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INHIBITION OF THE HUMAN LEUKOCYTE ENDOPEPTIDASES ELASTASE AND CATHEPSIN G AND OF PORCINE PANCREATIC ELASTASE BY *N*-OLEOYL DERIVATIVES OF HEPARIN

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Abstract—*N*-oleoyl-heparin derivatives differing in their oleic acid and sulfate contents were synthesized and studied for their abilities to inhibit human leukocyte elastase (HLE), human leukocyte cathepsin G (CatG) and porcine pancreatic elastase (PPE) at pH 8.0, ionic strength 0.05 M and 37°. Heparin (Hep) as well as *N*-oleoyl-heparins behaved as tight-binding, hyperbolic noncompetitive inhibitors of HLE ($K_{i\text{Hep}} = 75$ pM) and CatG ($K_{i\text{Hep}} < 25$ pM). The main driving force for the interaction between enzymes and glycosaminoglycans was electrostatic in nature. Under the condition [enzyme] $\gg K_i$, the stoichiometries of the interaction with Hep were 1:2 (Hep:HLE) and 1:4 (Hep:CatG). Coupling one oleic acid residue to three disaccharide units of partially N-desulfated Hep, $Ol_{1:3}$Hep, lowered HLE inhibition ($K_i = 0.3$ nM) and the stoichiometry of binding was reduced to 1:1. Re-N-sulfation of a similar derivative, $Ol_{1:5}Hep(SO_4)$, containing one fatty acid residue for five disaccharide units, led to a substance with similar HLE inhibitory characteristics as Hep ($K_i = 92$ pM) and stoichiometry 1:2. $Ol_{1:5}Hep(SO_4)$ was also a more efficient inhibitor of CatG ($K_i < 33$ pM) than $Ol_{1:3}$Hep ($K_i = 9.5$ nM). The residual activities of *N*-oleoyl-Hep complexes with CatG were much lower than the corresponding activities in the presence of Hep. While oleate and Hep could not inhibit PPE, *N*-oleoyl-Hep, independently of fatty acid substitution and sulfate content, could inhibit this enzyme with $K_i \approx 60$ nM and low residual activity. The efficient endopeptidase inhibitory characteristics of *N*-oleoyl-Hep derivatives, together with their non-anticoagulant properties and their capacity to interact with elastin, may be therapeutically useful in connective tissue degenerative diseases.

The endopeptidases from the azurophilic granules of polymorphonuclear neutrophils are involved in defense of the host against bacterial infections; however, when liberated in excess under the action of several stimuli, they can degrade extracellular matrix macromolecules in a synergistic way leading to diseases such as rheumatoid arthritis [1], lung diseases [2, 3] and periodontitis [4]. The use of synthetic inhibitors, fullfilling a set of criteria [5, 6], has been proposed as a strategic therapy under these circumstances [7–9].

It has previously been shown that glycosaminoglycans could bind electrostatically to human leukocyte elastase (HLE‖) [10–13] as well as to rat and hamster leukocyte elastases [14] and inhibit both the amidolytic and proteolytic activities of the enzymes. Also, HLE contains a secondary hydrophobic binding site that can accommodate a series of lipophilic substances, including unsaturated fatty acids, whose occupancy leads to enzyme inhibition [15–20]. Heparin fragments as well as oleoyl peptide conjugates were efficient in preventing emphysema induced in rodents by intratracheal HLE instillation [21–23]. In keeping with our earlier concept of designing bifunctional elastase inhibitors [22], i.e. substances that could bind to both enzyme and substrate (elastin), we synthesized *N*-oleoyl-heparin derivatives. In a preliminary investigation, we reported that an *N*-oleoyl-heparin derivative could indeed bind to elastin, providing to this extracellular macromolecule effective protection against HLE degradation [24].

The aim of the present contribution is to describe the mechanism of inhibition of *N*-oleoyl-heparin(s) with three different elastolytic enzymes: HLE, human leukocyte cathepsin G (CatG) [25] and porcine pancreatic elastase (PPE).

