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Inhibition of Human Neutrophil Elastase by NSAIDs and Inhibitors, and Molecular Pharmacological Mechanism of the Inhibition

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ABSTRACT

Human neutrophil elastases (HNElastase, EC 3.4.21.37), a causative factor of inflammatory diseases, are regulated by plasma proteinase inhibitors, alpha-proteinase inhibitor and alpha$_2$-macroglobulin. Under certain pathological conditions, however, released enzymes or abnormal function of inhibitors may cause various inflammatory disease. NSAIDs have been clinically applied for treatment of inflammatory diseases. Inhibition of cyclooxygenase is a known mechanism of action of NSAIDs in the treatment of inflammatory disease. In *in vitro* experiments, HNElastase was inhibited by naproxen, phenylbutazone, and oxyphenbutazone, but ibuprofen, ketoprofen, aspirin, salicylic acid, and tolmetin did not inhibit elastase. HNElastase was also inhibited by chelating agents, EDTA & EGTA, and tetracyclines. Removal of divalent metal ions by EDTA caused inhibition of elastase, and reconstitution of the metal ions recovered the enzyme activity to a certain level. Frequencies and contours in the Raman spectra of various conditions of human neutrophil elastase undergo drastic changes upon partial removal and/or reconstitution of calcium and zinc ions. The metal ion content dependent activities and change of the contour of the Raman spectrogram suggest us that the mechanism of action of a chelator or chelator-like agents on neutrophil elastase may be related to the conformational change at/or near the active site, especially $-C=O$ radical or $-COOH$ radical.

<Abbreviations>

SEC: sample after size exclusion chromatography; IEC: sample after ion exchange chromatography; PMDElastase: partial metal depleted elastase, CRElastase: Calcium reconstituted elastase, ZRElastase: Zinc reconstituted elastase, ZCRElastase: Zinc and calcium reconstituted elastase, NSAIDs: Nonsteroidal anti-inflammatory drugs.

Key Words: Inhibition of neutrophil elastase, NSAIDs, Chelators, Conformational change, Raman shift

INTRODUCTION

Human neutrophil elastase (HNE, EC 3,4,21,37) is known as an important factor in inflammatory diseases such as emphysema and rheumatoid arthritis (Janoff, 1972a, Starkey, 1980, Oholson & Olsson, 1977, Fritz *et al.*, 1986). The enzymatic activity of human neutrophil enzymes including elastase, cathepsin G, and collagenase are usually regulated by plasma proteinase inhibitors, alpha$_1$-proteinase inhibitor and alpha$_2$-macroglobulin (Salvesen *et al.*, 1980, Cohen, 1983). Under certain pathological conditions, however released enzymes or the abnormal function of inhibitors may cause various inflammatory diseases (Janoff, 1972a, Starkey, 1980, Oholson & Olsson, 1977, Fritz *et al.*, 1986). Nonsteroidal anti-inflammatory

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drugs (NSAIDs), so call, aspirin-like drugs, are known as clinically effective agents for treatment of inflammatory diseases, especially in the treatment of rheumatoid arthritis (Vane *et al.*, 1995, Insel, 1996). One of the known mechanisms of action of anti-rheumatic action of aspirin-like drugs is the inhibition of prostaglandin synthetases, cyclooxygenase (Vane, 1971). It is difficult to ascribe the anti-rheumatoid effects of aspirin-like drugs solely to the inhibition of prostaglandin synthesis (Insel, 1996). Therefore, it has been proposed that salicylate and certain other NSAIDs can directly inhibit the activation and function of neutrophils, perhaps by the inhibition of membrane-associate processes (Abramson *et al.*, 1989), or the inhibition of neutrophil migration by these drugs (Kim *et al.*, 1994). There have been several interesting reports that are related to the inhibition of neutrophil enzymes; i.e., ① certain antibiotics, including methicillin, cefamandole and oxytetracycline, inhibited human neutrophil elastase (Ghim *et al.*, 1989, Kim *et al.*, 1993), ② chelating agents inhibited leukocyte elastase (Kang, 1985, 1988, Tyagi *et al.*, 1991). ③ several NSAIDs inhibit the activity of cathepsin G which belongs to serine protease (Bae *et al.*, 1991). There was no report on the relationship between inhibition of human neutrophil elastase and the molecular conformational change after the binding of some chelators or NSAIDs. Therefore, in this paper, we report the *In vitro* experimental results of the inhibition of human neutrophil elastase by inhibitors and NSAIDs and molecular pharmacological mechanism of the inhibitions. We also report the technical possibility of the application of the Raman shift in *In vitro* study on molecular conformational change by drug-receptor interaction.

