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Effect of Very Low Molecular Weight Heparin-Derived Oligosaccharides on Lipoprotein Lipase Release in Rabbits

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Summary

Oligosaccharide fragments of heparin were prepared using flavobacterial heparinase. Following sizing, these oligosaccharide fractions were administered (i.v.) to rabbits and were examined for their ability to release lipoprotein lipase. The decasaccharides (dp = 10, M_r avg = 2 800) were the smallest oligosaccharides which resulted in substantial lipase release. The plasma lipase levels obtained with decasaccharides were comparable to low molecular weight heparin and one-third those obtained when heparin was administered at an equivalent dose. The peak plasma lipase concentration was observed 10 min following heparinization and fell off rapidly over the 60-min time course. The lipase release activity paralleled the in vivo pharmacokinetics of the heparin and decasaccharide sample as determined by monitoring their anti-Factor Xa activity. No activation of purified bovine milk lipoprotein lipase or plasma lipase was detectable at the concentrations studied, indicating that the increase in circulating lipolytic activity was due entirely to release. Lipoprotein lipase accounted for a major portion of the released activity with hepatic triglyceride lipase representing the remainder of the lipolytic activity. The sized decasaccharide sample was characterized with regards to its structure and anticoagulant activity. The decasaccharides exhibited reduced anticoagulant activity possibly making it a better drug candidate in the treatment of atherosclerosis.

Key words: *Anticoagulant – Atherosclerosis – Heparin – Lipase release – Lipoprotein lipase – Oligosaccharides – Rabbits*

Introduction

Heparin, a highly sulfated glycosaminoglycan, is currently being used as an anticoagulant and may have a role in the prevention of atherogenesis [1–3]. Following i.v. administration, heparin releases lipoprotein lipase (LPL) from the endothelium. This may result in increased triglyceride lipolysis occuring in the bloodstream thereby lowering the concentration of cholesterol-rich remnant particles in contact with the arterial wall

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[3]. In addition of LPL, hepatic triglyceride lipase (HTGL) is also released in the blood. It is, therefore, necessary to separate out the activities of LPL and HTGL [4–7] to assess the effect of heparin on each of these enzymes.

The effect of heparin on both the release and activation of LPL and HTGL has been studied in a number of different animal models including humans [8]. Heparin fractions having a low affinity for antithrombin (ATIII) have been tested for lipase release activity in an effort to eliminate potential hemorrhagic complications associated with heparin's anticoagulant activity [9,10]. These studies indicated that anticoagulant and lipase release activities are indeed separable. One approach towards lowering heparin's anticoagulant activity as well as improving its bioavailability, particularly when administered by a route other than i.v. [11], is to use very low molecular weight (VLMW) heparin obtained either through fractionation or depolymerization. Different low molecular weight (LMW)-heparin preparations (M_r 4000–6000) gave contradictory results, either showing markedly diminished [9,12] or similar [13,14] lipase release activity when compared to commercial heparin (M_r avg 14 000). The relationship of lipase releasing activity to the charge or degree of sulfation of heparins and heparan sulfates from various sources has also been examined [15]. The compounds examined contained between 1 and 3 sulfates per disaccharide unit and the degree of sulfation correlated linearly to the lipase release activity [15].

We have prepared oligosaccharide fragments from heparin by enzymatic depolymerization. These fragments are then fractionated into sized oligosaccharide mixtures of defined degree of polymerization (i.e., dp = 4,6,8,10). In addition, subfractions of commercial heparin have been prepared using affinity chromatography on immobilized purified bovine milk LPL. The effect of these oligosaccharide samples and heparin fractions on lipase release and activation as well as their pharmacokinetics are reported.

Materials and Methods

Heparin, its depolymerization and oligosaccharide preparation

Porcine mucosal heparin was from Hepar, Franklin, OH and LMW heparin was from Calbiochem, La Jolla, CA. The porcine heparin was depolymerized with flavobacterial heparinase [16]. The product mixture was fractionated by applying (0.1 g/2 ml) to a Fractogel TSK low pressure column (HW50S 17 × 2.5 cm and HW40F 80 × 2.5 cm run in series) and eluted at 0.3 ml/min with 1 M sodium chloride [17]. The different sized fractions (dp 4–10) were collected, dialyzed, freeze dried and then rechromatographed to obtain the sized tetra-, hexa-, octa-, and decasaccharides [18].

