INTERACTION OF HEPARIN WITH PROTEINS

Demonstration of different binding sites for antithrombin and lipoprotein lipase

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1. Introduction

Heparin is a polysaccharide with unique biological effects. It impedes the coagulation of blood by accelerating the reaction between antithrombin (a protease inhibitor in plasma) and a number of the proteolytic enzymes operating in hemostasis [1,2]. Furthermore, heparin has the ability to release lipases from tissue sites into the circulating blood (for review see ref. [3]). The antithrombin-stimulating effect of heparin presumably depends on specific binding between the polysaccharide and the protein, resulting in a conformational change in the inhibitor molecule [4,5]. The mechanism of lipase release is unclear but probably involves the formation of heparin–lipase complexes [3].

The relationship between the structure of heparin (for review see ref. [6]) and its biological properties has remained elusive. Recent studies have shown that heparin preparations may be separated with regard to affinity for antithrombin into two distinct fractions, one (in the following denoted HA-heparin) having high affinity for antithrombin and exceedingly higher anticoagulant activity while the other (LA-heparin) has low affinity for antithrombin and little or no anticoagulant activity [7,8]. No apparent structural dissimilarity between the two heparin species was noted. An oligosaccharide fraction thought to represent the antithrombin-binding site of heparin was isolated after digestion of the HA-heparin–antithrombin complex with bacterial heparinase [9]. The gross composition of this fragment was similar to that of HA- or LA-heparin; it was therefore concluded the binding of heparin to antithrombin may require a specific sequence of variously substituted sugar residues.

In the present study HA- and LA-heparins were compared on the basis of their ability to interact with lipases. Three types of experiments were conducted:

(a) Affinity chromatography of heparin on immobilized lipoprotein lipase.
(b) Heparin-induced release of lipoprotein lipase from immobilized heparin.
(c) Heparin-induced release of lipases into the circulating blood.

For technical reasons experiments (a) and (b) were performed with bovine enzyme whereas the in vivo experiment (c) was conducted with rats.

In all three systems the effects of HA- and LA-heparin were virtually indistinguishable, demonstrating that the specific structure which binds to antithrombin differs from that required for interaction with lipoprotein lipase (or hepatic lipase). In addition, these results demonstrate the occurrence of a native mammalian polysaccharide (LA-heparin) having

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potent lipase-releasing activity but negligeable effect on blood coagulation.

2. Materials and methods

Heparin (Stage 14, isolated from pig intestinal mucosa) obtained from Inolex Pharmaceutical Div., Park Forest South, Ill. USA) was purified by repeated precipitation with cetylpyridinium chloride [10] from 1.2 M NaCl. Radioactive heparin (specific activity 22.5 X 10^3 cpm/μg uronic acid) was isolated from mouse mastocytoma tissue after labelling *in vitro* with [^{3}H]galactose [9]. Unlabeled or radioactive heparin was fractionated by affinity chromatography on antithrombin–Sepharose as described [9]. The separation of unlabeled heparin is illustrated in fig.1; the [^{3}H]heparin showed an essentially similar elution pattern. The anticoagulant activities of unlabeled LA- and HA-fractions were 19 BP units/mg and 285 BP units/mg, respectively.

Lipoprotein lipase was purified from bovine skim milk essentially as described [11]; however, the treatment with rennin was exchanged for batch adsorption of the enzyme to heparin–Sepharose. The properties of the purified enzyme, were similar to those previously described [11].

The heparin–Sepharose used in the enzyme displacement experiments was prepared as described by Iverius [12], with the exception that only 40 μg heparin was added/ml cyanogen bromide-activated Sepharose gel.

Immobilized lipoprotein lipase was prepared by reacting the purified milk enzyme with activated Sepharose in the presence of acetylated heparin according to a method previously devised for the binding of antithrombin [8]. The final product contained about 0.9 mg protein/ml gel (50–60% recovery of the initial enzyme), as estimated by amino acid analysis.

Methods for the determination of uronic acid and radioactivity have been described [13].

Lipase activities were measured with ^{3}H-labeled triolein emulsified in gum arabic as lipid substrate [14]. Lipases from rat plasma were estimated as described in table 1.

