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INFLUENCE OF HEPARIN FRAGMENTS ON THE BIOLOGICAL ACTIVITIES OF ELASTASE(S) AND α_1 PROTEINASE INHIBITOR

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Abstract—The *in vitro* and *in vivo* effects of heparin fragments (CY 216; CY 222) towards elastase(s) and elastase inhibitor (α_1 P_i) were studied. Heparin as well as its lower M_r fragments were shown to inhibit rat leucocyte elastase. The interaction between this enzyme and heparins appears to occur via electrostatic forces. Porcine pancreatic elastase is unaffected by heparin(s) but CY 216 and CY 222 could partly abolish the hydrolytic activity of hamster serum on Suc-Ala-Ala-Ala-N-PhNO$_2$. N desulphated N acetylated CY 142 and CY 143 had no effect. CY 216 and CY 222 decreased *in vitro* the inhibitory potential of α_1 proteinase inhibitor (α_1 P_i) as well as the elastase inhibitory capacity of hamster serum. Maximum effect (30% decrease) was observed at ng concentrations of CY 216 and CY 222. Their N desulphated N acetylated counterparts (CY 142 and CY 143), but not heparin, exhibited similar effects.

CY 216 and CY 222 were administered daily subcutaneously to hamsters and blood was collected 1, 2, 4, 7 and 24 hr after treatment for determining both serum elastase activity (E.A.) and serum elastase inhibitory capacity (E.I.C.). E.A. levels dropped by 30% 2 hr after CY 216 or CY 222 injection but returned to original values 4–7 hr later. This effect is independent of the duration of the treatment. Hamster serum E.I.C. was significantly increased (>30%) after 3–4 weeks of treatment with CY 216 and CY 222. These findings point towards the potential use of these compounds in elastase-related diseases such as emphysema.

Heparin(s), a linear polyelectrolyte, can interact with several proteins and dyes via electrostatic forces [1]. Its anticoagulant effect is mainly mediated by the plasma protein antithrombin III which inactivates serine proteases involved in coagulation as thrombin, factors X_a, IX_a, XII_a and kallikrein [1, 2]. Thrombin is also inhibited by α_1 proteinase inhibitor (α_1 P_i), and it was shown that heparin could influence thrombin-α_1 P_i interaction [3].

Although α_1 P_i and antithrombin III both belong to the serpin superfamily of plasma proteinase inhibitors [4], kinetics data suggested that α_1 P_i acts primarily as an elastase inhibitor [5]. Elastases, non-specific neutral endopeptidases, originate from several cell types and tissues [6–9] and were shown to play an active role in many pathological conditions [6–8]. Polymorphonuclear neutrophil elastase (EC 3.4.21.37) was proposed to be involved in pulmonary emphysema, chronic bronchitis, cystic fibrosis, adult respiratory distress syndrome, rheumatoid arthritis, psoriasis, vasculitis and glomerulonephritis [10]. It has been also suggested that this leucocyte proteinase functioned in the degradation of fibrinogen and fibrin, both in normal conditions and disease states [11].

Previous studies pointed out that several oligosaccharides derived from glycosaminoglycans (GAG) could inactivate human leucocyte elastase [12–14].

The aim of the present investigations was to study the effect of heparin and its lower M_r fragments with variable degree of sulphation on the activities of several elastases and/or elastase-type proteases and also on α_1 P_i. These *in vitro* studies were completed by *in vivo* studies: heparin fragments (CY 216 and CY 222) were injected subcutaneously to hamsters and the levels of serum elastase activity (E.A.) and elastase inhibitory capacity (E.I.C.) were parallely quantified as a function of the duration of the treatment.

MATERIALS AND METHODS

Succinoyl-alanyl-alanyl-alanine paranitroanilide (Suc-Ala-Ala-Ala-N-PhNO$_2$) came from Institut Choay (Paris, France).