MATERIALS AND METHODS

Materials

N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (SAPNA), N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (SANA), naproxen, phenylbutazone, oxyphenbutazone, ibuprofen, ketoprofen, aspirin, salicylic acid, tolmetin, EDTA, EGTA, and tetracycline were Sigma products. Tris, glycine, and sodium dodecyl sulfate were Bio-Rad products. Spectrapor 2 dialysis membrane was the product of Spectrum Medical Industries INC, L.A., U.S.A. and ultrafiltration membrane was the product of Amicon Co.. All other chemicals were of the highest quality obtainable.

Methods

Purification of human neutral proteases: The method of purification of human neutrophil elastase used was that modified by Kang and Baugh & Travis (Kang *et al.*, 1987, Baugh *et al.*, 1976). In brief, Human neutrophils were separated from whole blood (acquired from blood bank) by Ficoll-hypaque step gradient centrifugation at 200 g for 40 minutes and washed three times with 50 mM Tris-Cl buffer, pH 7.3. Separated neutrophils were suspended in 0.5M Tris-Cl buffer, pH 7.3, containing 1.5 M NaCl and 0.05M $CaCl_2$, then homogenized with Polytron at 4℃ and centrifuged at 30,000 g for 60 minutes. The supernatant was then collected and the extract was chromatographed through an AcA54 gel filtration column equilibrated with 50 mM of Tris-Cl buffer containing 150 mM of NaCl. Elastase rich fractions were pooled, concentrated, and dialyzed against 50 mM of NaAc, pH 4.5, containing 150 mM NaCl. Ultrogel purified elastase was chromatographed again with CM-Sephadex ion exchange column equilibrated with 50 mM NaAc buffer, pH 4.5, containing 150 mM of NaCl. Bound protein was eluted by linear salt gradient (0.15~0.7 M). Purified elastase by ion exchange chromatography showed four bands of isozyme molecules in the SDS-PAGE (Baugh & Travis, 1976).

Metal analysis: Elemental composition was assayed by energy dispersive X-ray fluorescence spectrometry (EDXRF) system (Sky-peck *et al.*, 1981) and X-ray spectral response was measured and compared to standard elements.

Elastase assay: Elastase assay was carried in 200 ul of reaction medium containing 60~150 mM NaCl, 50 mM $CaCl_2$, pH 7.3 with or without inhibitor. After the substrate, SANA, was added to the reaction mixture, then it was incubated at 37℃ for an appropriate period usually 10~40 minutes). Elastase activity was measured spectrophotometrically at 410 nm by monitoring the concentration of liberated p-nitroaniline, using a Ti-

tertek Multiskan Spectrophotometer (MCC/340, Flow laboratories, Switzerland). Percent inhibition was determined by $100^{X}[1-(V_{\text{inhibitor present}}/V_{\text{inhibitor absent or control}})]$.

Preparation of partial-metal-depleted-Elastase (PMDElastase): Purified HNElastase was placed in the Spectrapor 2 membrane and dialyzed against 10~100 mM (depend on the design of the experiment) of Tris-EDTA or Tris-EGTA, pH 7.3, overnight, then dialyzed against pure water or 5 mM of Tris-Cl, pH 7.3, with change of water or buffer three times, for 6~12 hours.

