Differential Effects of Anti-Inflammatory Agents on Lysosomal Cysteine Proteinases Cathepsins B and H from Rat Spleen

Kenji YAMAMOTO, Osamu KAMATA* and Yuzo KATO

Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki 852, Japan
*Department of Pharmacology, Kyushu University School of Dentistry, Fukuoka 812, Japan

Accepted March 26, 1984

Abstract—The reactivity and specificity of commonly used anti-inflammatory agents with lysosomal cysteine proteinases cathepsins B and H purified from rat spleen have been investigated. Of the different agents tested, flufenamic acid and indomethacin were known to be potent inhibitors of cathepsin B. A half-maximal inhibition of the activity of cathepsin B was achieved at drug concentrations of $7.6 \times 10^{-5}$ M of flufenamic acid and $4.0 \times 10^{-4}$ M of indomethacin. The inhibition by these two agents was of a non-competitive type with benzyloxy-carbonyl-phenylalanyl-arginine-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) as a substrate. The maximal inhibitory potencies of these agents for the cathepsin B activity were observed at pH 7.0. At pH values between 4.5 and 6.5, the inhibitory potencies were less than at pH 7.0. No preincubation time was needed for the reaction between these agents and cathepsin B. In contrast, cathepsin H was not affected by these two drugs even at the drug concentration of $10^{-3}$ M at pH values between 4.5 and 8.0. Other anti-inflammatory agents including aspirin, sodium salicylate, phenylbutazone and prednisolone were found to be poorly or scarcely inhibitory for both cathepsins B and H.

The inflammatory condition involves a complex series of cellular responses. An integral part of the inflammatory responses is the tissue destruction by lysosomal proteolytic enzymes. Because of the potency of lysosomal proteinases, it has been speculated that inhibitors of these enzymes might be useful as therapeutic agents. Although it is generally agreed that inhibition of prostaglandin synthesis is an important aspect of anti-inflammatory therapy, few anti-inflammatory agents inhibiting lysosomal proteinases have been described, due in large part to the difficulty in determining the activities of these enzymes in the presence of anti-inflammatory drugs. As both pathways of the biosynthesis of prostaglandins and the tissue breakdown by proteolytic attack represent the major inflammatory responses, dual inhibitors of these pathways may have some advantages in the treatment of inflammatory diseases. The present work was undertaken to investigate the possibility that some of the drugs known to have an inhibitory effect on the biosynthesis of prostaglandins might have the ability to inhibit lysosomal proteinases.

Both cathepsins B and H are typical lysosomal cysteine (thiol) proteinases (1). Although these two enzymes are found to be apparently different enzymes (2–7), they are similar in several respects (6–8) such as their amino acid sequences, optimum pH (B, 6.0; H, 6.5–7.0), molecular weights (B, 29,000; H, 26,000–28,000), catalytic efficiencies with $\alpha$-N-benzoyl-DL-arginine-2-naphthylamide (BANA) and requirement of cysteine and EDTA for their full activation. In this paper, we examine and compare the effects of commonly used anti-inflammatory agents on cathepsins B and H purified from rat spleen by using the excellent substrates Z-Phe-Arg-MCA and L-arginine-4-methyl-7-coumarylamide (Arg-MCA), respectively, for the assays free from interference by these drugs.
Materials and Methods

Materials: Z-Phe-Arg-MCA and Arg-MCA were obtained from the Protein Research Foundation, Osaka, Japan. All other chemicals were of reagent grade from various commercial sources.

Enzyme preparation: The two lysosomal cysteine proteinases cathepsins B and H were purified from rat spleen as previously described (6, 7). As the rat spleen cathepsins B and H consist of two and three iso-enzymes, respectively, the enzymes used for the present study were the respective major forms (form I for cathepsin B and form II for cathepsin H).