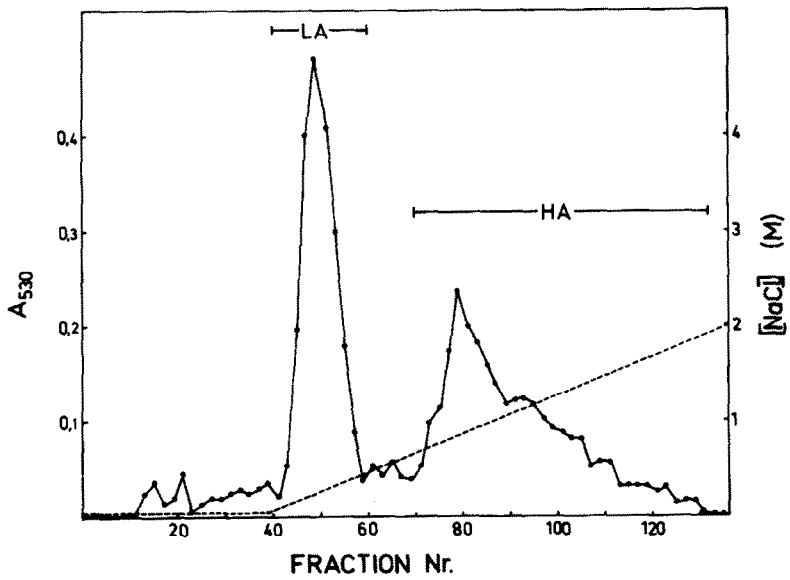

Fig.1. Fractionation of heparin by affinity chromatography on antithrombin–Sepharose [8]. Heparin (50 mg) was dissolved in 25 ml 0.05 M NaCl, 0.05 M Tris–HCl, pH 7.4 and applied to a 75 ml column of antithrombin–Sepharose, equilibrated with the same buffer. Gradient elution with sodium chloride (in 0.05 M Tris–HCl, pH 7.4) was started at the point indicated by the arrow. Effluent fractions of about 3 ml were collected, analyzed for uronic acid by the carbazole reaction (A_{530}) and pooled as indicated into low-affinity (LA) and high-affinity (HA) heparin, respectively. The fractions were desalted by dialysis and stored at −20°C. (– – –) NaCl concentration.

Table 1
Lipase activities in rat plasma after i.v. injections of HA- and LA-heparin

Heparin injected	Salt-resistant lipase[a] (^{3}H cpm × 10^{-4})					Serum-stimulated lipase[a] (^{3}H cpm × 10^{-4})				
	Plasma	Wash[b]	0.7 M eluate	1.5 M eluate	Recovery[c] (%)	Plasma	Wash[b]	0.7 M eluate	1.5 M eluate	Recovery[c] (%)
HA; 0.5 mg[d]	264	56	161	11	86	250	5	11	121	54
LA; 0.5 mg	213	47	123	15	86	271	24	6	126	57
HA; 10 mg	573	270	222	13	88	536	190	65	237	92
LA; 10 mg	490	277	196	9	99	553	188	17	199	72

[a]Salt-resistant lipase (considered to represent hepatic lipase) was determined in the presence of 1 M NaCl [14]. An approximate estimation of serum-stimulated lipase (considered to represent lipoprotein lipase) was obtained by subtracting the activity recorded at 1 M NaCl in the absence of serum from that recorded at 0.05 M NaCl in the presence of 5% human serum. These concentrations of salt and serum were selected on the basis of preliminary experiments; the concentrations previously found to be optimal for human or bovine lipoprotein lipase [14] were inhibitory to the rat enzyme. The enzyme activities are expressed as cpm [^{3}H]oleic acid liberated in 20 min at 37°C; all values are adjusted to represent 5 ml of plasma

[b]Lipases not retained by heparin–Sepharose should probably be at least partly ascribed to the presence of competitory heparin (the injected polysaccharide) in the liquid phase. However, see also Fielding, Shore and Fielding [16]

[c]Percent of initial enzyme activity in plasma sample

[d]Amount of heparin injected/kg body weight

Male Sprague-Dawley rats (250 g) were anaesthetized with ether and the heparin preparations (dissolved in 0.9% NaCl) were injected i.v. After 7 min blood was collected from the exposed abdominal aorta. A small amount of sodium citrate solution was added to prevent blood coagulation and plasma was obtained after centrifugation.