Bovine milk LPL purification and characterization

LPL was prepared from fresh bovine skim milk by affinity chromatography on heparin–Sepharose and stored in glycerol/buffered (10 mM Tris-hydrochloride, pH 7.4) 1 M sodium chloride (1 : 1, vol/vol) at −70°C [19,20]. Discontinuous SDS–polyacrylamide gel electrophoresis (PAGE) was performed in 10% polyacrylamide tube gels containing 1% SDS [21]. Molecular weight standard markers (Sigma Chem. Co., St. Louis, MO) were used and protein was measured both in gels and in solution by Coomassie dye [22].

Affinity fractionation of heparin

Bovine milk LPL (500 μg) was covalently immobilized [19] to 4 ml of CH-Sepharose 4B (Pharmacia, Uppsala, Sweden). Excess heparin (500 μg) was loaded onto the column (5 cm × 1 cm) packed with the immobilized enzyme and equilibrated with 150 mM sodium chloride, 20 mM Tris-hydrochloride, pH 7.2. The column was washed with 5 column volumes of the same buffer (low LPL-affinity heparin) followed by high salt wash with 1 M sodium chloride, 20 mM Tris-hydrochloride, pH 7.2 (high LPL-affinity heparin). The high and low affinity samples were dialyzed against 100 volumes of water and freeze-dried.

Characterization of heparin and sized oligosaccharides

The average molecular weight of heparin and the sized oligosaccharides was determined by HPLC-GPC [18]. Both HPLC-GPC and uronic acid determination by carbazole [23] were used to determine degree of polymerization (dp) of the sized oligosaccharides [18,24]. Sulfate determination, performed on pyrolyzed heparin and oligo-

saccharide samples by ion-chromatography [18] was used in combination with dp to determine average molecular weight. Heparin and the oligosaccharides (freeze-dried 3 times from D_2O) were examined by [^{1}H] nmr (360 MHz) and [^{13}C]nmr (90.6 MHz) at 25°C in D_2O using DSS as internal standard [24]. The anticoagulant activity of heparin fragments was measured by aPTT and factor Xa coagulation assays [25].

In vivo pharmacokinetic study of heparin and oligosaccharides and release of plasma lipases

Female New Zealand white rabbits weighing 4–5 kg were anesthetized using a 3-way mixture of ketamine, acepromizine and xylazine (0.22 ml/kg, i.m.). The femoral artery, just distal to the lateral circumflex offshoot, was isolated and cannulized with appropriate size polyethylene tubing. Blood samples were collected (over 4% sodium citrate, 9 : 1) at timed intervals following i.v. administration of either heparin or oligosaccharides (200 μg/kg) in the marginal ear vein (time = 0). Blood was centrifuged for 15 min (IEC Clinical Centrifuge, Needham, MA) and plasma stored at −70°C until assayed.

Enzyme assays

Lipase activity was measured by radioassay [19,26]. Plasma or bovine milk lipase (41 μl) and double distilled water (34 μl adjusted to pH 8.6) was incubated with 125 μl of the trioleoylglycerol emulsion containing: 4 mg/ml unlabeled trioleoylglycerol (Sigma, St. Louis, MO), 1.6 μCi/mL Triolene [9,10-[^{3}H](N)] (specific activity = 0.5 mCi/mL, New England Nuclear, Boston, MA), 61 mg/ml fatty acid-free bovine serum albumin (Sigma), and 0.06% [w/v] Triton X-100 (Sigma) in 0.3 M Tris-hydrochloride buffer, pH 8.6, containing 0.1 M sodium chloride. For a final concentration of 1 M sodium chloride the double distilled water was replaced with 5.3 M sodium chloride. The emulsion was vortexed vigorously to produce a homogeneous suspension. Following addition of samples to the emulsion substrate, the mixtures were incubated with shaking at 37°C for 50 min. The enzyme reaction was terminated by rapid addition of 3.25 ml of methanol/chloroform/heptane (1.41 : 1.25 : 1.00, v/v/v) and followed immediately with the addition of 1.05 ml of a certified potassium carbonate buffer solution, pH 10.0 (Fisher Scientific, Fairlawn, NJ). The capped tubes were vortexed briefly (3 s) and centrifuged at 1000 rpm for 15 min, 0.5 ml aliquots of the upper phase were transferred to counting vials containing 18 ml of scintillation liquid (3a70B Complete Cocktail, Research Products Int., Mount Prospect, IL) and counted for 10 min in a Beckman CS-100C Liquid Scintillation Counter.

