Enzymic Depolymerization of Macromolecular Heparin as a Factor in Control of Lipoprotein Lipase Activity

(gel filtration/intestinal depolymerase/inhibitors/activators)

ALAN A. HORNER*

Departments of Banting and Best Medical Research and Physiology, University of Toronto, Toronto, Ontario, Canada

Communicated by Charles H. Best, August 14, 1972

ABSTRACT Macromolecular heparin from rat skin shows poor lipoprotein lipase-releasing activity in vitro and is a potent inhibitor of rat-heart lipoprotein lipase in vitro. Rat-skin heparin is depolymerized by incubation with the 100,000 × g supernatant from a sonicated homogenate of rat small intestine. The depolymerized products, fractionated by gel filtration, range from inhibitors to activators of lipoprotein lipase as molecular size decreases. Depolymerized rat heparin in the same molecular size range as commercial heparin from pig intestinal mucosa has about two-thirds the activity of the commercial preparation, both in vitro and in vivo.

Hahn (1) first demonstrated the lipemia-clearing effect of heparin in vivo. The enzyme involved was characterized in heart muscle as a heparin-activated lipoprotein lipase (LPL) (2). This enzyme has since received great attention because of its important role in control of lipid transport.

Heparin appears to be involved in the activation, release, and stabilization of LPL and may actually link the enzyme to its lipid substrate (3). In all experiments with LPL, commercial heparins of bovine or porcine origin, with molecular weights about 10–20 × 10⁴ (4), have been used for convenience. It has been assumed that endogenous heparin, released into the circulation from the mast cells in which it is synthesized and stored (5), acts in the same way as exogenous heparin. The mechanism for the release of heparin is not understood.

A large proportion of the work on LPL has been done with rats. The present work was stimulated by my observation that heparin from rat skin (RS heparin) has a molecular weight of 1.1 × 10⁴ (6, 7). RS heparin is a multichain structure, composed of heparin chains of the same order of size as commercial heparins, covalently linked to a higher molecular weight polysaccharide core to form a macromolecular complex (7).

This paper shows that macromolecular RS heparin has poor LPL-releasing activity in vitro and actually inhibits LPL in vitro. Rat small intestine contains an enzyme(s) that can depolymerize RS heparin. Lower molecular weight heparin formed by this reaction shows good activity both in vivo and in vitro.

MATERIALS AND METHODS

Agarose gel granules (Bio-Gels A-50 m and A-1.5 m, both 100–200 mesh) were obtained from Bio-Rad, and agarose powder for gel electrophoresis was from Bausch and Lomb.

Abbreviations: LPL, lipoprotein lipase; RS heparin, rat-skin heparin; PM heparin, pig mucosal heparin; FFA, free fatty acids.

* Address reprint requests to Department of Physiology, University of Toronto, Toronto, Ontario, Canada.

Electrophoresis was performed in agarose gel, 0.7 g/100 ml in 0.87 M acetic acid, adjusted to pH 3.0 with LiOH. The gel was prepared as a thin layer on plastic film (8). Samples (1 μl) of heparin solutions (2 mg/ml) were run for 1 hr at 30 V/cm in a water-cooled thin-layer electrophoresis tank (Shandon). Gels were stained with an aqueous solution of toluidine blue (1 g/liter) and destained in cold water.

Commercial pig mucosal heparin (PM heparin), anticoagulant activity 142 IU/mg, was from the same batch used in earlier work (7, 9).

Macromolecular heparin was prepared from rat skin as published (7), except that the gel filtration step used Bio-Gel A-50 m instead of Sagavac 4F.

Assays of LPL Activity. The in vitro effect of heparin on LPL in rat heart acetone–ether powder was measured by a modification of the method of Mayes and Felts (10). Male Wistar rats (250–300 g) were bled by cardiac puncture under ether anaesthesia and were then killed by injection of air into the heart. Blood was allowed to clot in glass tubes and centrifuged for 20 min at 1,000 × g at room temperature; the serum was kept at 4°. Hearts were excised, cooled in ice, and extracted with acetone and ether (10). The heart powder was homogenized in 25 mM NH₄OH, adjusted to pH 8.6 with HCl, at a concentration of 10 mg/ml with a Ten Broeck glass homogenizer at 4°. The homogenate was stirred magnetically for 30 min at 4° and then left to stand in ice for 30 min, to allow connective tissue debris to settle out. The supernatant was then used immediately.

