Activation of Trypsin by Anti-Inflammatory Drugs (35146)

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The role of proteolytic enzymes in inflammation is uncertain. Localized proteases may contribute to the perpetuation or attenuation of an inflammatory response. Synovial fluids of human arthritides contain active proteases (1). Van Arman (2) demonstrated an inhibition of carrageenin edema in rat hindpaws by soybean trypsin inhibitor, suggesting that trypsin or a trypsin-like enzyme(s) may be involved in the onset and development of this inflammatory response. In addition, trypsin *per se* can induce a pronounced inflammation in rat hindpaws when injected into the subplantar region (2, 3). Thus, Domenjoz and Morsdorf (3) induced inflammation in rat hindpaws with trypsin (10 μg), α-chymotrypsin (100 μg) or a bacterial protease (10 μg). This response was paralleled by an increase in free amino acids in the paws. Only extremely high dosages of acetylsalicylic acid, indomethacin, or phenylbutazone inhibited this reaction. In partial agreement with those studies, Whitehouse (4) has shown that flufenamic acid, mefenamic acid, and phenylbutazone, but not indomethacin, inhibit the esterase activity of α-chymotrypsin *in vitro*. The effect of these anti-inflammatory drugs on the proteolytic enzyme activity of trypsin and similar proteases *in vitro* has not been studied in detail. Such a study forms the basis of this report.

Materials and Methods. Crystalline trypsin (Sigma Chemical Co.) was assayed by adding 0.2 ml of enzyme (40 μg/ml) dissolved and stored in cold 0.001 *N* HCl to 1.8 ml of 0.025 *M* Tris·HCl buffer, pH 8.0 (or buffer containing the drugs under study) and 20 mg insoluble diazotized collagen (azocoll, Calbiochem). Reaction was initiated after preincubation of the buffer or drug solutions at 40° for 5 min and was terminated by addition of 4.0 ml distilled water followed immediately by filtration through Whatman No. 40 or 43 filter paper. Identical assays were employed for α-chymotrypsin (0.2 ml of 400 μg/ml), crystalline subtilisin (0.2 ml of 10 μg/ml), or thrombin (0.2 ml of 400 NIH units/ml); all enzymes were obtained from Sigma Chemical Co. When α-*N*-benzoyl-DL-arginine *p*-nitroanilide·HCl (BAPNA) was used as substrate, the reaction mixture contained 3.6 ml drug solution (in 0.025 *M* Tris·HCl buffer, pH 8.0) or buffer, 2.0 ml substrate (1 mg/ml dissolved by warming to 85–90° in buffer) and 0.4 ml trypsin; reaction was terminated by adding 2.0 ml of 0.2 *N* HCl. Hydrolysis of azocoll was expressed as the increase in absorbance (OD) at 520 mμ and hydrolysis of BAPNA as increase in absorbance at 383 mμ. A Bausch and Lomb Spectronic 20 colorimeter was used for absorbance measurements. Drug solutions were prepared by dissolution in a small volume of 1.0 *N* sodium hydroxide, dilution to slightly less than 50 ml, neutralization with 1.0 and/or 0.1 *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 and stirred into solution. Appropriate blanks and controls were assayed in all experiments.

Results. Activation of tryptic hydrolysis of azocoll by acetylsalicylic acid and indomethacin was apparent within 3 min after addition of enzyme and did not diminish with time of incubation (Fig. 1). The concentrations of these and other anti-inflammatory drugs required to produce a 50% increase in activity after a 15-min incubation period (Table I) were similar in degree and thus did not satisfactorily parallel rank order of

