.4 ml of 30% acetic acid, and the mixture left at 4°C for 1 hr. After removing the precipitate, the reaction product was read at 405 nm. Enzyme activity was expressed as a unit calculated from the molar extinction of p-nitroaniline at 405 nm (E = 10,500). One unit was expressed as the amount of enzyme that would hydrolyze 1 mM BAPNA.

Immobilization of Papain (11): 8 mg papain (Type III, Sigma Chemical Co.) was dissolved in 6 ml H_2O, and 2 ml 0.4 M carbonate buffer (pH 9.5) was added. Coupling to 1 ml CNBr-activated Sepharose 4B was done at 4°C for 20 hr and the remaining active sites blocked with 1 M glycine at 20°C for 2 hr. The papain-Sepharose 4B was washed alternately with 0.1 M acetate buffer (pH 4.0) and

0.1 M borate buffer (pH 8.0), each containing 1 M NaCl. The final product was suspended in water (7 ml). The complex showed BAPNA hydrolyzing activity of 5.6 μ/ml.

In Vivo Study of Cathepsin L and Papain: Two-day-old rats were injected intradermally with 0.05 ml of EPI, cathepsin L, papain, or Sepharose-coupled papain. In some experiments EPI was mixed with one of the enzymes and injected, and control animals were injected with buffers. Concentration of EPI used was 50 μg/ml; cathepsin L was prepared as 0.4 U/ml in 10 mM potassium phosphate buffer (pH 5.8) containing 0.5 M KCl, 0.8 mM EDTA and 0.4 mM DTT. Papain was diluted to 7, 14, or 21 U/ml of H_2O. Before use, 0.1 ml of 0.2 M Tris-HCl (pH 7.5) containing 4 mM DTT and 8 mM EDTA to each ml of papain solution was added. The rats were sacrificed at various intervals after injection; the injected skin sites were removed and quickly frozen. They were cut at 4 μ with a cryostat and fixed with 100% methanol. The sections were reacted at room temperature for 30 min with rabbit anti-EPI IgG. They were washed in phosphate buffered saline (pH 7.4), dried, and stained with fluorescein conjugated goat anti-rabbit IgG (Hyland Co.) (F/P ratio 2.8).

RESULTS

Crude lysosomal fraction of the rat liver after Sephadex G-75 column chromatography showed hydrolytic activity to both casein and BAPNA in the second peak; EPI inhibited 30% of casein hydrolysis and 10% BAPNA hydrolysis (Fig. 1). By application of the second peak on CM Sephadex column, proteins eluted with 0.2 M KCl had BAPNA hydrolytic activity and EPI inhibited up to 30% of the enzyme activity (most probably cathepsin B) (Fig. 2). Cathepsin L eluted with 0.5 M KCl showed the greatest casein hydrolytic activity, which

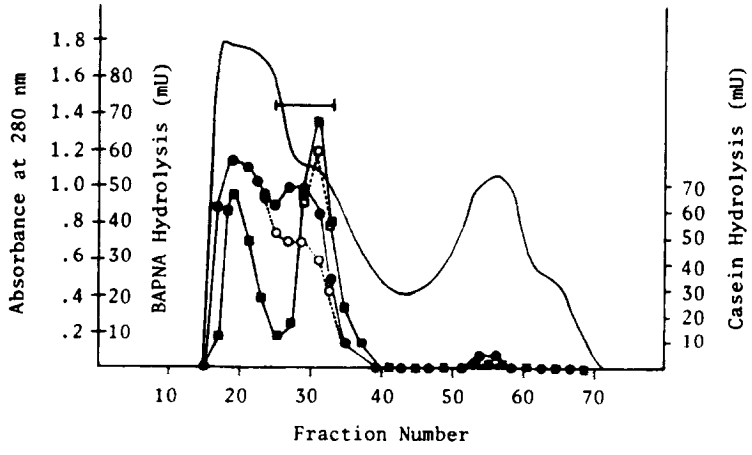

Figure 1: Sephadex G-75 chromatography of the crude lysosomal extract and effect of EPI on enzyme activities. 350 mg of protein were applied to a 2.5 x 88 cm column equilibrated with 10 mM potassium phosphate buffer (pH 6.9) in 0.15 M KCl. Flow rate was 40 ml/hr. In each fraction, 7.8 ml were collected. Absorbance at 280 nm (——); casein hydrolyzing activity without (●—●) and with (○····○) EPI; BAPNA hydrolyzing activity without (■—■) and with (□····□) EPI. Fractions indicated with a bar (M.W. 20,000 to 30,000) contained cathepsin L.