MATERIALS AND METHODS

Enzymes and substrates. HLE (EC 3.4.21.37) was obtained from Elastin Products Co. (Owensville, MO, U.S.A.), CatG (EC 3.4.21.20) was purified from human leukocytes as detailed elsewhere [26],

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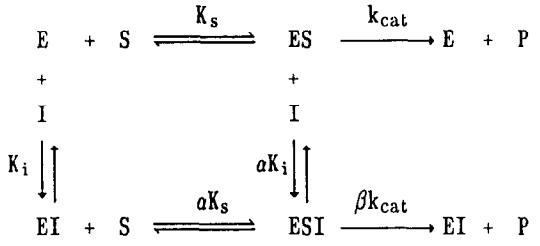
‖ Abbreviations: HLE, human leukocyte elastase; CatG, human leukocyte cathepsin G; PPE, porcine pancreatic elastase.

Names of amino acids and substituents are abbreviated in accordance with IUPAC–IUB recommendations [*Biochem J* **219**: 345–373, 1984].



Scheme 1. The general modifier mechanism. E = enzyme, S = substrate, I = inhibitor, P = product, K_i = [E][I]/[EI], K_s = [E][S]/[ES], αK_i = [ES][I]/[ESI], αK_s = [EI][S]/[ESI], k_{cat} = catalytic constant, α and β = coefficients.

PPE (EC 3.4.21.36) was Type IV from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The active site concentration of HLE was determined by titration with MeO-Suc-Ala-Ala-Pro-Val-chloromethane and residual activity was measured with Suc-Ala-Ala-Val-*p*NA. HLE of known concentration was used to titrate a sample of human recombinant α1-proteinase inhibitor (a gift of Dr H. P. Schnebli, Basle, Switzerland), with which the active site concentration of cathepsin G was then determined using Suc-Ala-Ala-Pro-Phe-*p*NA to measure residual activity. The concentration of PPE, not critical for this study, was calculated from weight and M_r = 25,900. All substrates and the chloromethane derivative used to titrate HLE were from Bachem (Liestal, Switzerland).

Heparin and derivatives. Heparin (average M_r = 12,000) was a gift from M. Petitou (Sanofi, Gentilly, France) and was N-desulfated in dimethyl sulfoxide containing 5% (v/v) methanol [27]. The pyridinium salt (250 mg) of the desulfated product was dissolved in 4 mL of dimethyl sulfoxide and 200 μL triethylamine. Then, 400 μL of oleoyl chloride (Merck, Darmstadt, Germany) was added in portions to the stirred solution during 10 min and the reaction mixture was kept for 2 hr at 20° under constant stirring. Oleylated heparin was precipitated by adding 30 mL of ethyl acetate, isolated by centrifugation, washed twice with 30 mL of ethyl acetate and dried *in vacuo*. The dried material was dissolved in 5 mL of 2 M sodium acetate and the sodium salt was precipitated with 40 mL of ethanol, redissolved in 5 mL H_2O and dialysed against distilled water. This compound was designated as the oleoyl-heparin derivative $Ol_{1:3}$Hep. The ratio of the sulfate to carboxyl residues was evaluated by conductometric titration [28] and was equal to 2.1 for heparin and 1.1 for $Ol_{1:3}$Hep. The number of oleoyl residues was estimated by NMR spectrometry carried out on a Varian YXR 400 apparatus in D_2O at 50° using tetramethylsilane as the external standard. The degree of oleylation was calculated from the area of the CH_2 (1.25–1.4 ppm) and the CH_3 (0.84–0.92 ppm)