Enzyme assay: The activities of cathepsins B and H were measured by fluorimetric methods using Z-Phe-Arg-MCA and Arg-MCA as substrates, respectively. Both assays were performed by a modification of the method of Barrett (9). Except where otherwise noted, the assay buffers were 100 mM sodium phosphate buffer containing 1 mM EDTA and 5 mM cysteine added freshly before use (at pH 6.0 for cathepsin B and at pH 6.5 for cathepsin H). Both substrates dissolved in dimethylsulfoxide as 10 mM solutions were diluted with 0.1% Brij 35 to 0.02 mM. The agents to be tested were each dissolved in dimethylsulfoxide at concentrations that required the addition of 10 to 0.5 ml enzyme solutions to achieve the desired concentrations. This concentration of dimethylsulfoxide does not inhibit the enzymes. The enzyme solutions (0.5 ml, 200 mM sodium phosphate buffer containing 2 mM EDTA and 10 mM cysteine, pH 6.0 or 6.5) were preincubated at 25°C for 10 min with or without the agent (10 μl) before adding 0.5 ml of the substrate solutions to start the reaction. The reaction was carried out at 40°C for 10 min and stopped by addition of 1 ml of 100 mM sodium acetate buffer, pH 5.0, containing 100 mM sodium iodoacetate. The liberated product, 7-amino-4-methylcoumarin, was estimated by measurement of the fluorescence intensity at 460 nm using a Hitachi fluorescence spectrophotometer, model 650-40. The excitation wavelength was 380 nm. One unit of the enzyme activity was defined as the amount of each enzyme required to release 1 μmol of 7-amino-4-methylcoumarin per min. The percent inhibition was calculated on the basis of control mixtures which were incubated in the absence of agents followed by addition of them after terminating the reaction. The inhibition assays were also conducted by changing preincubation conditions, e.g., pH, temperature and time.

Data analysis: The kinetic parameters of the inhibited enzyme were determined by using five different substrate concentrations ranging from 10 to 160 μM. The Lineweaver-Burk equation was used to analyze the type of inhibition and to determine Ki values. Data are presented as the means of at least two pairs of duplicates.

Results

Titration of the activities of cathepsins B and H with anti-inflammatory agents: The 6 anti-inflammatory agents listed in Table 1 were employed as effectors of the activities of cathepsins B and H purified from rat spleen in experiments with Z-Phe-Arg-MCA and Arg-MCA as substrates, respectively. The enzyme concentrations were kept constant in all experiments (3.4×10^-13 M for cathepsin B and 5.8×10^-13 M for cathepsin H). Table 1 demonstrates that flufenamic acid and indomethacin inhibit the cathepsin B activity at the concentration of 10^-4 M. The rank order of inhibitory potencies of the drugs tested for cathepsin B was flufenamic acid > indomethacin > phenylbutazone. Aspirin, sodium salicylate and prednisolone were relatively ineffective inhibitors under these conditions. On the other hand, none of the agents inhibited the cathepsin H activity at drug concentrations up to 10^-4 or 10^-3 M. As shown in Fig. 1, the inhibitory potencies of flufenamic acid and indomethacin for cathepsin B activity were in a dose-dependent manner. A half-maximal inhibition of the cathepsin B activity was observed at drug concentrations up to 10^-4 or 10^-3 M. As shown in Fig. 1, the inhibitory potencies of flufenamic acid and indomethacin for cathepsin B activity were in a dose-dependent manner. A half-maximal inhibition of the cathepsin B activity was observed at drug concentrations of 7.8×10^-15 M for flufenamic acid and 3.8×10^-4 M for indomethacin.