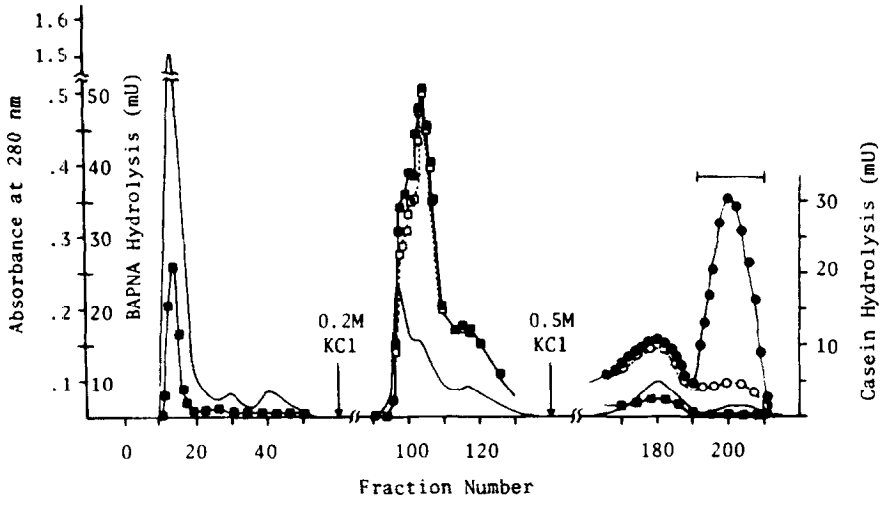

Figure 2: CM Sephadex C-50 chromatography of crude cathepsin L fraction and effect of EPI on enzyme activity. 50 mg of protein was applied to a 1.5 x 55 cm column eluted stepwise with increasing concentrations of 0.2 M KCl to 0.5 M KCl in 10 mM phosphate buffer (pH 5.8). Flow rate was 10 ml/hr. 2.4 ml fractions were collected. Absorbance at 280 nm (——), BAPNA hydrolyzing activity without (■—■) and with (□····□) EPI, casein hydrolyzing activity without (●—●) and with (○····○) EPI. A bar indicates cathepsin L fractions that showed EPI inhibited casein hydrolytic activity, but no BAPNA hydrolytic activity.

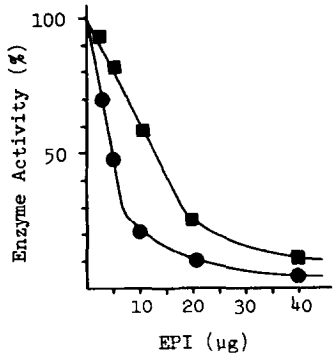

Figure 3: Effect of EPI on casein hydrolyzing activity of cathepsin L and papain. Inhibition assays were carried out under the conditions described in "Methods," using cathepsin L (0.4 μ/ml) and papain (7 μ/ml). 50% inhibition was seen by addition of 13 μg EPI to cathepsin L (■—■) and 3.2 μg EPI to papain (●—●).

was effectively inhibited by EPI, and no BAPNA hydrolytic activity. The inhibitory activities of EPI to cathepsin L and papain were compared in Fig. 3. Fifty percent inhibition of 1 U cathepsin L required 325 μg EPI, whereas only 50 μg EPI was needed for 50% inhibition of 1 U papain.

After injection of cathepsin L, the skin of newborn rat became erythematous and the superficial capillary vessels dilatated during the first 6 to 8 hr

returning to normal after 12 hr. Similar changes were seen with papain injection, but not in the skin sites where EPI was injected with or without enzymes. Formation of blisters was not observed.

Immunofluorescence microscopy demonstrated that EPI localizes in all cell layers of the epidermis as reported (Figs. 5a, 6a). Decrease in the immunoreac tivity of anti-EPI IgG with the living epidermal cells, but not in keratinized cells, was detected from 3 hr after injection of cathepsin L (Fig. 5b). By 6 hr after injection, almost no reactivity was found in the basal to granular cells, whereas keratinized cells continued to show the staining (Fig. 5c). The reaction products were not observed in the dermis before or after injection. The following possibilities were considered for the decrease of the immunological reaction: (1) EPI enzyme complex does not react with anti-EPI IgG, or (2) EPI was released from the epidermis and acted as an inhibitor to the injected enzymes and the complex was removed from the skin sites. In order to test the first possibility, agar diffusion was used: Immunologic reactivity of EPI complex and anti-IgG was seen (Fig. 4). Skin excised from injection sites of EPI was mixed with cathepsin L and studied by immunofluorescence microscopy. The antigen was detected, as shown in Figure 6b, in the dermis in the specimen taken immediately after injection of the mixture (the EPI-cathepsin complex) but not in the biopsy taken 6 hr after injection. The complex seemed to be removed from the skin sites during the 6 hr. No significant changes were seen in the skin vessels and EPI distribution in the