Preparation of partial-metal-reconstituted-HNElastase: PMDElastase was placed in the Spectrapor 2 membrane and was dialyzed against 20 mM of Tris-buffer containing 5 mM of Ca^{++} or Zn^{++} or Ca^{++} and Zn^{++} overnight, then dialyzed against 5 mM of Tris-Cl, pH 7.3, with change of buffer three times, for 6 hours.

Detection of molecular conformational change with Raman spectrophotometer: The sample was placed in the middle of the capillary Pyrex tube and scanned by Laser Raman Spectrophotometer (Laser Raman Spectrophotometer System: Sepex 1403, 0.85m double spectrometer equipped by RCA-C 31034 phototube and DM 3000 software). A 514.5-nm excitation line of an argon ion laser was used. Data were collected at 1 cm^{-1} intervals with 0.3^{-S} integration time and 4 cm^{-1} spectral slit width.

RESULTS AND DISCUSSION

Elemental analysis

Table 1 is the result of elemental analysis. Crude extract (data not shown), SEC-HNElastase, and IEC-Elastases contained high quantities of Fe^{++}, Ca^{++} and Zn^{++} ions. The quantity of Fe^{++} was highest in the extract and decreased as the purity of HNElastase increased. However, quantities of both Ca^{++} and Zn^{++} ions proportionally increased with the increased purity of HNElastase. The quantity of Ca^{++} ion dramatically decreased when size exclusion chromatography was performed with high concentration of salt in the mobile phase (HS-SEC). The quantity of Zn^{++} ions in the sample purified by HS-SEC, however, stayed at the same level as that of IEC prepared elastase.

Table 1. Elemental analysis

	Mass ratio(ug/g protein)		
Element	SEC	IEC	After dialysis in EGTA
Ca^{++}	776.3	5181.7	102.8
Fe^{++}	485.2	704.3	24.6
Cu^{++}	34.9	25.0	4.0
Zn^{++}	103.8	5806.4	13.0

SEC: sample after size exclusion chromatography; IEC: sample after ion exchange chromatography; EGTA dialysis: Purified HNElastase was dialyzed against 100 mM of Tris-EGTA.

Table 2. Inhibition of HNElastase by different concentrations of NSAIDs

Drugs	Relative enzyme activity at different concentrations of drugs		
	0(mM)	0.5 (mM)	1 (mM)
Control	0.48	—	—
Ibuprofen		0.53	0.55
Ketoprofen		0.497	0.528
Naproxen		0.422	0.36
Phenylbutazone		0.345	0.338
Oxyphenbutazone		0.308	0.339
Aspirin		0.502	0.547
Salicylic Acid		0.478	0.537
Tolmetin		0.657	0.452

Elastase assay was done as described in the Methods except addition of drug in the medium 20 minutes before addition of substrate. Final concentration of NaCl was 60 mM.

Almost all of the divalent metal ions were removed after dialysis of the enzymes against 100 mM of Tris-EGTA but trace amounts of Ca^{++}, Fe^{++}, Zn^{++} were detected.

Inhibition of HNElastase by NSAIDs

HNElastase was inhibited effectively by 0.5 and 1 mM of naproxen, phenylbutazone, and oxyphenbutazone at our specific experimental condition, however, other agents such as ibuprofen, ketoprofen, aspirin, salicylic acid, and tolmetin did not inhibit HNElastase (Table 2).

Inhibitions of HNElases by different concentra-

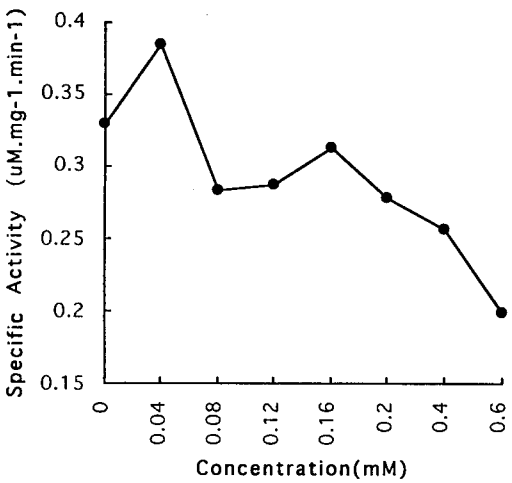

Fig. 1. Inhibition of HNElastase by different concentrations of naproxen: Elastase assay was done as described in the Methods. Final concentration of NaCl was 60 mM.