Results

Porcine mucosal heparin was depolymerized using flavobacterial heparinase to prepare fragments (Fig. 1) which were then fractionated on the basis of dp into sized oligosaccharides. The heparin and heparin-derived oligosaccharides were characterized with regards to their degree of polymerization (dp), average molecular weight and sulfation level (Table 1).

LPL was purified from bovine milk by heparin affinity chromatography [19,20]. The center cut of the activity peak eluting from this column was analyzed for purity by SDS-PAGE and showed a single band with $M_r = 66000$. This purified enzyme was then immobilized on CH-Sepharose 4B [19]. The LPL column (4 ml containing 500 μg protein, 100 μg of heparin binding capacity) was used to prepare high LPL-affinity heparin.

In vitro studies were performed to measure anticoagulant activity and lipase activation by heparin and oligosaccharides. The in vitro activity, of the sized oligosaccharides and high LPL-affin-

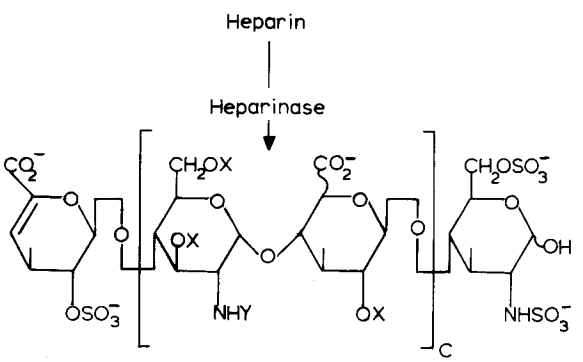

Fig. 1. The structure of heparin fragments formed using Flavobacterial heparinase: X = H or SO_3; Y = H, SO_3, or $COCH_3$; and C = 0, 1, 2, 3, 4,...(for decasaccharide (dp = 10), C = 4).

TABLE 1

STRUCTURAL CHARACTERISTICS OF HEPARIN AND HEPARIN-DERIVED OLIGOSACCHARIDES

	Degree of polymerization [a] (dp)	Sulfates per disaccharide repeating unit [b]	Average molecular weight [c] (M_r avg)
Tetrasaccharides	4	2.0	1157
Hexasaccharides	6	1.7	1556
Octasaccharides	8	1.6	2033
Decasaccharides	10	2.1	2804
Low molecular weight heparin	avg 13	2.7	3700
Heparin	16–94 avg 46	2.7	14000

[a] By nmr, gpc or FAB-MS [17,18,24].

[b] By ion-chromatography following pyrolysis [18].

[c] To a saccharide backbone of the appropriate dp the measured level of sulfation is added and the molecular weight is calculated for the heparin fragments [18].

ity heparin samples, against thrombin and factor Xa was determined by coagulation assays (Table 2). No significant effect on the lipase activity contained in plasma or a 5 nM solution of purified bovine LPL was observed when either heparin or the sized oligosaccharide mixtures were added at concentrations up to 14 μg/ml in the assay mixture.