Effect of sulfhydryl compounds: To eliminate the possibility that the inhibition of cathepsin B by flufenamic acid and indomethacin might be ascribed to an effect of these drugs on the assay component
Table 1. Effect of anti-inflammatory agents on lysosomal cysteine proteinases cathepsins B and H purified from rat spleen

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (M)</th>
<th>% of activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cathepsin B</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Aspirin</td>
<td>$10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>95</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>$10^{-9}$</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$</td>
<td>105</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>$10^{-5}$</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>70</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>$10^{-6}$</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>63</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>$10^{-6}$</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>41</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>$10^{-5}$</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>90</td>
</tr>
</tbody>
</table>

The purified cathepsins B ($3.4 \times 10^{-13}$ M) and H ($5.8 \times 10^{-13}$ M) were preincubated with each agent at respective concentration for 10 min at 25°C, before the 10 min incubation with Z-Phe-Arg-MCA and Arg-MCA as substrates, respectively, at 40°C. The values are means of at least three determinations.

Fig. 1. Dose-response relationship for the inhibitory activities of flufenamic acid (A) and indomethacin (B) for rat spleen cathepsins B and H. The purified cathepsin B (---) or H (---) was preincubated with different concentrations of the agents at 25°C for 10 min. The residual activities were measured as described in "Materials and Methods" and are expressed as percentages of the respective controls which were assayed in the absence of agents. Each point represents the mean value of three determinations.

Fig. 2. Effect of cysteine on the inhibition of the cathepsin B activity by flufenamic acid and indomethacin. The purified cathepsin B was preincubated at 25°C for 10 min in the presence of different concentrations of cysteine with (-- -- --) or without (-----) $7.8 \times 10^{-6}$ M of flufenamic acid (○) and $2.8 \times 10^{-4}$ M of indomethacin (●). The residual activities were measured and are expressed as percentages of the control activity which was determined at the cysteine concentration of 5 mM in the absence of each agent. Each point represents the mean value of three determinations.
cysteine rather than the enzyme, the inhibitory potencies were investigated by addition of increasing concentrations of cysteine. As shown in Fig. 2, the inhibitory effects of these two drugs on the cathepsin B activity were not affected by cysteine concentrations between 2 and 100 mM. Similar results were obtained with other sulfhydryl compounds such as dithiothreitol, β-mercaptoethanol and reduced glutathione (data not shown). These results indicate that the inhibition by these two agents is exerted through a drug-induced modification of the conformation of cathepsin B since these agents appear to have no effect on the small molecular weight substrate Z-Phe-Arg-MCA.

**Effect of temperature:** The effects of anti-inflammatory agents on the activities of cathepsins B and H were measured at 4°C, 25°C and 37°C. The change of temperature during preincubation had a rather small effect on the results obtained in Table 1 and Fig. 1. Accordingly, subsequent experiments were always carried out at 25°C.

**Effect of pH:** The inhibitory potencies of flufenamic acid and indomethacin for the cathepsin B activity were studied at various pH values (100 mM sodium acetate, pH 4.5–5.5; 100 mM sodium phosphate, pH 6.0–8.0). Figure 3 shows that the maximal inhibition of cathepsin B by flufenamic acid (2.8×10^{-4} M) and by indomethacin (5.6×10^{-4} M) was observed at pH 7.0. At pH values below 6.5, the inhibitory potencies by these two agents were less than at pH 7.0. At pH values over 7.5, it was difficult to determine the inhibitory potencies since cathepsin B is very unstable above pH 7.5. In contrast, the cathepsin H activity was not affected at pH values between 4.5 and 8.0, not only by these two agents, but also by the...
Other drugs tested.

Effect of preincubation time: The effect of preincubation time on the inhibitory potencies of flufenamic acid and indomethacin for the cathepsin B activity was investigated. Cathepsin B was preincubated with these agents up to 40 min at 37°C at pH 5.0 or 7.0, and the remaining activity was measured by the usual assay. Data given in Fig. 4 show that the inhibition of cathepsin B by these agents occurs immediately upon addition of them to the enzyme solution. At pH 5.0, no significant change in the inhibitory potencies of these two agents was observed up to 40 min. At pH 7.0, the inhibitory activities by both agents increased gradually up to 10 min after preincubation and attained constant values.