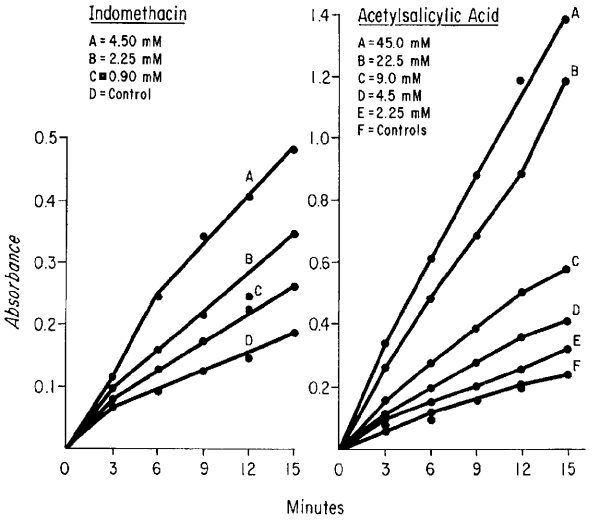

FIG. 1. Activation of azocoll hydrolysis by trypsin.

clinical potency as manifested in other *in vitro* or *in vivo* systems (5). Moreover, structurally related compounds, *p*-aminosalicylic acid or *p*-chlorophenoxyisobutyric acid, and ε-aminocaproic acid (EACA), all containing acid functions similar to those in the anti-inflammatory drugs, induced a similar degree of activation, Table I. Steroidal antiarthritic drugs, cortisone acetate and dexamethasone, were ineffective.

Using calcium-activated trypsin much less drug-induced activation of the enzyme was obtained, Table II. Nonetheless activation in excess of that produced by 1.0 m*M* calcium was observed with the three anti-inflammatory drugs studied.

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

Compound	AC_{50}[a] (m*M*)
Acetylsalicylic acid	3.0
Flufenamic acid	2.8
Indomethacin	1.2
Phenylbutazone	1.2
Cortisone acetate	Ineffective
Dexamethasone	Ineffective
p-Aminosalicylic acid	1.2
p-Chlorophenoxyisobutyric acid	1.8
ε-Aminocaproic acid	1.2

[a] Concentration of drug that induces 50% increase in enzyme activity in 15 min interpolated from a plot of % activation *vs* log drug concentration. Concentration of drugs studied were 0.9, 2.25, and 4.5 m*M*.

TABLE II. Activation of Trypsin in the Presence of Calcium.

Compound	Conen (m*M*)	OD (520 mμ)	% Change in enzyme activity
None	—	0.745[a]	—
Acetylsalicylic acid	2.25	0.725	— 2 (+ 37)[b]
	4.50	0.785	+ 5 (+ 72)
	9.00	0.845	+13 (+143)
Indomethacin	0.90	0.875	+17 (+ 37)
	2.25	0.877	+18 (+ 62)
	4.50	0.985	+32 (+118)
Phenylbutazone	0.90	0.735	— 1 (+ 36)
	2.25	0.923	+24 (+101)
	4.50	1.22	+67 (+231)

[a] In the absence of 1 m*M* Ca^{2+}, the OD was 0.200 ± 0.002. Values represent the mean of two experiments; 15-min incubation.

[b] Values in parentheses indicate activation in the absence of Ca^{2+}.

With BAPNA as substrate very little enzyme activation was observed, Table III. Cortisone acetate and dexamethasone again produced no change in enzyme activity.

In contrast to trypsin, hydrolysis of azocoll by subtilisin was inhibited slightly by the anti-inflammatory drugs and structurally related compounds (Table IV). Only the

TABLE III. Activation of Trypsin Using BAPNA as Substrate.

Compound	Concn (m*M*)	Activation[a] (%)
Acetylsalicylic acid	1.5	12
	3.0	21
	6.0	28 (72)[b]
ε-Aminocaproic acid	1.5	16
	3.0	23 (122)
p-Aminosalicylic acid	1.5	16
	3.0	25 (72)
p-Chlorophenoxyisobutyric acid	1.5	12
	3.0	15 (48)

[a] Mean of two experiments; 30-min incubation.

[b] Values in parentheses indicate activation of azocoll hydrolysis by 4.5 m*M* acetylsalicylic acid or 2.25 m*M* *p*-aminosalicylic acid, ε-aminocaproic acid, or *p*-chlorophenoxyisobutyric acid. Dexamethasone and cortisone acetate did not affect hydrolysis of BAPNA at 0.06 m*M* concentrations.

effects of the highest concentration of drugs are listed.