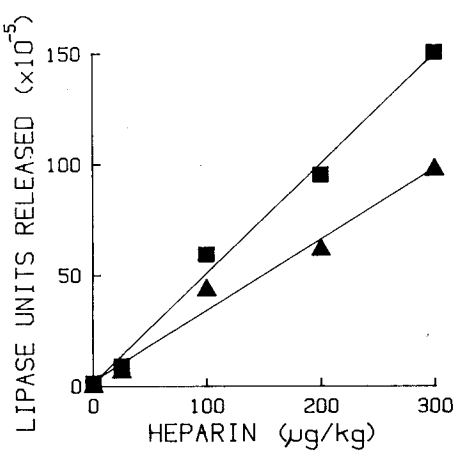

Fig. 2. Dose–response curve for heparin-induced release of lipase. Heparin was administered i.v. to rabbits (n = 4) at doses ranging from 25 to 300 μg heparin/kg body weight. Lipolytic activity (1 unit = 1 μmol free fatty acid formed min^{-1} mg^{-1} total plasma protein) was determined in a plasma sample taken 10 min after administering heparin in assay tubes containing low salt (0.1 M NaCl to measure total lipolytic activity LPL + HTGL) (■) and high salt (1 M to measure HTGL only) (▲) buffers.

TABLE 2

LIPASE RELEASE AND ANTICOAGULANT ACTIVITIES OF HEPARIN-DERIVED OLIGOSACCHARIDES

Sample	Wt% of heparin's activity [a]		Wt% of heparin's lipase release activity [b]
	aPTT	anti-Xa	
Tetrasaccharides	0.8	3	8
Hexasaccharides	2.8	29	10
Octasaccharides	15	35	9
Decasaccharides	55	51	30
Low molecular weight heparin	39	46	32
High lipase affinity heparin	208	173	119
Heparin	100.0	100	100

[a] Measured against a standard curve constructed with heparin (USP 167 U/mg) [16,25].

[b] Increase in lipolytic activity following i.v. administration of 200 μg/kg sample, where one lipase unit = 1 μmole of free fatty acid formed min^{-1} mg^{-1} total plasma protein. Peak values measured in 0.1 M sodium chloride 10 min after administering the samples. The plasma lipase level before sample administration was 5×10^{-5} lipase units and after administration of 200 μg/kg heparin was 1×10^{-3} lipase units. Control studies in which either saline or heparin-derived disaccharide were administered showed no elevation of plasma lipase levels. Average value of 10 experiments for heparin; 8 experiments for decasaccharides; 4 experiments for the commercial low molecular weight heparin; 2 experiments for tetra, hexa and octasaccharides and 1 experiment for the high lipase-affinity heparin.

The in vivo studies, on heparin induced lipase release, were begun by constructing a dose-response curve in rabbits (Fig. 2). A dose (200 μg/kg) in the center of this curve was selected for further study. Each of the samples were administered at this dose and their pharmacokinetics were examined. These experiments (Fig. 3A) showed that each sample gave a peak plasma concentration (as measured by anti-FXa activity) 10 min following i.v. administration except of the tetrasaccharides which peaked at 22 min. Each sample was cleared (> 90%) from the circulation within 1 h of administration.

