Inhibition of Elastase and Collagenase by Anti-Inflammatory Drugs (35145)

JOHN H. BROWN AND STANLEY H. POLLOCK

Department of Pharmacology, Louisiana State University School of Medicine, New Orleans, Louisiana 70112

Polymorphonuclear leukocytic granules contain elastase and collagenase activities, both of which may be involved in the lysis of vascular basement membranes at physiologic pH (1, 2) and, therefore, related to the pathogenesis of vasculitis (2). Abnormally high levels of collagenase are found in synoviocytes of patients with rheumatoid arthritis in tissue culture; levels of the latter correlate positively with the severity of the disease (3). Collagenase or elastase inhibitors are, therefore, of potential therapeutic utility, especially since these may attenuate the development or perpetuation of connective tissue diseases. The purpose of this report is to describe the effects of currently used anti-inflammatory agents on crystalline pancreatic elastase and *C. histolyticum* collagenase, both of which are similar to the above-mentioned leukocytic or synovial enzymes.

Materials and Methods. Achilles tendon collagen and chromatographically purified collagenase (*C. histolyticum*) were purchased from Worthington Biochemical Corporation. Twice-crystallized pancreatic elastase (porcine) and elastin–orcein were obtained from Sigma Chemical Company. Collagenase was assayed by incubating 2 μg of enzyme and 25 mg collagen in 0.025 *M* Tris–HCl buffer, pH 8.0, with or without drugs for 20 hr at 37°, followed by filtration through Whatman No. 40 filter paper. The total reaction volume was 3.0 ml; test tubes were covered with parafilm to avoid evaporation. The filtrate (0.2 ml) was assayed for liberation of amino acids by the ninhydrin procedure of Moore and Stein (4); leucine was employed as a reference amino acid. Blank (no enzyme) and control (enzyme, no drug) absorbance values were, respectively, 0.126 ± .005 ($n = 15$) and 0.611 ± .026 ($n = 22$). Collagenase (4 μg) was also assayed using 20 mg of insoluble diazotized collagen (azocoll, Calbiochem) as substrate in 2.0 ml 0.025 *M* Tris–HCl buffer, pH 8.0, 37°, for 30 min followed by addition of 3.0 ml distilled water and filtration through Whatman No. 40 filter paper. Absorbance was determined at 520 mμ. Elastase activity was measured by incubating 0.1 mg/ml enzyme in a 2.0-ml total reaction volume containing 20 mg elastin–orcein and 0.025 *M* Tris–HCl buffer, pH 8.0, with or without drugs for 60 min at 37°. Reaction was terminated by addition of 3.0 ml distilled water followed by filtration through Whatman No. 40 or 43 filter paper. Absorbance was determined at 590 mμ. Blank (no enzyme) and control (enzyme, no drug) absorbance values were, respectively, 0.027 ± .002 ($n = 3$) and 0.204 ± .006 ($n = 9$). Drug solutions were prepared by dissolution in a small amount of 1.0 *N* sodium hydroxide, dilution to slightly less than 50 ml, neutralization with 1.0 *N* hydrochloric acid, and final dilution in 0.05 *M* Tris–HCl buffer, pH 8.0, to yield the final drug solution at pH 8.0 in 0.025 *M* Tris buffer. Addition of sodium hydroxide did not appreciably aid the dissolution of cortisone acetate or dexamethasone. Thus, the latter drugs were slowly triturated into solution. The substrate and buffer were preheated to 37° prior to addition of enzyme. Appropriate blanks and controls were assayed in all experiments.

Results. Of the nonsteroidal and steroidal drugs[1] tested only phenylbutazone inhibited elastase (Fig. 1B). Steroids had no effect on the enzyme while other drugs induced activation (Table I). That the activation observed

[1] Structures of the drugs used in this study can be found in the "Merck Index", 8th Ed., Merck and Co., Inc., Rahway, New Jersey, 1968.

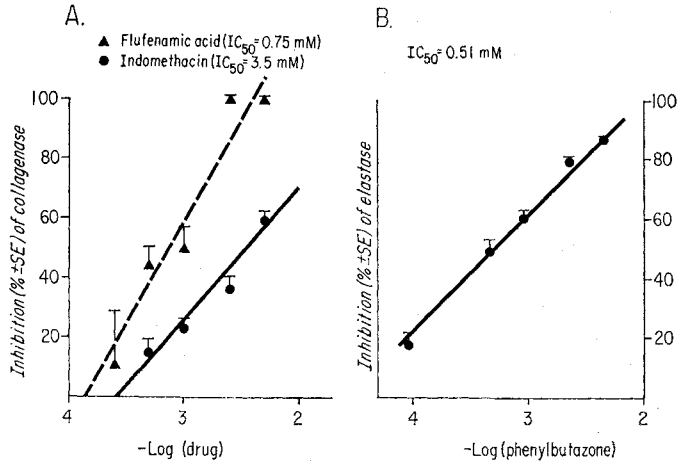

FIG. 1. A. Inhibition of collagenase by flufenamic acid and indomethacin. B. Inhibition of elastase by phenylbutazone. Each point on either curve represents the mean (±SE) of three to six experiments.