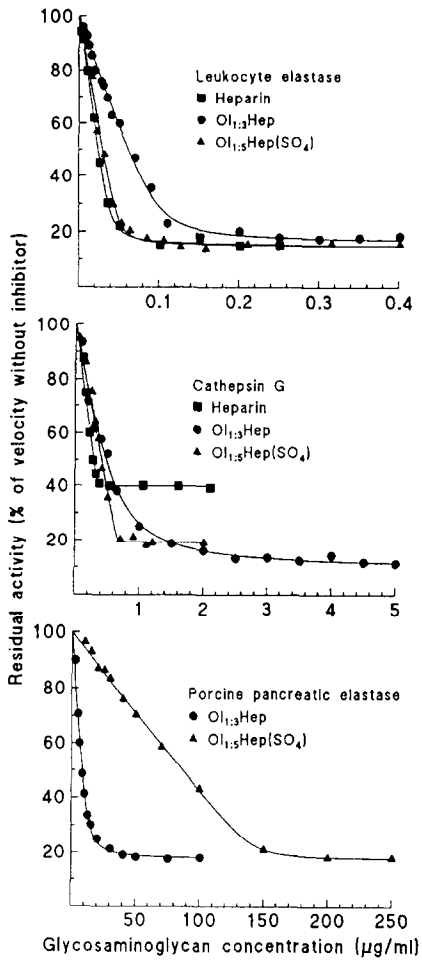


Fig. 1. Inhibition profiles of HLE, CatG and PPE by heparin and its oleylated derivatives $Ol_{1:3}$Hep and $Ol_{1:5}$Hep(SO_4). Tris–HCl (0.1 M) pH = 8.0, ionic strength 0.05 M, 37 ± 1°. The substrates, their concentrations and the concentrations of the enzymes were: 0.10 mM MeO-Suc-Ala-Pro-Val-*p*NA for HLE (8.3 nM of titrated active sites); 0.20 mM Suc-Ala-Ala-Pro-Phe-*p*NA for CatG (130 nM of titrated active sites); 0.20 mM Suc-Ala-Ala-Ala-*p*NA for PPE (77 nM as protein). Points are experimental and lines represent best-fit curves obtained by nonlinear regression analysis using Eqn 1.

$$v_i = \frac{v_o}{2}(1-\beta)\left\{\sqrt{\left[\left(\frac{K_i + [I]_t}{[E]_t} - 1\right)^2 + 4\,\frac{K_i}{[E]_t}\right]} + \left[\frac{1+\beta}{1-\beta} - \frac{Ki + [I]_t}{[E]_t}\right]\right\}$$

Equation 1.

Table 1. Kinetic parameters for inhibition of HLE, CatG and PPE by heparin and two oleylated heparins [$Ol_{1:3}$Hep and $Ol_{1:5}Hep(SO_4)$]

	HLE	CatG	PPE
Heparin			
K_i	0.9 ± 0.5 (0.075)	<0.3 (<0.025)	
β	0.15 ± 0.02	0.40 ± 0.02	No inhibition
EP	2.08	4.3	
$Ol_{1:3}$Hep			
K_i	3.6 ± 1.3 (0.3)	115 ± 40 (9.5)	790 ± 300 (65.8)
β	0.16 ± 0.01	0.09 ± 0.02	0.17 ± 0.01
EP	1.06	2.6	—
$Ol_{1:5}Hep(SO_4)$			
K_i	1.1 ± 0.5 (0.092)	<0.4 (<0.033)	750 ± 520 (62.5)
β	0.15 ± 0.01	0.19 ± 0.02	0.17 ± 0.01
EP	2.7	2.4	—

Tris–HCl (0.1 M) pH 8.0, ionic strength 0.05 M, 37 ± 1°.

The significance of K_i and β is explained in Scheme 1. For K_i and β the numbers indicate best-fit parameters ± SE from nonlinear regression to a tight-binding, hyperbolic noncompetitive inhibition mechanism, Eqn 1. Units of K_i are in ng/mL and values in parentheses indicate the K_is recalculated in nM units for M_r = 12,000.

EP is the 'equivalence point', calculated as the ratio of the effective molar enzyme concentration to the best-fit value of $[E]_t$ from Eqn 1.

proton signals of the oleoyl residues as compared to the signals of the CH and CH_2 protons of the sugar residues (3.7–4.5 ppm). The results were in agreement with the decrease of the free NH_2 residues in the oleylated derivatives as assayed by trinitrobenzene sulfonic acid [29]. $Ol_{1:3}$Hep contained an average of one fatty acid residue for three disaccharide units of heparin and more than 90% of the non-oleylated amino groups were free. Using lower amounts of oleoyl chloride (300 μL for 250 mg of the pyridinium salt of N-desulfated heparin) in the chemical coupling of fatty acid to heparin led to derivatives containing less oleic acid residues per disaccharide of heparin. One of them, containing about one oleate residue per five disaccharide units of heparin, was re-N-sulfated from its tributylammonium salt with pyridine sulfur dioxide complex in dimethylformamide (SO_3^-/COO^- = 1.7) [30] and was designated $Ol_{1:5}Hep(SO_4)$.