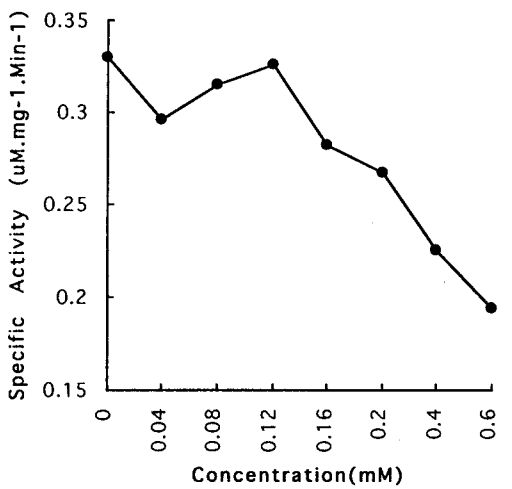

Fig. 2. Inhibition of HNElastase by different concentrations of phenylbutazone: Elastase activities was assayed by the method described in the Methods. Final concentration of NaCl was 60 mM.

tions (0~0.6 mM) of naproxen and phenylbutazone were concentration dependent manner as seen in Fig. 1 and 2, respectively.

Table 3. Activity of HNElastase

Sample	Protein (mg/ml)	Specific activity (mM. mg^{-1}. $minute^{-1}$)	(%)
1	0.777	0.242	(100%)
2	0.911	0.207	(86%)
3	0.489	0.156	(64%)
4	0.711	0.255	(105%)
5	0.533	0.129	(52%)

Elastase assay was done as described in the Methods. Final concentration of NaCl was 150 mM. 1: control; 2: PMDElastase; 3: CRElastase; 4: ZRElastase; 5: ZCRElastase.

Activity and Raman frequencies of human neutrophil elastases at various enzymatic conditions

Specific activity of partial metal depleted elastase (PMDElastase) was 85.5~64% of the activity of control elastase at our specific experimental condition (Table 3). Reconstitution of calcium ion or a mixture of zinc and calcium with the PMDElastase did not recover the enzyme activity at all. Actually activities of these two elastase were further decreased rather than increased. However, reconstitution of zinc ion to the PMDElastase recovered the elastase activity to as much as 105% of the control elastase activity (Table 3).

Frequencies and contours in the Raman spectra of various conditions of human neutrophil elastase undergo drastic changes upon partial removal and/or reconstitution of calcium and zinc ions. Native elastase gives rise to intense and sharp lines at 316, 620, 1084, 1392, 1616 and 1660 cm^{-1} (Fig. 3-A). Wave number 1616 cm^{-1} may be related to the ionized and coordinated $-COO^-$ stretching band, and wave number 1660 cm^{-1} may be a signal of strong carbonyl stretching, and indicates that control elastase has considerable β-turn and β-sheet structure. In the PMDElastase (Fig. 3-B), Raman frequencies at 316, 620, 1084, 1392 and 1660 cm^{-1} disappeared or gave rise to smaller intensities, and the enzyme activity also diminished (Table 2). The features at 1616 and 1664 cm^{-1} seen in the spectrum of the PMDElastase most likely correspond to bands at 1616 and 1660 cm^{-1} in the native elastase. Especially, the band 1660 cm^{-1}