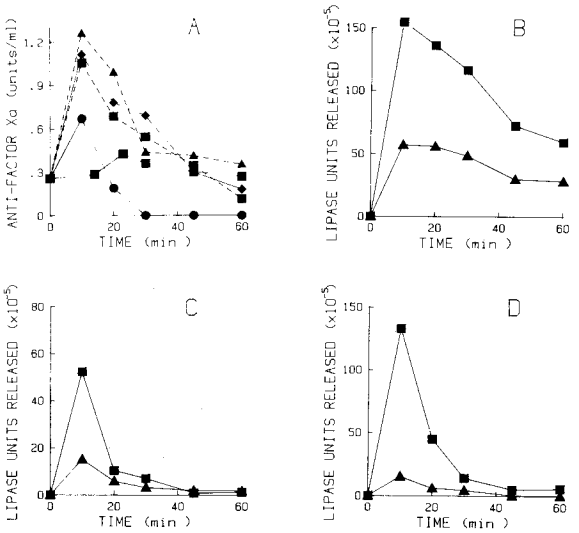

Fig. 3. Pharmacokinetics of heparin and oligosaccharides administered i.v. to rabbits. *A*: The plasma anti-factor Xa activity in units/ml (determined by coagulation assay against a heparin standard curve) is plotted against time in minutes following the i.v. administration (at t = 1 min) of 200 μg/kg of each drug to rabbits (n = 2). Heparin (▲) and the decasaccharides (■) are drawn as dotted lines while tetrasaccharides (■), hexasaccharides (●), and the octasaccharides (◆) are drawn as solid lines. *B*: Lipase activity released into the plasma following i.v. administration of heparin (200 μg/kg) to rabbits (n = 4). *C*: Lipase activity released into the plasma following i.v. administration of decasaccharides (200 μg/kg) to rabbits (n = 4). *D*: Lipase activity released into the plasma following i.v. administration of high LPL-affinity heparin (200 μg/kg) to a single rabbit. The lipolytic activity plotted in *B*, *C*, and *D* were based on assays performed in the presence of 0.1 M sodium chloride (■) to determine total lipolytic activity and in the presence of 1 M sodium chloride (▲) to determine the activity associated with HTGL.

The peak lipolytic activity released into the circulation following the administration of heparin, the sized oligosaccharides, and high LPL-affinity heparin and LMW heparin are given in Table 2. Of the oligosaccharide fragments only the decasaccharide sample showed substantial lipase release activity. The plasma lipase levels obtained with the decasaccharide sample was the same as that obtained using a commercial LMW heparin. The high LPL-affinity heparin resulted in high levels of circulating lipolytic activity. Also shown in Table 2 is the anticoagulent activity measured by aPTT and anti-Xa coagulation assays.

The samples having the highest lipase release activity, heparin, the decasaccharides, and high LPL-affinity heparin were explored further by examining the relation between their circulating concentration and the level of lipolytic activity measured over time (Figs. 3B–3D). In all cases the peak lipolytic activity in the circulation corresponds to the peak concentration of the drug administered. The lipolytic activity was also assayed at high salt concentrations which inhibit LPL activity while leaving HTGL activity intact [4]. The results of these assays indicate that about half of the released lipolytic activity is due to LPL. Activation studies were performed in vitro by measuring the effect of exogenously added heparin and oligosaccharides to plasma which contained lipolytic activity. These studies gave no measurable lipase activation at the doses used to study lipase release. The clearance of lipolytic activity from the circulation paralleled the clearance of drug in the case of heparin (Figs. 3A and B) and high lipase-affinity heparin (Figs. 3A and D). Lipolytic activity released by the decasaccharides is cleared from the circulation at a faster rate than is the drug itself (Figs. 3A and C).

Discussion

The effect of heparin on lipase release and activation have been studied by a number of workers [8,27]. However, heparin is a highly polydisperse (M_r = 5 000–40 000) [28], heterogeneous mixture [29] making it difficult to get precise information regarding its mode of lipolytic activity. In addition, heparin is such a potent anticoagulant that this activity can mask its other biological activities [30]. Affinity chromatography has been used to fractionate heparin in an effort to separate heparin's lipase release activity from its anticoagulant activity. Bianchini et al. [10] have fractionated heparin using ATIII affinity chromatography and have examined the ability of these fractions to release lipase in vivo. Heparin with a high affinity for ATIII also showed a higher lipase release activity than did the fraction with a low affinity for ATIII [10]. We have fractionated heparin using LPL-affinity chromatography and then tested the high LPL-affinity fraction for its ability to release lipolytic activity in vivo.

An alternative approach to separating heparin's

activities involves its enzymatic depolymerization to low molecular weight oligosaccharides. Heparinase cleaves at specific sites in the heparin polymer [24] including its ATIII binding site [31]. Using this technique we have prepared VLMW heparin fragments and separated sequences possessing various activities including: anticoagulant and antithrombotic activities [25,31]; anti-angiogenic [32] and angiogenic activity towards endothelial cells [33–35]; effect on smooth muscle cell proliferation [36]; and fragments which inhibit complement activation [17,37]. Towards these ends we have separated the oligosaccharide components of this mixture into fractions with dp 4–10, characterized them by chemical and spectroscopic techniques and have examined their ability to increase the circulating level of lipolytic activity.