Enzyme kinetics. Enzymatic activities were determined at 37 ± 1° in thermostatted polystyrene cuvettes using 0.10 M Tris–HCl buffer, pH = 8.0, ionic strength = 0.05 M, by following the release of *p*-nitroaniline at 410 nm from the substrates MeO-Suc-Ala-Ala-Pro-Val-*p*NA (HLE), Suc-Ala-Ala-Pro-Phe-*p*NA (CatG) and Suc-Ala-Ala-Ala-*p*NA (PPE). Exact conditions are specified in the legend of Fig. 1. The interaction of elastases and CatG with glycosaminoglycans was analysed according to the general modifier mechanism [31] shown in Scheme 1. In the case of classical binding, i.e. when the free inhibitor concentration can be approximated to its total concentration, the specific velocity plot is a suitable method for both diagnostic and quantitative analysis [32]. When the tight-binding condition applies, a more rigorous treatment must take into account the depletion of inhibitor concentration [33]. When the inhibitory mechanism has a noncompetitive character, the coefficient α in Scheme 1 equals 1 and the appropriate rate equation is given by Eqn 1 [33]. Equation 1 has been written explicitly using the nomenclature of Scheme 1. $[I]_t$ and $[E]_t$ represent the total concentrations of inhibitor and enzyme, respectively.

RESULTS

Inhibition profiles of HLE, CatG and PPE by heparin and its oleoyl derivatives $Ol_{1:3}$Hep and $Ol_{1:5}Hep(SO_4)$ are shown in Fig. 1. In separate experiments, profiles as shown in Fig. 1 were obtained at three more substrate concentrations and the data analysed according to the specific velocity plot [32]. This graphical method can be used for quantitative purposes only in the case of 'classical' inhibition, i.e. when a tight-binding condition between enzyme and inhibitor does not exist. However, the plot retains its powerful diagnostic character also in the case of tight-binding, but in this latter case the kinetic parameters cannot be calculated with accuracy, because, the free inhibitor concentration cannot be approximated with its total concentration. For HLE and CatG the specific velocity plots (not shown) consisted of a family of lines parallel to the abscissa, indicative of a noncompetitive inhibition mechanism ($\alpha = 1$ in Scheme 1), and replots indicated a hyperbolic character. For calculating the relevant kinetic parameters, a tight-binding, hyperbolic non-competitive inhibition mechanism (Eqn 1) was fitted by nonlinear regression to the experimental points obtained at a fixed substrate concentration and variable inhibitor concentrations (Fig. 1). PPE was not inhibited by heparin and interacted with the oleoyl derivatives only at high concentrations. The kinetic parameters are summarized in Table 1. For the inhibition of CatG by heparin and $Ol_{1:5}Hep(SO_4)$ K_i could not be determined precisely because of very

high affinity. The values of $K_i < 0.3$ ng/mL and $K_i < 0.4$ ng/mL mean that the actual values may be lower, but not higher than those shown. All measurements reported here were performed in 0.1 M Tris–HCl buffer, pH 8.0 and ionic strength 0.05 M. Increasing the ionic strength by addition of NaCl resulted in a progressive reduction of the inhibitory power for all enzymes and glycosaminoglycans, i.e. both K_i and β increased. At physiological ionic strength the K_i values shown in Table 1 increased roughly by an order of magnitude and inhibition was completely abolished at an ionic strength of 0.4 M.

When using Eqn 1 for nonlinear regression analysis of experimental data, v_o and $[E]_t$ are known constants and $[I]_t$ is the independent variable, while β and K_i are parameters. For mechanisms with a noncompetitive character ($\alpha = 1$ in Scheme 1) the percentage of inhibition does not depend on the substrate concentration and results can be plotted considering the velocity in the absence of inhibitor as 100% (Fig. 1). The interaction between the enzymes and the concerned inhibitors is obviously electrostatic and the number of positively charged amino acid residues on the enzymes that interact with the inhibitors is not known *a priori*. Further complicating factors are the polydispersity of heparin and its derivatives and the indeterminateness of the position of the oleoyl residues along the heparin backbone. For these reasons, inhibitor concentrations were expressed as w/v and $[E]_t$ in Eqn 1 was therefore also considered as a parameter to be fitted. Due to the three-parameter fitting with a single set of data, the standard errors of K_i (Table 1) are apparently high despite very good fittings of the model to data. Of the three parameters fitted, namely K_i, β and $[E]_t$, the latter represents the effective enzyme concentration involved in the interaction with the inhibitor. This best-fitted parameter (recalculated as an equivalent molar concentration, based on $M_r = 12{,}000$ for the glycosaminoglycans) was compared with the actual enzyme concentration and is shown in Table 1 as the 'equivalence point', i.e. as the ratio of the effective to the best fit enzyme concentration. Since all of the curves for HLE and CatG shown in Fig. 1 indicate tight binding, this ratio can be considered as a measure of the stoichiometry of the enzyme/inhibitor interaction under the condition $[E]_t \gg K_i$. One molecule of heparin was needed for the saturation of two molecules of HLE and roughly four molecules of CatG. One molecule of $Ol_{1:3}Hep$ bound one molecule of leukocyte elastase and 2.6 molecules of cathepsin G, whereas $Ol_{1:5}Hep(SO_4)$ had a stoichiometry between 2 and 3 with both enzymes.