To about 5 ml plasma were added 1.5 mmol sodium chloride and 1 ml sedimented heparin–Sepharose gel (1–2 mg heparin/ml gel) at 4°C. After 90 min the mixture was poured into a small column (i.d. 10 mm). After collection of the effluent the column was washed with 20 ml 0.3 M NaCl in 5 mM sodium veronal buffer, pH 7.4, followed by 10 ml 0.5 M NaCl in the same buffer; the values in columns 'Wash' represent enzyme activity in the combined initial effluent and wash fractions. It was then eluted first with 22 ml 0.7 M NaCl and then with 22 ml 1.5 M NaCl in the veronal buffer. All buffers contained 1% bovine serum albumin.

3. Results

3.1. *Affinity chromatography of heparin on immobilized lipoprotein lipase*

The chromatography of [^{3}H]heparin on lipoprotein lipase–Sepharose is shown in fig.2A. The polysaccharide was essentially quantitatively retained by the gel and was eluted over a wide range of salt concentrations, with a recovery of more than 90% of the radioactivity applied to the column. Rechromatography of fractions eluted at low and at high salt concentration, respectively, yielded clearly separated elution patterns (fig.2B), suggesting that heparin molecules may differ with regard to affinity for lipoprotein lipase.

Chromatography of LA- and HA-heparin on immobilized lipoprotein lipase gave virtually identical elution profiles (fig.2C). This result thus stands in marked contrast to the separation obtained on antithrombin–Sepharose, where LA-heparin is eluted as a distinct peak at a lower salt concentration than is HA-heparin (fig.1).

3.2. *Displacement of lipoprotein lipase from immobilized heparin*

Lipoprotein lipase was incubated with suspensions of heparin–Sepharose, resulting in essentially complete binding of the enzyme to the immobilized polysaccharide. On addition of heparin to the suspensions lipoprotein lipase was displaced from the gel into the liquid phase (fig.3). Judging from the dose-response curves shown in fig.3, HA-, LA- and unfractionated heparin had about equal enzyme-displacing ability.

3.3. *Release of lipases by heparin in vivo*

The interaction of lipoprotein lipase with HA- and LA-heparins in vivo was studied by measuring lipase activities in blood plasma after intravenous (i.v.) injection of the polysaccharides into rats (see table 1