with indomethacin, acetylsalicylic acid, or flufenamic acid was not specific for non-steroidal anti-inflammatory agents is suggested by activation of elastase in the presence of structurally related compounds,

TABLE I. Activation of Elastase by Anti-Inflammatory Drugs.

Drug	Concn (m*M*)	No. expts.	% Activation[a] (± SE) of elastase
Acetylsalicylic acid	2.25	4	10 ± 13
	4.50	6	16 ± 8
	9.00	5	49 ± 17
Flufenamic acid	0.90	4	21 ± 4
	2.25	6	36 ± 10
	4.50	6	43 ± 14
Indomethacin	0.90	4	40 ± 8
	2.25	6	33 ± 6
	4.50	7	68 ± 14
Cortisone acetate	0.09	5	4 ± 8
Dexamethasone	0.09	3	(−) 3 ± 4
	0.22	3	1 ± 4
p-Aminosalicylic acid	2.25	2	20 ± 8
	4.50	3	64 ± 12
p-Chlorophenoxyisobutyric acid	2.25	2	34 ± 4
	4.50	3	69 ± 8
ε-Aminocaproic acid	2.25	1	14
	4.50	2	32 ± 7

[a] Minus sign indicates inhibition.

TABLE II. Effects of Anti-Inflammatory Drugs on Collagenase.

Drug	Concn (m*M*)	No. expts.	% Inhibition[a] (± SE)
Acetylsalicylic acid	0.25	3	7.0 ± 3.2
	0.50	6	34.0 ± 13.6
	1.00	6	28.0 ± 5.7
	5.00	7	53.5 ± 8.8
	7.50	3	46.0 ± 4.4
	10.00	7	47.0 ± 3.3
Phenylbutazone	0.50	4	6.0 ± 6.5
	1.00	6	49.0 ± 11.2
	2.50	7	(+) 3.1 ± 9.1
	5.00	7	(+)19.0 ± 10.9
Cortisone acetate	0.1	3	(+)75.0 ± 15.0
Dexamethasone	0.1	9	31.6 ± 3.3

[a] Plus signs indicate activation.

viz. p-chlorophenoxyisobutyric acid, *p*-aminosalicylic acid, or ε-aminocaproic acid (EACA).

Collagenase was found to be inhibited by acetylsalicylic acid, flufenamic acid, and indomethacin (Table II and Fig. 1A). Phenylbutazone produced variable effects which, however, were reproducible. Although dexamethasone caused only slight inhibition of enzyme activity, cortisone acetate, to the contrary, induced 75% activation. The drugs at the concentrations employed in this study had no solubilizing effects *per se* on col-

lagen or elastin–orcein. They also did not interfere with the ninhydrin assay.

When assayed in duplicate with azocoll as substrate, no inhibitory or activating influences of the nonsteroids or steroids (at least three concentrations that were effective when tendon collagen was used as substrate) could be found.

Discussion. The variable effects induced by both nonsteroidal and steroidal anti-inflammatory drugs on collagenase or elastase activities defy generalization. If, indeed, augmented collagenase levels are causally related to the severity of connective tissue diseases and accompanying deposition of granulation tissue (3), acetylsalicylic acid, indomethacin, and flufenamic acid may very well exert their therapeutic effects, at least in part, by inhibition of collagenase. The usefulness of phenylbutazone, however, cannot be explained on this basis unless one can achieve the rather singular plasma or synovial fluid concentration of 1.0 m*M*.

The activating influence of cortisone acetate on collagenase activity suggests that an augmentation of connective tissue disease would occur if collagenase is involved in degenerative changes therein and cortisone is used to treat the disease. High chronic dose levels of glucocorticoids do lead to necrotizing vasculitis (5) and aseptic bone necrosis (6). Dexamethasone, however, inhibited collagenase activity. Moreover, activating or inhibitory influences of anti-inflammatory drugs are absent when natural collagen is not used as substrate. Thus, no effect of any of the drugs studied herein was noted when azocoll was employed as substrate. This suggests that influences on collagenase noted with natural collagen as substrate are not due to blockade of the active site of the enzyme, but blockade of the interaction of the enzyme with its substrate.