DISCUSSION

The tight binding, hyperbolic noncompetitive inhibition mechanism of HLE and CatG by heparin [12], was confirmed. In the present study we investigated whether coupling oleic acid residues to heparin could alter the inhibitory characteristics of the parent molecule, heparin, towards leukocyte proteinases. Both of the two oleoyl-heparin derivatives synthesized behaved as tight-binding, hyperbolic noncompetitive inhibitors of HLE and CatG. From our data it is not easy to understand the relative inhibitory roles of the heparin backbone and of the oleoyl residues. HLE contains a hydrophobic binding pocket, located near the active center, that is able to interact with a series of lipophilic substances, the most extensively studied being long chain unsaturated fatty acids. Oleate binding to this site brings about conformational changes in elastase and modulates the enzyme activity by two inhibitory modes; a high affinity mode ($K_i = 48$ nM, partial noncompetitive with 87% residual activity) and a competitive mode of much lower affinity (16 μM) [18]. Globally, our results suggest that the main driving force in the interaction of HLE and CatG with *N*-oleoyl-heparins is electrostatic in nature, the contributions of the fatty acid chains being less important. The inhibitory efficiency correlates well with the structural characteristics of the glycosaminoglycans; a higher degree of sulfation is expected to be accompanied by lower K_i values, and this is indeed the case, since $Ol_{1:5}Hep(SO_4)$ is more sulfated than $Ol_{1:3}Hep$. The inhibition of cathepsin G is somewhat puzzling; while heparin and $Ol_{1:5}Hep(SO_4)$ bind extremely tightly, the interaction with $Ol_{1:3}Hep$ is apparently anomalous for its relatively high K_i and its low β (some 90% inhibition at saturation).

The results for PPE should be considered from a qualitative point of view only, to indicate low affinity and the necessity of oleoyl residues for inhibition. Both *N*-oleoyl-heparin derivatives could inhibit about 80% of the PPE activity, although with scarce efficiency when compared with the leukocyte proteinases. Neither heparin, nor oleate had any influence on the activity of PPE and we could recently demonstrate that oleate had no effect on its conformation, as assessed by Raman spectroscopy (K. Marx *et al.* in preparation).

In conclusion, covalently coupling oleic acid to heparin leads to a molecule with novel specificity towards serine endopeptidases, since *N*-oleoyl-heparin can also inhibit urokinase and plasmin [34]. The oleoyl-heparin conjugates synthesized in the present study behave as bifunctional modulators of the elastase activity, i.e. they directly inhibit the enzyme activity and also bind to elastin. In fact, we found that 1 mg of insoluble calf neck ligament elastin can be saturated with 5.3 μg of $Ol_{1:3}Hep$, $K_{diss} = 4.6$ μM (data not shown). As compared with heparin, the oleoyl derivatives display new characteristics that could be potentially useful during inflammation, where several serine endopeptidases, including leukocyte elastase, cathepsin G and plasmin, act synergistically in the degradation of extracellular matrix macromolecules. These enzymes also play a role as activators of matrix metalloproteinases [35] and CatG can induce platelet activation [36]. In favor of the potential pharmacological use of *N*-oleoyl-heparins, it is worthy of mention that these substances do not possess anticoagulant properties and, as other lipophilic derivatives, may have higher half-lives in the circulation [37].

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