Porcine pancreatic elastase (Type IV), α_1 proteinase inhibitor (α_1 antitrypsin A-9024) bovine trypsin and benzoyl arginine paranitroanilide were purchased from Sigma Chemical Co. (St. Louis, MO).

Rat leucocyte elastase was purified to homogeneity as recently described [15]. α_1 proteinase inhibitor was titrated using an active site titrated human leucocyte elastase preparation kindly provided by Drs C. Boudier and J. Bieth (Université L. Pasteur, Strasbourg, France). Assuming that porcine pancreatic and rat leucocyte elastases react stoichiometrically with α_1 P_i [16], the molar amounts of active enzymes in the porcine pancreatic and rat leucocyte elastase preparations were then determined.

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§ Abbreviations used: α_1 P_i, α_1 proteinase inhibitor; E.A., elastase activity; E.I.C., elastase inhibitory capacity; Suc-Ala-Ala-Ala-N-PhNO$_2$, succinoyl alanyl-alanyl-alanine paranitroanilide.

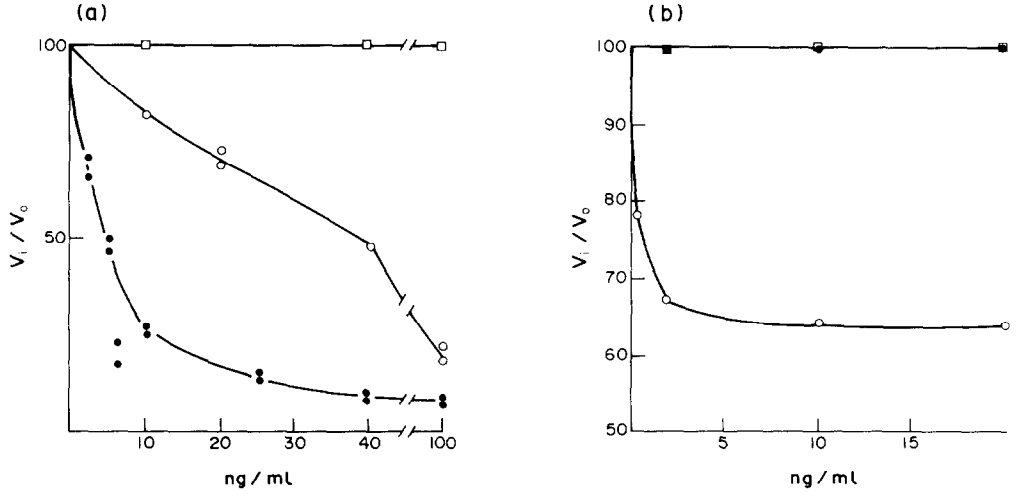

Fig. 1. Influence of heparin and its fragment (CY 216) and derivative (CY 142) on the activities of rat leucocyte elastase (80 nM) (a) and hamster serum elastase-type protease (b). Substrate: Suc-Ala-Ala-Ala-N-$PhNO_2$ (1.25 mM); heparin (●——●); CY 216 (○——○); CY 142 (□——□); abscissa: concentration of heparin, its fragment and derivative in ng/mL. Ordinates: ratio of the rate of hydrolysis of the substrate in presence (v_i) and absence (v_o) of heparin(s).

Heparin and its fragments. CY 216 (M_r = 2500–8000) and CY 222 (M_r = 1500–8000) were obtained by chemical depolymerization of porcine mucosal heparin (160 USP units/mg and 155 anti factor X_a units/mg) and further gel filtration [17]. The corresponding *N* desulphated–*N* acetylated compounds were obtained as described [17, 18].