With respect to elastase activity, inhibition by phenylbutazone may provide a basis for theoretical clinical usage in vasculitis or related pathologic phenomena, provided other hydrolases which can produce destructive changes in blood vessesl are also inhibited. Such hydrolases include hyaluronidase, collagenase, and other proteases. Hyaluronidase isolated from rat-liver lysosomes is not inhibited by phenylbutazone (7), while collagenase is inhibited at a singular concentration shown herein. Activation of elastase by acetylsalicylic acid, flufenamic acid, or indomethacin would preclude a basis for theoretical clinical usage if elastase is involved in the pathogenesis of vasculitis. Similarly, the lack of effect of the glucocorticoids would preclude their usage. Although pancreatic elastase was employed in the experiments reported herein, this enzyme has been shown to be quite similar biochemically to that isolated from granules of polymorphonuclear leukocytes (2).

Finally, Houck *et al.* (8) demonstrated that cortisol, prednisolone, indomethacin, or oxyphenbutazone can, after a single oral dose of 3 mg/kg, induce a significant increase in collagenase or protease activity in rat skin. Prednisone or cortisone were ineffective in the latter experiments. The inhibition of collagenase reported here could very well be counterbalanced by induction of a similar enzyme *in vivo*. A number of tissues (9–11) have been shown to possess collagenolytic activity, but characterization of these enzymes and the products of their action on collagen is very incomplete. Thus, the biophysical similarity between bacterial collagenase and collagenases of various human tissues is unknown. Interpretation of the mechanism of action of bacterial collagenase *per se* is complicated by the fact that different reaction products are formed from, for example, rat- and calf-skin collagen, presumably because rat-skin collagen contains two α_1 and one α_2 chains, while calf-skin collagen is composed of α_1, α_2, and α_3 chains (12, 13). Bacterial collagenase does differ from tissue collagenases, however, in that it attacks the collagen molecule from both ends at temperatures as low as 4–10°. Tissue collagenases split the collagen molecule by hydrolyzing a single position which results in limited breakdown (14). The pertinence of the data reported herein, therefore, using bacterial collagenase, must await further clarification of the mechanism and structure of other "collagenases." The experiments of Houck (8) also raise the question of whether the correla-

tion between augmented collagenase levels in cultured synoviocytes and the severity of rheumatoid arthritis results from the pathology or the pharmacodynamics of the situation.

Summary. *C. histolyticum* collagenase has been shown to be inhibited by acetylsalicylic acid, flufenamic acid, and indomethacin. Effects with phenylbutazone were variably manifested as inhibition or activation depending upon the concentration of drug used. Cortisone acetate induced activation of collagenase while slight inhibition was found with dexamethasone. Crystalline pancreatic elastase (porcine) was shown to be inhibited by phenylbutazone but cortisone acetate and dexamethasone had no effect. Flufenamic acid, acetylsalicylic acid, indomethacin, and some structurally related compounds caused activation of the enzyme.

1. Lazarus, G. S., Brown, R. S., Daniels, J. R., and Fullmer, H. M., Science **159**, 1483 (1968).

2. Janoff, A., and Scherer, J., J. Exp. Med. **128**, 1137 (1968).

3. Lazarus, G. S., Decker, J. L., Oliver, H., Daniels, J. R., Nultz, C. V., and Fullmer, H. M., N. Engl. J. Med. **279**, 914 (1968).

4. Moore, S., and Stein, W. H., J. Biol. Chem. **176**, 367 (1948).

5. Smyth, C. J., and Gum, O. B., Arthr. Rheum. **4**, 1 (1961).

6. Sutton, R. D., Benedek, T. G., and Edwards, G. A., Arch. Intern. Med. **112**, 190 (1963).

7. Anderson, A. J., Biochem. Pharmacol. **17**, 2253 (1968).

8. Houck, J. C., Sharma, V. K., Patel, Y. M., and Gladner, J. A., Biochem. Pharmacol. **17**, 2081 (1968).

9. Evanson, J. M., Jeffery, J. J., and Krane, S. M., J. Clin. Invest. **47**, 2639 (1968).

10. Eisen, A. Z., Jeffery, J. J., and Gross, J., Biochim. Biophys. Acta **151**, 637 (1958).

11. Riley, W. B., and Peacock, E. E., Proc. Soc. Exp. Biol. Med. **124**, 207 (1967).

12. Stark, M., and Kuhn, K., Eur. J. Biochem. **6**, 534 (1968).

13. Stark, M., and Kuhn, K., Eur. J. Biochim. **6**, 542 (1968).

14. Manahan, J., and Mandl, I., Arch. Biochem. Biophys. **128**, 6 (1968).

Received June 8, 1970. P.S.E.B.M., 1970, Vol. 135.