Biochemical Pharmacology, Vol 23, pp 2077–2081 Pergamon Press, 1974 Printed in Great Britain

INHIBITION OF HUMAN LEUCOCYTE COLLAGENASE BY SOME DRUGS USED IN THE THERAPY OF RHEUMATIC DISEASES

E. WOJTECKA-ŁUKASIK and A. M. DANCEWICZ

Department of Biochemistry, Institute of Rheumatology, Warsaw, Poland, and Department of Radiobiology and Health Protection, Institute of Nuclear Research, Warsaw, Poland

(*Received* 14 *July* 1973; *accepted* 4 *December* 1973)

Abstract—Seven anti-inflammatory agents were tested for their possible inhibition of human leucocyte collagenase. Acetylsalicylic acid, indomethacin, phenylbutazone, sanocrisin and flufenamic acid were found to inhibit collagenase at concentrations which might be present in tissue during therapy Hydrocortisone acetate and cyclophosphamide were ineffective.

LEUCOCYTE infiltration in inflammatory conditions is frequently associated with collagen degradation. It has been suggested that leucocytes are engaged in the degradation of basement membranes seen in the Arthus reaction and in the arteritis of serum sickness.[1] Janoff[2] has shown that an extract of polymorphonuclear leucocytes degrades human renal basement membranes and damages vessel walls in rabbit skin.

Lazarus *et al.*[3,4] have demonstrated that polymorphonuclear human leucocyte granules contain collagenase acting at a physiological pH. The authors have suggested that this collagenase may influence the degradation of collagen in many pathological conditions in which case the recognition of leucocyte collagenase inhibitors could contribute to the therapeutic problems of connective tissue diseases.

Brown and Pollock[5] demonstrated that anti-inflammatory agents inhibit the activity of collagenase isolated from *Clostridium histolyticum*. However, there are no reports on the effect of such agents on tissue collagenase. Since tissue collagenase differs from the bacterial enzyme,[6] we have extended our study of the properties and the role of human leucocyte collagenase[7] and report in this paper the effect of anti-inflammatory and analgetic drugs used in the therapy of rheumatoid diseases on the enzyme activity.

MATERIALS AND METHODS

Isolation and characterization of the enzyme. Collagenase was isolated from leucocytes separated from human blood. Homogenized leucocytes were mixed with dioxan. The resulting precipitate was removed by centrifugation and the supernatant was further purified by molecular-sieve filtration on a Sephadex G-200 column.[8] The most active effluent fractions were pooled and lyophylized. By this method the enzyme was purified about 40-fold. Its activity was characterized by a 35 per cent decrease in the intrinsic viscosity of the substrate collagen solution after incubation at 26° for 6 hr. The preparation had a caseinolytic activity per mg protein equivalent to 0·5–1·0 μg trypsin. Chelation of Ca^{2+} by adding an equimolar amount

of EDTA to the suspension resulted in 95 per cent inhibition of the collagenase activity. The enzyme was completely inhibited by 10^{-2} M EDTA.

Polyacrylamide gel disc electrophoresis of the collagen degradation products resulting from the action of collagenase, gave a separation pattern similar to that seen with other tissue collagenases (Fig. 1).[9]

Substrate. Calf skin collagen, soluble in 0·5 M acetic acid, was isolated and purified according to Kang *et al.*[10] The final lyophylized product was dissolved in 0·05 M acetic acid to give a concentration of 0·2%. Before use the solution was dialyzed against 0·05 M Tris–HCl buffer, pH 7·5 containing 0·005 M $CaCl_2$ and 0·2 M NaCl and clarified by centrifugation for 1 hr at 10,000 *g*.

TABLE 1. THE SUSCEPTIBILITY OF THE COLLAGEN FIBRILS TO THE ACTION OF COLLAGENASE AND TRYPSIN

Enzyme added to the system	% Collagen solubilized during 18 hr incubation
None	0
Collagenase 80 μg	23
Trypsin 600 μg	5
Trypsin 60 μg	2

The assay system contained approx. 6 mg of collagen fibrils suspended in 3 ml of 0·05 M *Tris*-HCl buffer pH 7·5 containing 0·005 M $CaCl_2$ and enzyme as indicated.

The collagen fibrils were reconstituted by thermal precipitation of the collagen solution for 12 hr at 37°. The fibrils were spun down, washed with 0·05 M Tris–HCl buffer pH 7·5 containing 0·005 M $CaCl_2$ and then resuspended in the same buffer. The susceptibility of the substrate to collagenase and trypsin action was checked and the results are presented in Table 1.

Assay of collagenolytic activity. Collagenase activity was determined by the release of soluble hydroxyproline-containing collagen degradation products in a reaction mixture which contained enzyme and reconstituted collagen fibrils. The reaction system contained 2 ml of the suspension of reconstituted collagen fibrils (approx. 6 mg of collagen), 0·5 ml of enzyme preparation (approx. 80 μg of protein) and 0·5 ml of the drug solution to be tested. After incubation for 18 hr at 37° the medium was filtered through filter paper to remove non-solubilized fibrils.