Enzyme assays. Elastase activity was determined using Suc-Ala-Ala-Ala-N-$PhNO_2$ as substrate. Active site titrated elastases or serial dilutions of hamster serum were allowed to react with different concentrations of heparin derivatives for 15 min in 100 mM Tris/HCl, 0.02% sodium azide pH 8.0. Ten to 30 μL of a stock solution of substrate (125 mM) dissolved in *N*-methyl pyrrolidone were added to all polystyrene tubes and the variation of absorbance at 410 nm was recorded as a function of time in a spectrophotometer type Acta C III (Beckman, France). Experiments were performed at 37°. Linear kinetics were obtained (in the presence or absence of heparin derivatives) up to 24 hr of incubation and the dose–response curves were also linear for serum concentrations ranging from 5 to 50 μL [18]. Appropriate blanks were always included to take into account the spontaneous hydrolysis of the substrate and also the absorbance of the different serum dilutions at 410 nm. The results were expressed as nanomoles of substrate hydrolyzed per hour (Units) per mL of serum (ε nitroaniline = 8800 $M^{-1} cm^{-1}$). V_i/V_o ratio referred to the rate of hydrolysis of this substrate in presence (V_i) or absence (V_o) of inhibitor.

Rate association constants between porcine pancreatic elastase and α_1 proteinase inhibitor were quantitated as previously described [19].

The elastase inhibitory capacity of hamster serum (E.I.C.) was determined using porcine pancreatic elastase (2.31 pmol) and Suc-Ala-Ala-Ala-N-$PhNO_2$ (1.25 mM) as substrate. Briefly, the enzyme and 20 μL of serial dilutions of hamster serum were preincubated at 20° for 30 min in 100 mM Tris/HCl, 0.02% sodium azide pH 8.0 before determining the residual activity hydrolysing Suc-Ala-Ala-Ala-N-$PhNO_2$. Inhibition curves were drawn for each experiment. The inhibition of porcine pancreatic elastase by hamster serum was linear up to 85% of inhibition and, as observed with human serum, increased elastase activity was observed with increasing serum concentrations probably reflecting the activity of α_2 macroglobulin (α_2M)–elastase complexes on Suc-Ala-Ala-Ala-N-$PhNO_2$ [16]. The straight lines were extrapolated to 100% inhibition in order to evaluate the E.I.C. of hamster serum expressed as μg of proteinase inhibited per mL of serum.

In vivo studies. Three groups of two months old male Golden Syrian hamsters (100 g body weight) from Charles River Laboratories (Massassuchetts, U.S.A.) were used: (i) an untreated group (N = 10); (ii) a group treated with CY 216 injected subcutaneously at the dosage of 2 mg/100 g body weight in 200 μL saline, 6 days a week, during 5 weeks (N = 10); (iii) a group treated similarly with CY 222 (N = 10).

During this treatment, at the beginning of each week, blood samples were taken from the jugular vein of each hamster. In order to investigate the kinetics of variation of serum elastase activity and elastase inhibitory capacity in response to injection of CY 216 and CY 222, blood samples were taken in duplicate at 1, 2, 4, 7 and 24 hr after the injection of the heparin derivatives.

RESULTS

At concentrations ranging from 1 to 100 μg/mL heparin(s) as well as its fragments (CY 216 and CY 222) did not affect the activity of either bovine trypsin or porcine pancreatic elastase. In contrast, purified rat leucocyte elastase was inhibited by heparin and CY 216 and CY 222. Heparin was a better inhibitor than CY 216 and CY 222 but with all these com-

pounds a complete inhibition of leucocyte elastase could not be reached. Increasing the ionic strength to 0.5 M NaCl completely abolished the interaction between leucocyte elastase and heparin and its fragments. The role of ionic interactions in leucocyte elastase–heparin(s) interactions was further substantiated by comparing the effect of N-desulphated–N-acetylated compounds to those of their unmodified counterparts. Figure 1(a) shows that *N*-desulphated–*N*-acetylated heparin derivatives had no inhibitory potential towards rat leucocyte elastase. Similar results were obtained for human leucocyte elastase (not shown). CY 216 and CY 222 could also partly suppress the elastase activity