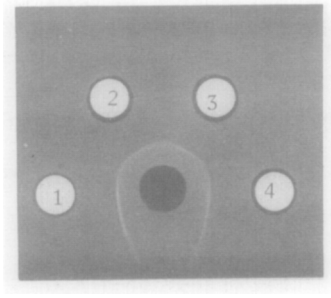

Figure 4: Agar double diffusion analysis of papain-EPI complex with rabbit anti-EPI IgG. Center well, IgG (2 mg/ml), (1) EPI (25 µg/ml), (2) papain (21 U/ml) + EPI (50 µg/ml), (3) papain (14 U/ml) + EPI (50 µg/ml), (4) papain (7 U/ml) + EPI (50 µg/ml).

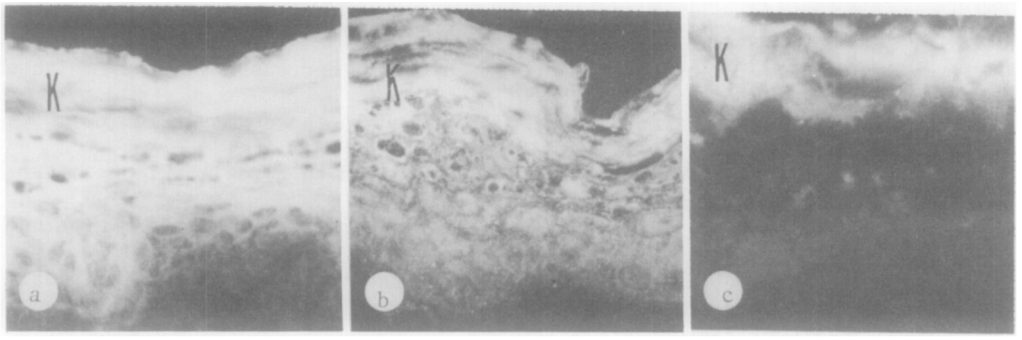

Figure 5: Reactivity of anti-EPI IgG in newborn rat epidermis. Before injection of cathepsin L EPI was found in all epidermal cells (1) but EPI in the living cells decreased greatly by 3 (b) and 6 hr (c) after injection of cathepsin L. EPI in keratinized cells (K) did not show the change. Rabbit anti-EPI IgG goat fluorescein-bound protein was diluted to approximately 85 μg/ml and 17 μg/ml respectively.

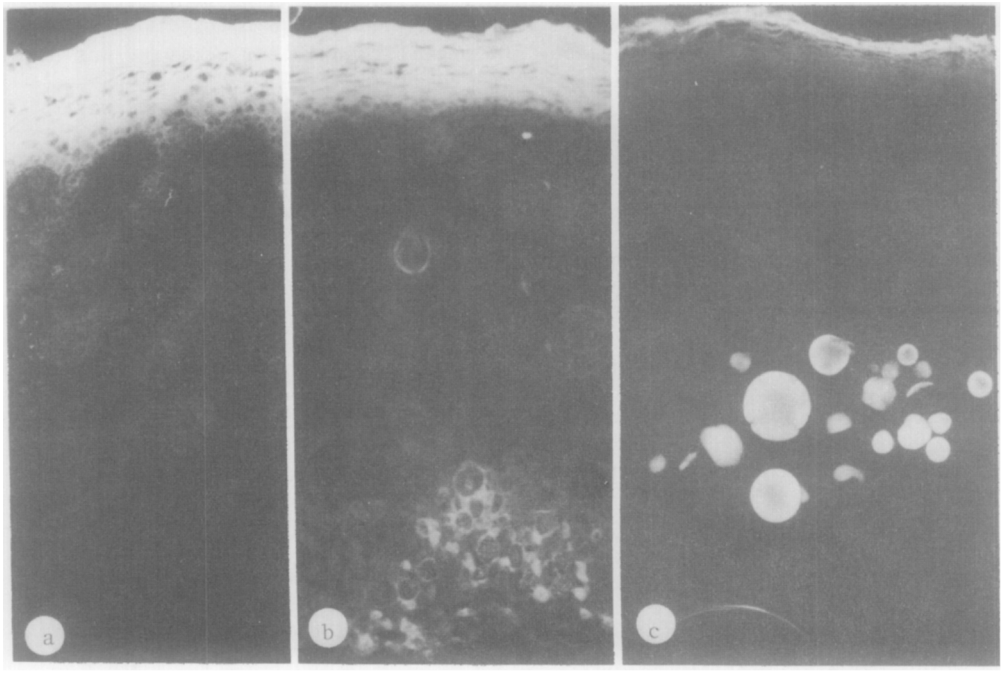

Figure 6: Reactivity of anti-EPI IgG was not detected in the dermis (weak reaction was seen in the hair) of nontreated newborn rats (a), but it was seen in the dermis immediately after injection of a mixture of cathepsin L and EPI (b) or 6 hr after injection of Sepharose 4B-conjugated papain (c). The epidermal change shown in Figure 5 occurred in (c) but not in (b), indicating that EPI detected in the dermis in (c) was of epidermal origin, whereas that in (b) was exogenous from the injection.