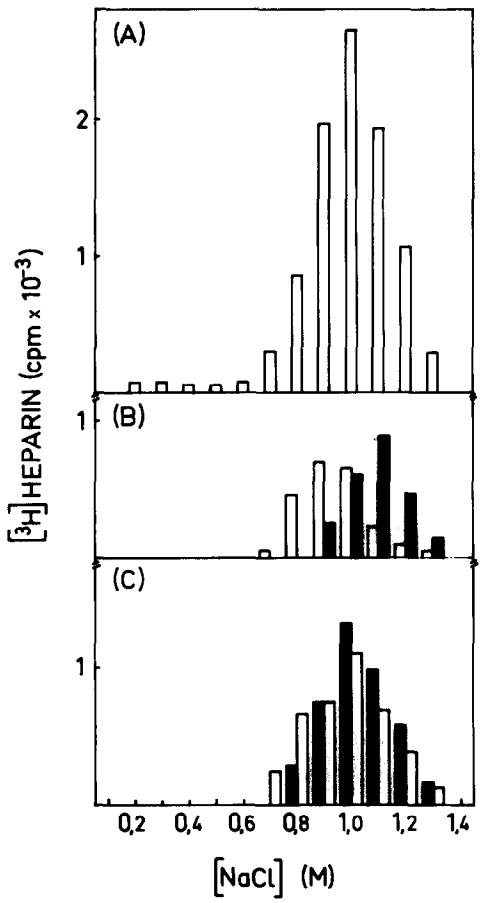

Fig.2. Affinity chromatography of [^{3}H]heparin on immobilized lipoprotein lipase. [^{3}H]Heparin (26 X 10^3 cpm or less) was dissolved in 0.2 ml 5 mM sodium veronal buffer, pH 7.4, containing 0.2 M NaCl, and was then applied to a small (0.8 ml) column of lipoprotein lipase–Sepharose, equilibrated with the same buffer at 4°C. After an equilibration period of 15 min the column was first washed with 4 X 2 ml of NaCl–veronal buffer and was then eluted in a stepwise manner with sodium chloride at increasing concentrations in 5 mM veronal buffer. At each salt concentration the effluent was collected in two successive 3 ml fractions; the radioactivity of the first fraction always exceeded that of the second one. The values recorded in the figures represent the sum of the two fractions. All runs were performed with the same column. (A) Unfractionated heparin. Two-thirds of the eluates at 0.7–0.9 M and at 1.1–1.3 M NaCl, respectively, were pooled, diluted with 5 mM veronal buffer and reapplied to the column (B), yielding the patterns represented by open and closed bars, respectively. (C) LA-Heparin (open bars) and HA-heparin (closed bars).

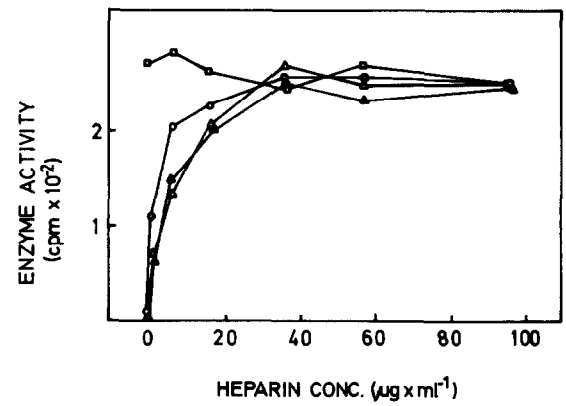

Fig.3. Release of lipoprotein lipase from heparin–Sepharose by addition of heparins to the liquid phase. Lipoprotein lipase (about 12 µg) was added to suspensions of heparin–Sepharose (100 µl sedimented gel) in 5 mM sodium veronal buffer, pH 7.4, containing 0.32 M NaCl and 1% bovine serum albumin. The mixtures (total vol. 1 ml) were placed in an oscillating shaker at 4°C in order to keep the gels suspended. After about 2 h the tubes were centrifuged (2000 X *g* for 2 min) and samples of the supernatants were assayed for lipoprotein lipase. Heparin solution (1 mg/ml) was added to the gel suspensions to give the desired concentration. The tubes were then shaken for another 20 min and centrifuged; samples of the supernatants were assayed for enzyme activity. The procedure was repeated with further additions of heparin. During the entire experiment a total volume of 35 µl was withdrawn for lipase assay while 100 µl of heparin solution was added. A control was carried out by incubating enzyme in the absence of gel or heparin. Unfractionated heparin (○———○), HA-heparin (△———△), LA-heparin (▲———▲), and control (□———□).

for details). On the basis of preliminary experiments with unfractionated heparin two dose levels were selected, corresponding to near maximal (10 mg heparin/kg body wt) and half-maximal (0.5 mg/kg), respectively, release of lipases. Injection of either HA- or LA-heparin resulted in the appearance of salt-resistant as well as serum-stimulated lipase in the blood (table 1). The identities of the two lipases (considered to represent hepatic lipase and lipoprotein lipase, respectively) were ascertained by chromatography on heparin–Sepharose.

The salt-resistant lipase was largely recovered in a 0.7 M NaCl eluate whereas the serum-stimulated lipase appeared in a 1.5 M fraction (table 1), in agreement with previous separations of these enzymes [14,15]. With both heparins more lipase activity was released

by the higher dose. HA- and LA-heparin did not differ significantly with regard to type or amount of lipase released.

4. Discussion

In all systems tested HA- and LA-heparin showed equal ability to interact with lipoprotein lipase. Since the two types of heparin are differentiated solely on the basis of affinity for antithrombin these results demonstrate that the specific structure required for interaction with antithrombin differs from that required for interaction with lipoprotein lipase. This conclusion is further supported by the finding that the anticoagulant activity of heparin may be selectively eliminated by chemical modification of the polysaccharide [17]. The occurrence in mammalian tissues of a native polysaccharide (LA-heparin) that affects lipoprotein lipase but lacks effect on blood coagulation may have important physiological and therapeutic implications. The selective interaction between LA-heparin and lipoprotein lipase has recently been utilized in purification of the enzyme from human plasma [18]. The detailed structural features of the heparin molecule responsible for its diverse biological effects remain to be elucidated.

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