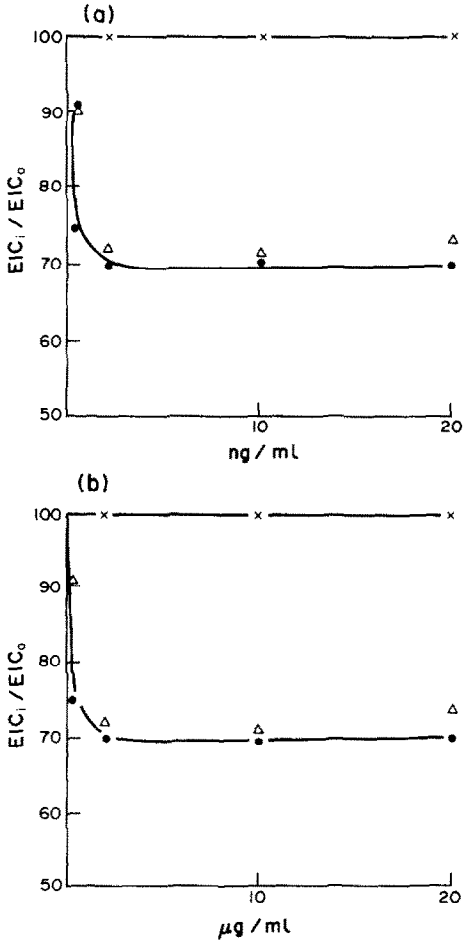

Fig. 2. Effect of heparin, its fragment (CY 216) and derivative (CY 142) on the porcine pancreatic elastase inhibitory capacity (E.I.C.) of α_1 proteinase inhibitor (a) and hamster serum (b). Porcine pancreatic elastase (2.31 pmoles) was titrated by α_1 P_i or hamster serum in the presence or absence of heparin, fragment and derivative at different concentrations. In both cases linear inhibition was obtained which allowed definition of the quantity of α_1 P_i and/or of serum necessary (elastase inhibitory capacity) to suppress the activity of the enzyme. The substrate used was Suc-Ala-Ala-Ala-N-PhNO$_2$. Abscissa: concentration of heparin (×——×), its fragment CY 216 (△——△) and its derivative CY 142 (●——●); ordinates; ratio of the elastase inhibitory capacity in presence (E.I.C.$_i$) and in absence (E.I.C.$_o$) of heparin(s).

expressed by hamster serum. A 35% maximal inhibition was attained and in contrast with that obtained with leucocyte elastase, heparin was not able to appreciably modify the elastase-type activity exhibited by hamster serum. *N*-desulphated–*N*-acetylated compounds (CY 142 and CY 143) were also ineffective in modifying hamster serum elastase activity (Fig. 1(b)).

These compounds were also tested for their ability to interfere with elastase–α_1 proteinase inhibitor interactions. Porcine pancreatic elastase was used since, as previously mentioned, this proteinase did not interact with heparin and its derivatives. Therefore, any variation in the extent of inhibition of porcine pancreatic elastase by α_1 P_i in the presence of heparin derivatives could be attributed to α_1 P_i–heparin interactions. When using Suc-Ala-Ala-Ala-N-PhNO$_2$ as a substrate, the inhibition of porcine pancreatic elastase by α_1 P_i was linear up to 85% inhibition. Extrapolation of the straight line to 100% inhibition allowed us to confirm the stoichiometry of the reaction between the proteinase and α_1 P_i. Heparin at concentration as high as 100 µg/mL had no influence on this inhibition. CY 216, as well as its *N*-desulphated counterpart, could decrease the extent of inhibition of porcine pancreatic elastase by α_1 P_i. This effect was found to be dose related, and the maximal decrease of the inhibitory capacity of α_1 P_i towards pancreatic elastase (30%), and also bovine trypsin (40%) (not shown) was reached for concentrations of CY 216, or CY 142 in the 0.2–2 ng range (Fig. 2(a)). We also demonstrated that the rate constant of association (k_{on}) between porcine pancreatic elastase and α_1 P_i is decreased from $2.8\,10^5\,M^{-1}\,sec^{-1}$ to $1.23\,10^5\,M^{-1}\,sec^{-1}$ in the presence of 1 µg/mL of CY 216. As also shown in Fig. 2(b), heparin fragments, but not heparin itself, could affect the elastase inhibitory capacity of hamster serum. This effect was also found dose related but maximal decrease of E.I.C. was reached at CY 216 concentrations one order of magnitude higher than necessary to obtain the same inhibition with pure α_1 P_i. This difference is probably related to the binding of heparin fragments to several other hamster serum proteins.