Kinetic studies: The kinetics of inhibition of cathepsin B by flufenamic acid and indomethacin were studied. The enzyme activity at various concentrations of Z-Phe-Arg-MCA as a substrate was measured in the absence and presence of fixed concentrations of these agents. As shown in Fig. 5, the Lineweaver-Burk reciprocal plots showed that the inhibition of cathepsin B by these two agents were non-competitive and that the inhibition constant (K_i) values were 9×10^{-5} M for flufenamic acid and 1.4×10^{-4} M for indomethacin. The K_m value for this substrate was 2.86×10^{-4} M.

Discussion

The present findings showed that two non-steroidal anti-inflammatory agents, flufenamic acid and indomethacin, inhibited the cathepsin B activity in vitro in a dose-dependent manner and that the inhibition of the enzyme by these two agents is a non-competitive type with respect to the small synthetic substrate Z-Phe-Arg-MCA. The K_i values obtained by Lineweaver-Burk plots were in good agreement with those by Dixon plots (data not shown). The inhibition of cathepsin B by these two agents was exerted through a modification of conformation of the enzyme rather than at the active site of the enzyme. This conformational change of the enzyme is probably due to the binding of these agents to the protein lysyl ε-amino groups. This contention may be supported by the observations that 2,4,6-trinitrobenzene sulfonic acid (TNBS) strongly inhibited the cathepsin B activity in the same way as flufenamic acid and indomethacin did and that there existed additional effects between TNBS and these agents (data not shown). TNBS is known as a high affinity compound for the lysyl ε-amino groups of proteins (10). However, since salicylates and phenylbutazone are thought to bind to protein lysyl ε-amino groups (10), flufenamic acid and indomethacin do not exert all of their inhibitory effects on cathepsin B through binding the ε-amino groups. The inhibition of cathepsin B by these agents is due to binding to the protein lysyl ε-amino groups and to an additional, as yet unknown, effect.

Although lysosomal cysteine proteinases are thought to play an important role in a number of physiological and pathological events in inflammatory sites, little information is available concerning the mechanisms of inflammatory tissue destruction and the function of these enzymes. At present, we cannot decide which proteinase or proteinases are more responsible for the tissue breakdown at inflammatory sites. Granting that cathepsin

![Fig. 5. Lineweaver-Burk double reciprocal plots of the cathepsin B activity at different substrate concentrations. Cathepsin B was preincubated at 25°C for 10 min at pH 6.0 with or without 7.8×10^{-5} M flufenamic acid (O) and 2.8×10^{-4} M indomethacin (Δ). Then the residual activities were measured with different concentrations of Z-Phe-Arg-MCA. Closed circles (●) represent the uninhibited enzyme reaction. Data points presented are the means of at least two pairs of duplicates.](image-url)
B catalyzes the tissue proteolysis in injury and inflammatory states, the inhibition of this enzyme appears to be important from the therapeutic point of view. As both flufenamic acid and indomethacin are strong inhibitors for the prostaglandin-forming process (11), these agents appear to have dual advantages in the treatment of inflammatory diseases. In contrast, aspirin and sodium salicylate which are also inhibitors for the biosynthesis of prostaglandins had much less or no effects on the cathepsin B activity under these conditions. These data suggest the variation in therapeutic effectiveness of these anti-inflammatory agents. Interestingly, another lysosomal cysteine proteinase, cathepsin H, was not inhibited by any of the anti-inflammatory agents tested. According to Takio et al. (8), there exists a striking homology in the amino acid sequences between rat liver cathepsins B and H. The lack of inhibitory potencies of flufenamic acid and indomethacin for cathepsin H probably represents the differences between these two enzymes in drug-induced conformational changes and in the accessibility of the substrates to the catalytic sites of enzymes.

From the present observation, anti-inflammatory agents may be of value in examining their effects on other lysosomal proteinases, e.g., cathepsins D and E as an aspartyl proteinase, cathepsins N, L and T as a cysteine proteinase and cathepsin G as a serine proteinase. Studies along this line are in progress in our laboratory.

References