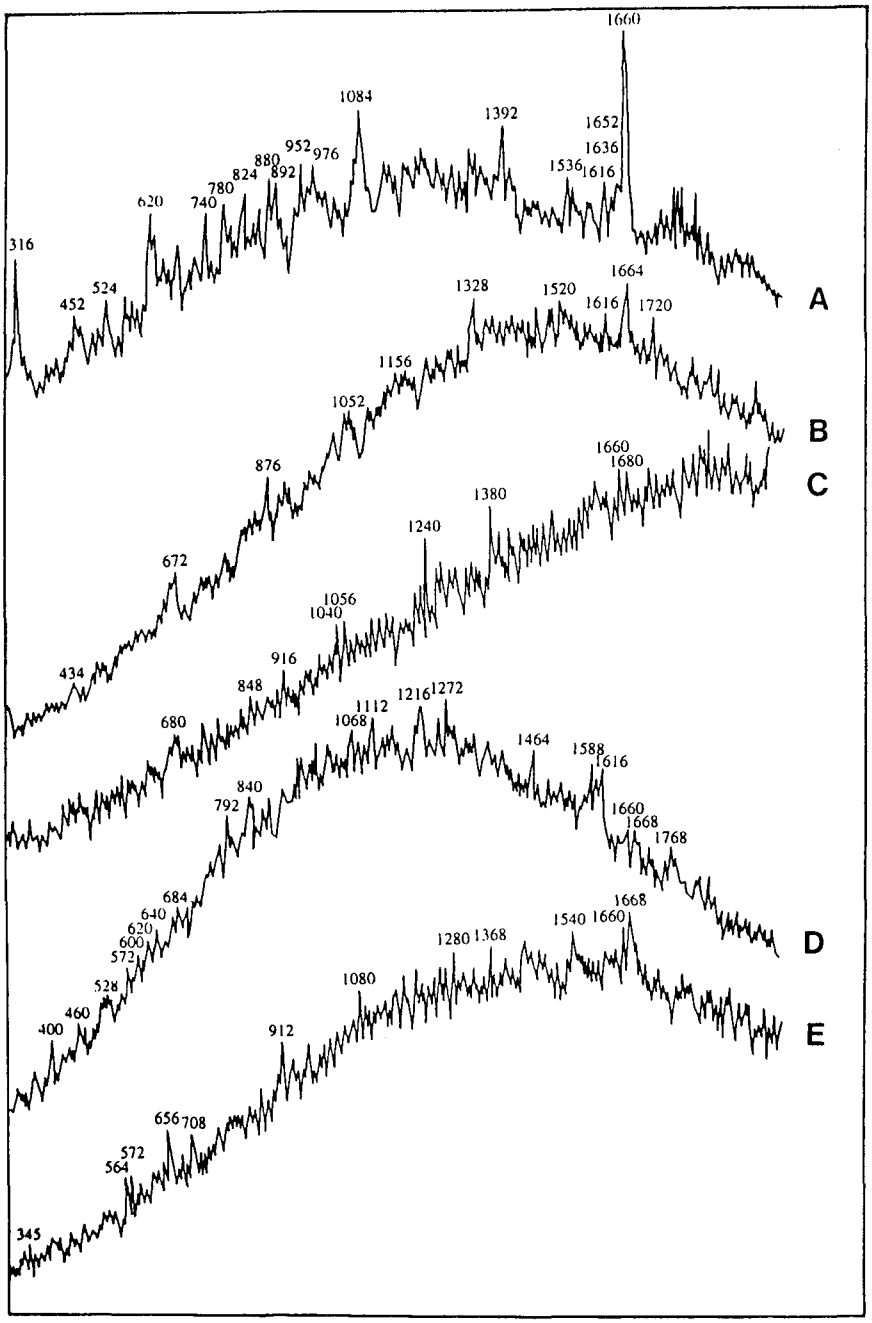

Fig. 3. Raman spectra of human neutrophil elastase: A: control, B: Partial metal depleted elastase, C: Calcium reconstituted elastase, D: Zinc reconstituted elastase, E: Calcium and zinc reconstituted elastase. Metal depletion and reconstitution were done as described in the Methods. Detection of molecular conformational change with Raman spectrophotometer was done as described in the Methods.