There are conflicting reports in the literature as regards the effect of heparin's molecular weight on lipase activation and release [9,12–14]. Our heparin-derived oligosaccharides vary in average molecular weight from 1 100 to 3 000. We observe that at a dose of 200 μg/kg the decasaccharides (M_r avg 2 800) result in an increase in circulating lipase levels comparable to that associated with LMW-heparin (M_r avg 3 700) and about one-third of the lipase release activity of heparin (M_r avg 14 000). Oligosaccharides smaller than decasaccharides showed only slight increases in circulating lipase when administered at the same dose. The smallest heparin previously reported to show a comparable lipase releasing activity was a 4 000–6 000 molecular weight fraction [13].

Casu et al. [14] showed that for heparins from different sources, having 1 to 3 sulfates per disaccharide repeating unit, the lipase activity increases with degree of sulfation. Our heparin oligosaccharides (dp = 4–10) have 1.6–2.1 sulfates per disaccharide repeating unit (Table 2). Although the decasaccharide sample, with the highest degree of sulfation, results in the highest lipase release, no clear correlation is apparent between degree of sulfation and lipase release activity for the oligosaccharides examined. A minimum size requirement for lipase release activity may be a more likely explanation than the degree of sulfation for the large activity difference between the octasaccharide and decasaccharide mixtures.

An initial screen of heparin and the various sized oligosaccharides (Table 2) clearly indicated that heparin, the decasaccharides, and high LPL-affinity heparin, warranted a more detailed examination as these samples all induce high levels of circulating lipolytic activity.

In vivo pharmacokinetics for heparin, decasaccharides and high lipase-affinity heparin were measured in the rabbit. The parallel disappearance of circulating drug and circulating lipase activity, for heparin and high lipase-affinity heparin, supports the view that heparin simply acts by exchanging lipase off the endothelial surface and that as the drug is cleared, the circulating lipase may then simply be removed by metabolism in the liver [38]. In the case of the decasaccharide, however, the lipolytic activity is cleared at a faster rate than the drug is removed. This might be the result of differences in either the mechanism or rate of clearance or the catalytic stability of the circulating heparin-lipase and decasaccharide-lipase complexes. Olivecrona et al. [38] have shown that bovine milk LPL is a dimer and has 2 heparin binding sites. Clarke et al. [39] have shown that heparin chains of average molecular weight greater than 10 000 form a 1 : 1 complex with LPL while heparin chains with average molecular weight less than 10 000 form a 2 : 1 complex. The different stoichiometry of these complexes suggests that the role of polymer chain size in heparin stabilization of LPL requires further investigation.

The activation of inhibition of serum lipases by heparin has been reported [8,27]. Heparin activation of purified milk LPL has also been reported by Shirai et al. [40] although Olivecrona et al. [41] report no activation and ascribe the observed effect to heparin stabilization of LPL. To determine whether the increase in circulating lipolytic activity was due primarily to release, as opposed to activation, in vitro studies were performed. Heparin or the oligosaccharide mixtures were added to both purified bovine milk lipase and plasma sample containing lipolytic activity and the change in lipase activity was measured. No heparin or oligosaccharide activation or inhibition of lipase was observed at the concentrations used in the release studies.

Lipase activity was assayed in the presence of high and low salt to distinguish the relative amounts of LPL and HTGL released by heparin

[4]. From the results of these assays, the lipolytic activity released by heparin, decasaccharides, and high LPL-affinity heparin can be equally ascribed to LPL and HTGL.

This work has shown for the first time that a component as small as a decasaccharide has substantial lipase release activity and that heparin with high LPL-affinity is similar to unfractionated heparin in its LPL releasing activity. Further reduction in the anticoagulant activity of the decasaccharides may result in a better drug candidate than heparin for the treatment of atherosclerosis. Recently we have been able to separate the sized heparin decasaccharides into a large number of components by SAX HPLC [18] and are in the process of identifying and characterizing [24–31] the components of the decasaccharide mixture which exhibit high lipolytic and low anticoagulant activities. The capacity of continuously infused heparin fragments to maintain elevated plasma lipase levels also needs to be examined.

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