The filtrate was hydrolyzed in 6 N HCl and the content of hydroxyproline in the hydrolyzate was determined by the method of Stegemann and Stalder.[11] In each series of experiments appropriate controls and blanks were included. The enzyme used in the controls were denatured by heating for 10 min at 100°. The activity of the enzyme was expressed in μg of hydroxyproline formed. The activity in the presence and absence of anti-inflammatory drugs was compared.

Drugs. The following drugs were used in the experiments: acetylsalicylic acid, 1,2 diphenyl-3,5 dioxo-4*n*-butylpyrazolidin (Phenylbutazone), indomethacin (Indocid) and hydrocortisone acetate, all manufactured by Polfa, Poland; sodium aurothiosulphate (Sanocrisine) produced by Ferrosan, Denmark; cyclophosphamide (Endoxan) from Asta-Werke, DDR; flufenamic acid from Parke/Davis, England. Drugs not soluble in 0·05 M Tris–HCl buffer were first dissolved in a small volume

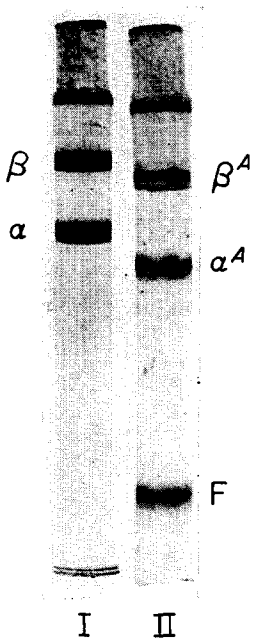

FIG. 1. Acrylamide gel electrophoresis pattern of thermally denatured enzyme-calf skin collagen. (I) Control preparation, not digested with collagenase. (II) After digestion with collagenase for 6 hr at 26°. α,β—collagen components; α^A,β^A—collagen component degradation products; F—buffer front.

of 0·1 N NaOH then diluted to slightly less than 50 ml, neutralized with 1 N HCl and diluted to 100 ml with 0·1 M Tris–HCl buffer, pH 7·5.

RESULTS AND DISCUSSION

The effect of anti-inflammatory drugs on the activity of partly purified human leucocyte collagenase is presented in Table 2. Acetylsalicylic acid, indomethacin

TABLE 2. EFFECT OF ANTI-INFLAMMATORY DRUGS ON COLLAGENASE ACTIVITY

Drug	Concentration (mM)	Inhibition (%)
Acetylsalicylic acid	0·1	4
	0·5	20
	1·0	35
	5·0	55
	10·0	63
Phenylbutazone	0·1	0
	0·5	15
	1·0	40
	5·0	68
	10·0	95
Indomethacin	0·1	8
	0·5	23
	1·0	50
	5·0	68
	10·0	85
Sanocrisine	0·1	0
	0·5	20
	5·0	30
	10·0	42
Flufenamic acid	0·01	10
	0·1	19
	1·0	25
	10·0	38
Cyclophosphamide	1·0	0
	10·0	0
Hydrocortisone acetate	0·5	0
	5·0	0

The assay system contained 2 ml of a suspension of reconstituted collagen fibrils (6 mg of collagen), 0·5 ml of enzyme preparation (80 μg of protein) and 0·5 ml of drug solution.

and phenyl-butazone were the most potent inhibitors. Sanocrisine and flufenamic acid were less potent, while hydrocortisone acetate and endoxan had no inhibitory effect on collagenase activity *in vitro*, even at a drug concentration of 10 mM.

Acetylsalicylic acid is widely used in the therapy of rheumatoid diseases owing to its analgesic action. Its therapeutic effect is attained at a concentration in blood of 15–20 mg% which is equivalent to 1 mM. *In vitro*, leucocyte collagenase activity is inhibited 35 per cent by this concentration.

The therapeutic concentration of phenylbutazone and indomethacin in blood is difficult to estimate since it is not proportional to the dose of drug administered. Ninety per cent of these drugs bind to alpha globulin proteins in serum. On the other hand the concentration of phenylbutazone in the tissue increased 2–4-fold.[12] Thus, it is difficult to judge whether the concentrations of these drugs inhibiting collagenase in *in vitro* are attained *in vivo* during therapy.

It is known that in *in vitro* systems salicylates inhibit some transaminases and dehydrogenases and at higher concentrations proteases.[13] Burns *et al.*[14] noted that phenylbutazone also inhibits oxidative phosphorylation. *In vivo*, phenylbutazone was shown to decrease the metabolism of collagen in rat skin and in granuloma tissue.[15] It seems likely therefore that the therapeutic action of phenylbutazone is connected to some extent with the inhibition of the activity of some enzymes and among them leucocyte collagenase.