Thrombin-induced hydrolysis of azocoll was likewise not affected by acetylsalicylic acid (22.5 m*M*), flufenamic acid (4.5 m*M*), indomethacin (4.5 m*M*), or phenylbutazone (4.5 m*M*).

When chymotrypsin was assayed with azocoll as the substrate in the presence of 0.9, 2.25, or 4.5 m*M* drugs, variable inhibitory or activating effects of less than 18% were obtained. Cortisone acetate (0.09 m*M*) and dexamethasone (0.09 m*M*) were ineffective.

When assayed in triplicate in 0.30 *M* sodium phosphate buffer, pH 7.5 (20 min, 40°, 20 mg azocoll, 2.0-ml volume), trypsin (8 μg/ml) was not affected by 5.0 m*M* acetylsalicylic acid or flufenamic acid (dissolved in 0.3 *M* phosphate buffer); phenylbutazone yielded slightly less than 30% activation.

TABLE IV. Inhibition of Subtilisin by Anti-Inflammatory Drugs.

Compound	Concn (m*M*)	Inhibition[a] (%)
Acetylsalicylic acid	9.0	—29
Flufenamic acid	4.5	—23
Indomethacin	4.5	—20
Phenylbutazone	4.5	—34
p-Aminosalicylic acid	4.5	—12
p-Chlorophenoxyisobutyric acid	4.5	—20
ε-Aminocaproic acid	4.5	—20

[a] Mean of two separate experiments. Cortisone acetate (0.09 m*M*) and dexamethasone (0.09 and 0.22 m*M*) had no effect; 15-min azocoll hydrolysis.

Discussion. The results reported herein do not permit generalizations about the effects of anti-inflammatory drugs on "proteases"; except that the activity of trypsin was the only enzyme affected to any great extent. Slight and variable effects were noted with other proteolytic enzymes, namely, subtilisin, thrombin, and chymotrypsin. These enzymes are similar to trypsin in that they are inhibited by diisopropylfluorophosphate, have molecular weights of 20,000–30,000 and act optimally at alkaline pH values of 8.0–10.0 (6–8).

In contrast to inhibition of hydrolysis of the small molecular weight synthetic esterase substrate acetyltyrosine ethyl ester (ATEE) by chymotrypsin (4), little or no effect on hydrolysis of a large, insoluble protein substrate (azocoll) was produced by the anti-inflammatory drugs. Using ATEE, competition between drug and substrate of similar molecular size prevents initial binding of substrate, its acylation, and subsequent hydrolysis (4). Thus, it must be concluded that with a more natural substrate, such as azocoll, neither the overall structure of chymotrypsin nor its active center is influenced when bound to azocoll.

The activation of tryptic hydrolysis by nonsteroidal anti-inflammatory drugs in Tris buffer but not phosphate buffer may be mediated by the Tris–hydroxymethyl groups of Tris (in salt complex, *viz.*, carboxylate–ammonium ion interaction, with the acidic drugs). Such groups may mimic the serine hydroxymethyl moiety of the active center when bound to the enzyme via the acidic drugs or analogs studied. Alternatively, this complex when bound to the enzyme could lead to a better fixation of the enzyme onto the surface of azocoll. Such an effect would not be observable with a small molecular weight, soluble substrate, which fits directly into or near the active center. In agreement with this hypothesis, BAPNA hydrolysis was increased much less than that of

azocoll in the presence of the anti-inflammatory drugs.

Since inflammation induced in rat hind-paws by trypsin or other proteases probably involves hydrolysis of peptides and proteins (3), blockade of this hydrolysis by anti-inflammatory drugs cannot be explained by inhibition of trypsin since activation was observed. It is more likely that the well-known capillary-stabilizing effects of the anti-inflammatory drugs account for their rather weak inhibitory effects in trypsin-induced edema.

Summary. Trypsin in the absence of calcium is activated as much as 575% by non-steroidal anti-inflammatory compounds when assayed with azocoll as substrate. Using a small synthetic compound, *N*-benzoyl-DL-arginine *p*-nitroanilide, as substrate much less activation was noted. Chymotrypsin was variably affected by less than 18% while no effect of the drugs was found on thrombin. Subtilisin was found to be inhibited slightly (less than 30%).

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