epidermis after injection of the complex. The supposition was tested by injection of immobilized papain. (The amount of cathepsin L obtainable was too small to use for this purpose.) In the skin taken immediately after injection of Sepharose 4B coupled papain, we detected neither changes in EPI distribu-

tion in the epidermis nor appearance of the antigen in the dermis. However, by 3 and 6 hr after injection, EPI of the epidermis decreased and the implanted Sepharose 4B showing the immunoreaction was found in the dermis (Fig. 6c). Loss of immunoreactivity from the epidermis returned to normal by 24 hr after injection of cathepsin L. This finding seems to coincide with the [^{3}H]glycine labeling study in which EPI was shown to be synthesized continuously in basal and spinous cells of the epidermis (6).

DISCUSSION

EPI inhibited casein hydrolysis by "cathepsin L" purified from rat liver by the method of Kirschke (8). Inhibitory activity to BAPNA hydrolysis by other liver fractions was also seen, but specific inhibitor activity was not determined, because no further purification of the thiol proteinases was attempted. Cathepsin L has not been purified from skin but it is highly probable that the enzyme exists in the lysosomes of epidermal cells. As lysosomal enzymes, acid phosphatase and aryl sulphatase were demonstrated in the epidermis by electron microscopy (12). A highly sensitive technique using [^{3}H]casein and [^{3}H]hemoglobin demonstrated cathepsin D and neutral proteinase activity in human skin (13) and cathepsin D has been detected in the epidermis by immunohistochemical techniques (14). Fräki and Hopsu-Havu (15) also isolated human skin cathepsin B1 and found it to be almost identical to the purified human liver enzyme. In this study we show cathepsin L evokes vessel dilatation and erythema. EPI appears to be released from the epidermis and to act as inhibitor in the dermis and epidermis during the first 3 to 6 hr. This proposal was supported by (1) the disappearance of erythema by 12 hr; (2) the considerable reduction of the inflammatory reaction after injection of EPI along with cathepsin L; and (3) detection of EPI on injected papain-Sepharose 4B into the dermis. EPI and enzyme interaction did not block the antigenic site of EPI and the complex reacted immunologically with rabbit anti-EPI IgG both *in vitro* and *in vivo*: The complex was soluble and shown to be removed from the tissue site. These findings suggest that EPI is a thiol

proteinase inhibitor which modulates dermal inflammation, and which is involved in the protective function of the skin against exogenous attack.

ACKNOWLEDGMENT

This study was supported by the National Institutes of Health grant no. AM-12433, and Biomedical Research Support Grant no. RR-05355 (Dr. Fukuyama)

REFERENCES

1. Järvinen, M., Räsänen, O., and Rinne, A. (1978) J. Invest. Dermatol. 71, 119-121.
2. Udaka, K., and Hayashi, H. (1965) Biochim. Biophys. Acta 97, 251-261.
3. Hayashi, H. (1975) International Review of Cytology, pp. 101-151, Academic Press, New York.
4. Tokuda, A., Hayashi, H., and Matsuba, K. (1960) J. Exp. Med. 112, 249-255.
5. Tokaji, G. (1971) Kumamoto Med. J. 24, 68-86.
6. Hibino, T., and Fukuyama, K. (1979) J. Invest. Dermatol. 72, 209 (abst.).
7. de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Biochem. J. 60, 604-617.
8. Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., and Bohley, P. (1977) Eur. J. Biochem. 74, 293-301.
9. Arnon, R., and Shapira, E. (1967) Biochem. 6, 3942-3956.
10. Arnon, R. (1965) Immunochem. 2, 107-114.
11. March, S.C., Parikh, I., and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152.
12. Rowden, G. (1975) J. Invest. Dermatol. 64, 4-8.
13. Levine, N., Hatcher, V.B., and Lazarus, G.S. (1976) Biochim. Biophys. Acta 452, 458-467.
14. Lazarus, G.S., and Poole, A.R. (1975) Arch. Dermatol. 111, 1150-1153.
15. Fräki, J.E., Hopsu-Havu, V.K. (1972) Arch. Dermatol. Forsch. 243, 52-66.