Our results have shown that the activity of leucocyte collagenase is inhibited to a similar extent by acetylsalycilic acid, indomethacin and phenylbutazone at the same concentration (35–50 per cent inhibition at 1 mM concentration).

In their therapeutic action, especially in inflammatory states, all three drugs have a similar pharmacodynamic mechanism.

In this respect it is interesting to note that the action of phenylbutazone on bacterial collagenase differs from that towards leucocyte collagenase. At concentrations below 1 mM phenylbutazone inhibits, while at higher concentration activates bacterial collagenase.[5] Hydrocortisone acetate also acts differently on the two enzymes as it inhibits the activity of bacterial collagenase[5] but has no effect on the activity of leucocyte collagenase (Table 2).

Sanocrisin and flufenamic acid had less inhibitory effect on leucocyte collagenase activity *in vitro*. It is possible that the mechanism of their therapeutic action differs from that of the first group of drugs described. Flufenamic acid has a weak anti-inflammatory effect and a distinct analgesic property but its mechanism of action is not known. *In vitro*, inhibition of collagenase activity can be demonstrated only at concentrations many times higher than the concentration of this drug in blood of patients during therapy.[12] The same can be shown in case of sanocrisin, a drug containing gold.

According to Janoff[16] gold is concentrated in granules of lysosomes in phagocytic leucocytes. Collagenase is known to be located also in granules of leucocytes.[4] Although the granules concentrating gold and containing collagenase have not been identified one would expect that concentration of sanocrisin in lysosomal granules might reach the level effective also *in vivo* for inhibition of collagenase.

Peltemaa[17] has shown that gold salts inhibit the activity of typical lysosomal enzymes, such as acid phosphatase, β-glucuronidase and cathepsins in synovial fluid. This observation can be extended now to human leucocyte collagenase. The action of sanocrisin or other gold preparations is not simple. Adam[18] has shown that both *in vitro* and *in vivo* the structure of collagen becomes more stable when gold salts are applied probably due to the formation of additional cross-linking bonds between collagen molecules. It is possible that such collagen would be more resistant to the attack of collagenase *in vivo*. Such an effect would cooperate with the demonstrated inhibition of leucocyte collagenase.

In conclusion the inhibition of collagenase by drugs used in the therapy of rheumatoid diseases might be a factor in the mechanism of their therapeutic action.

REFERENCES

1. G. G COCHRANE, *J. exp. Med.* **124,** 733 (1966).
2. A. JANOFF, *Lab. Invest.* **22,** 228 (1970).
3. G. S. LAZARUS, R. S. BROWN, J. R. DANIELS and H. M. FULLMER, *Science, N.Y.* **159,** 1489 (1968).
4. G. S. LAZARUS, J. R. DANIELS, R. S. BROWN, H. A. BLADEN and H. M. FULLMER, *J. clin. Invest.* **47,** 2622 (1968).
5. J. H. BROWN and S. H. POLLOCK, *Proc. Soc. exp. Biol. Med.* **135,** 792 (1970).
6. E. A. BAUER, A. Z. EISEN and J. J. JEFFREY, *J. Invest. Dermatology* **55,** 859 (1970).
7. D. KRUZE and E. WOJTECKA, *Biochim. biophys. Acta* **285,** 436 (1972)
8. E. WOJTECKA-ŁUKASIK, Ph. Thesis, Warsaw (1973)
9. Y. TOKORO, A. Z. EISEN and J. J. JEFFREY, *Biochim. biophys. Acta* **258,** 289 (1972).
10. A. H. KANG, Y. NAGAI, K. A. PIEZ and J. GROSS, *Biochemistry* **5,** 509 (1966).
11. H. STEGEMANN and K. STALDER, *Clin. Chim. Acta* **18,** 267 (1967).
12. J. WAWRZYŃSKA-PAGOWSKA, in *Leczenie Farmakologiczne Chorób Reumatycznych* (Ed. I. KRZEMIŃSKA-LAWKOWICZOWA), p. 22. PZWL Warszawa (1972).
13. W. G. SPECTOR and D. A WILLOUGHBY, *The Pharmacology of Inflammation,* p. 72. English Universities Press, London (1968).
14. J. J. BURNS, R. K. ROSE, S. GOODWIN, J REICHENTHAL, E. C. HORNING and B. B. BRODIE, *J. Pharm. exp. Ther.* **113,** 481 (1955)
15. K. FEGELER and U GERLACH, *Z. Rheumatorsch.* **29,** 107 (1970)
16. A. JANOFF, *Biochem. Pharmac.* **19,** 626 (1970).
17. S. PELTEMAA, *Acta Rheum. Scand.* **14,** 161 (1968).
18. M. ADAM, *XII Congr. Rheum. Intern.* Abstracts No. 389. Prague (1969).