In vivo *experiments*. The results shown in Table 1 indicate that daily subcutaneous injections of CY 216 and CY 222 were able to influence the E.A. expressed by hamster serum. As early as at the first hour following subcutaneous injection of these heparin fragments, a decrease of serum E.A. was observed. This decrease was maximal (24%) 2 hr after the injection but returned to the original uninhibited values 7 hr later; this effect was independent of the duration of the treatment.

In our experimental conditions, 1 mL of hamster serum was capable of abolishing the activity of 59.8 ± 0.26 µg of porcine pancreatic elastase. This figure is in agreement with those reported in the literature [20] and indicates that hamster serum has lower E.I.C. than human serum [21]. When CY 216 and CY 222 were injected subcutaneously into hamsters, the serum samples progressively exhibited an increased tendency to inhibit a constant amount of pancreatic elastase.

This variation of the E.I.C. of hamster serum was

Table 1. Influence of subcutaneous injection of heparin fragments (CY 216 and CY 222) upon the elastase activity (E.A.) and elastase inhibitory capacity (E.I.C.) of hamster serum.

	Untreated hamsters (N = 10)	Hamsters treated with CY 216 or CY 222 (N = 20)			
		2 weeks treatment (after injection)		4 weeks treatment (after injection)	
		2 hr	7 hr	2 hr	7 hr
Serum elastase activity (Units E.A.)*	25 (± 0.4)	19 (± 0.7)	25 (± 1.4)	20 (± 3)	25 (± 4)
% Variation		24	0	20	0
Statistical significance (compared to the control group)		$P < 0.01$	N.S.	$P < 0.05$	N.S.
Serum elastase inh. capacity (units E.I.C.)**	59.8 (± 0.26)	60.3 (± 2.33)	62.8 (± 2.99)	70.2 (± 1.69)	79 (± 2.98)
% Variation		0.8	5	17.4	32.1
Statistical significance (compared to the control group)		N.S.	N.S.	$P < 0.05$	$P < 0.01$

* 1 unit E.A. = 1 nmol Suc-Ala-Ala-Ala-N-$PhNO_2$ hydrolysed/hr/mL of hamster serum.
** μg of pancreatic elastase inhibited per mL of hamster serum.
The values in brackets referred as standard error of the mean.
Statistical analysis performed according to the Fisher Student *t*-test. N.S.: not significant.

influenced by the duration of the treatment. It was significant after 2–3 weeks and a 30% increase in E.I.C. was reached after 4 weeks of treatment with the heparin derivatives. This level of increase was still found 24 hr after the last injection (Fig. 3) The same modifications in the levels of hamster serum E.A. and E.I.C. were observed whether the animals were treated with CY 216 or CY 222.