was shifted to a 4 cm^{-1} higher wave number and the band 1616 cm^{-1} remained at almost the same intensity in the PMDElastase. This phenomenon might be related to the conformational change toward the inactive form after partial removal of metal ions from the intact elastase. CRElastase gives rise to bands at 680, 1240, 1380, 1660, 1680, and 1852 cm^{-1} (Fig. 3-C). The features at 1380 and 1660 cm^{-1} seen in the spectrum of the CRElastase most likely correspond to bands at 1392 and 1660 cm^{-1} in the native elastase, especially, band 1660 cm^{-1} was extreamly weak. ZRElastase gives rise to bands at 328, 400, 1272, 1588, 1616, 1660, and 1668 cm^{-1} (Fig. 3-D). The features at 328, 1616, and 1660 cm^{-1} seen in the spectrum of ZRElastase most likely correspond to the same wave numbers in the native elastse, and the activity of elastase also recovered 90~105% of control activity. ZCRElastase gives rise to bands at 572, 656, 912, 1080, 1280, 1368, 1540, 1660, 1668, and 1789 cm^{-1} (Fig. 3-E). The bands at 1616, 1660, and 1668 cm^{-1} are most likely to correspond to the same bands in the native elastase. The band 1660 cm^{-1} was weak and band 1668 cm^{-1} was strong in the ZCRElastase. At or near the active site, wave number 1616 cm^{-1} may be related to the ionized and coordinated $-COO^-$ stretching.

From the above results, we strongly suggest that zinc ion may be an important element in keeping the natural conformation and in the reformation of the active molecular conformation from PMDElastase. Calcium ion, however, is not the specific element for keeping or constructing the active conformation of elastase, and is rather an active element for reformation of minor parts of the molecular conformation. Especially, noticible wave number 1660 cm^{-1} which is related to the strong carbonyl stretching.

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=국문초록=

비스테로이드성 항염증제와 효소 억제제에 의한 사람 중성구 Elastase의 활성도 억제 및 분자약리학적 기전

고신대학교 의과대학 약리학교실 및 경북대학교 화학교육과*

강구일 · 김우미 · 홍인식 · 이무상*

염증 질환의 원인이 되는 사람 중성구 elastases는 혈액에 존재하는 α_1-PI나 α_2-macroglobulin과 같은 단백질 분해효소 억제제에 의하여 조절되어진다. 그러나 특수한 병리적 상황에서 과다하게 분비되는 효소나 또는 단백질 분해효소 억제제의 비정상적 작용으로 말미암아 다양한 염증질환이 유발된다. 비스테로이드성 항염증제는 염증 질환을 치료하기 위하여 이미 임상에 적용하고 있으며, prostaglandin 합성하는 효소인 cyclooxygenases의 활성도를 억제하는 것이 그 작용 기전으로 잘 알려져 왔다. 사람 중성구 elastase의 활성도는 naproxen, phenylbutazone, oxyphenbutazone 등에 의하여 억제되었으나, ibuprofen, ketoprofen, aspirin, salicylic acid, tolmetin 등에 의하여서는 억제되지 않았다. 또한 사람 중성구 elastase의 활성도는 EDTA, EGTA, tetracycline 등에 의하여서도 억제되었다.

EDTA에 의하여 2가 이온 Ca^{++}나 Zn^{++}등을 elastase 분자로부터 일부 제거함으로 효소 활성도가 억제되었고 Raman spectra의 변화도 강하게 일어났으며, 금속이온 Zn^{++}를 새로 충진함으로 그 활성도는 원래대로 회복되고 Raman spectrum도 원래 상태와 유사한 상태로 회복되었다. 이런 현상은 chelator나 chelator-like agents가 효소분자안에 존재하는 Zn^{++} 이온을 제거하거나 chelation함으로 활성 부위나 그 인접 부위의 4차원 구조의 변화를 일으켰음에 기인하며 특히 $-C=O$나 $-COOH$기의 관여에 의한 것으로 생각된다.