DISCUSSION

Heparin and derivatives of lower M_r were found to inhibit the activity of leucocyte elastase. Similarly as observed with other glycosaminoglycans, the interaction between the proteinase and heparin(s) occurred via electrostatic forces [12–14]. The mechanism of inhibition was kinetically analysed using heparin fragments and derivatives of defined M_r; the data obtained were consistent with the classification of the inhibition type as tight binding hyperbolic and non-competitive [17]. Porcine pancreatic elastase and bovine trypsin did not interact with heparin(s), but CY 216 could suppress part of the elastase-type activity of hamster serum. In human serum, a significant portion of the elastase activity is associated with lipoproteins [22]. High density lipoproteins carry a metallo elastase-type proteinase able to hydrolyze apolipoprotein Al and fibronectin. The enzyme associated with low density lipoproteins was shown to be a serine proteinase and accounts for 20–40% of the overall elastase activity of human serum [21, 22]. Although the potential role of leucocyte elastase (EC 3.4.21.37) inflammatory reactions is now well documented, serum elastase(s) could also be involved in pathological modifications of elastic tissue. Elastase activities were found elevated in the sera of patients suffering from emphysema and sarcoïdosis [21].

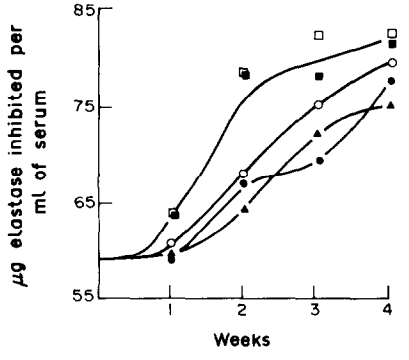

Fig. 3. Variations of elastase inhibitory capacity (E.I.C.) of hamster serum following subcutaneous injections of CY 216 or CY 222: ▲——▲, 1 hr after injection; ●——●, 2 hr; ○——○, 4 hr; ■—■, 7 hr; □——□, 24 hr. Abscissa: weeks of treatment; ordinates: E.I.C., μg elastase inhibited per mL of hamster serum.

We also provided evidence that heparin fragments, but not heparin, interact with α_1 P_1, the major elastase inhibitor present in the circulation. Maximal effect was observable at low concentration of CY 216 and was not related with the degree of sulphation of the heparin derivatives.

These *in vitro* findings prompted us to investigate the influence of subcutaneous injections of CY 216 and CY 222 to hamsters on their serum E.A. and E.I.C. Repeated injections to these animals of either of these heparin derivatives significantly lowered the amount of their circulating E.A. This effect is transient, being maximal 1–2 hours after injection and normal E.A. levels were found 7 hr after CY 216 injection. It is also reproducible over the 4 weeks treatment. Such *in vivo* inhibition of E.A. is in keeping with the *in vitro* effect of CY 216 and CY 222 towards serum E.A.

In these investigations we found that CY 216 and CY 222 administered to hamsters for time periods exceeding 3 weeks induced a 30–40% increase in serum E.I.C. Since low molecular weight substrate (Suc-Ala-Ala-Ala-N-PhNO$_2$) was used for E.I.C. determinations, such variations probably represented quantitative modifications of α_1 P_i levels in hamster serum. This *in vivo* result is directly opposed to the *in vitro* effect of these substances on α_1 P_i-porcine pancreatic elastase interactions. One plausible explanation is that CY 216 and CY 222 administration to hamsters stimulated the biosynthesis of α_1 P_i or its secretion by liver cells. Danazol, a synthetic androgen, was recently shown to possess similar characteristics [23]. Although side effects of this compound were minimal, muscle cramps and increase in the level of transaminases were observed in patients treated with this drug [23].

It can be stated that heparin fragments of low M_r possess a dual beneficial effect on the elastase–elastase inhibitor balance; they can inhibit leucocyte elastase and serum elastase and also increase the elastase inhibitory capacity of the blood, when injected to animals. Besides its beneficial influence on endothelial cells, heparin(s) possesses multiple other functions on blood constituents. It corrects hypercoagulability, enhances fibrinolysis, inhibits excessive complement activation, increases HDL levels, displaces lipoprotein lipase activity and lowers the serum triglycerides [2].

Our findings suggest that heparin derivatives could also correct the elastase-α_1 P_i imbalance observed in several pathological conditions.

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