The assay system contained: 0.50 ml of rat-heart homogenate, 0.50 ml of bovine serum albumin (Cohn fraction V, Sigma, 15 g/100 ml, pH 8.0), 0.20 ml of 1.35 M Tris-HCl, pH 8.0, 0.10 ml of activated triglyceride substrate (3 volumes of 10% Intralipid emulsion (A.B. Vitrum, Stockholm, Sweden) mixed with 7 volumes of rat serum and incubated for 30 min at 37°), and 0.20 ml of 0.15 M NaCl containing heparin. Heparin-free controls were extracted at zero time or after incubation.

Reaction mixtures were incubated with continuous gentle shaking at 37° for 1 hr and then cooled in ice. Free fatty acids (FFA) were extracted from 1-ml aliquots by the method of Dole (11), as modified by Trout et al. (12). 2-ml Aliquots of the final acid-washed heptane extracts were titrated with 0.10 M tetraethylammonium hydroxide in ethanol, with thymol-phenol as indicator, in a titration cell of the type described by Salaman and Robinson (13).

The in vivo effect of heparin on LPL activity in plasma was measured by taking small blood samples at suitable intervals after injection of heparin. A simple procedure involving
minimal surgery was adopted. Male Wistar rats (250-300 g) were anaesthetized by intraperitoneal injection of sodium pentobarbital (4 g/100 ml, about 2 ml/kg body weight), and a small piece of skin was removed to expose the xiphoid process and lower ribs. An injection of heparin in 0.15 M NaCl (2.5 ml/kg body weight) was made directly through the rib cage into the heart, and four samples of blood (about 1.5 ml each) were taken 1, 3, 7, and 12 min later, with 23-gauge needles and plastic syringes. 1.35 ml of each blood sample was immediately mixed with 0.15 ml of 0.13 M sodium citrate in a calibrated tube chilled in ice and centrifuged (500 × g for 20 min at 4°C). Samples of the plasmas were assayed for LPL activity.

The assay for LPL activity in plasma was essentially similar to the in vitro assay of rat heart-plug homogenate. The assay mixture consisted of: 0.50 ml of plasma, 0.50 ml of albumin solution, 0.20 ml of 1.35 M Tris-HCl, pH 8.0, 0.10 ml of activated triglyceride substrate, and 0.20 ml of 0.15 M NaCl. Controls containing plasma from rats injected with 0.15 M NaCl were extracted at zero time or after incubation. Conditions for incubation, extraction, and titration were identical to the heart-plug homogenate assay.

**Table 1. LPL-releasing activity of preparations of rat heparin in vivo:**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dosage (μg/kg)</th>
<th>No. of observations (n)</th>
<th>Mean peak activity (± SD) (μmol FFA/ml assay mixture per hr)</th>
<th>Activity, relative to PM heparin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS heparin</td>
<td>500</td>
<td>5</td>
<td>1.38 ± 0.38</td>
<td>13</td>
</tr>
<tr>
<td>A-4</td>
<td>125</td>
<td>4</td>
<td>1.70 ± 0.40</td>
<td>62</td>
</tr>
<tr>
<td>B-4</td>
<td>125</td>
<td>6</td>
<td>1.64 ± 0.46</td>
<td>59</td>
</tr>
<tr>
<td>C-3</td>
<td>125</td>
<td>5</td>
<td>1.84 ± 0.34</td>
<td>66</td>
</tr>
<tr>
<td>C-4</td>
<td>125</td>
<td>6</td>
<td>1.78 ± 0.32</td>
<td>65</td>
</tr>
</tbody>
</table>

**Intestinal Depolymerase Preparation.** Small intestines from male Wistar rats (200-250 g) were rinsed with ice-cold 0.15 M NaCl, cut into about 1-cm segments, strained on cheese cloth, and suspended in 50 mM Tris-HCl-1 mM EDTA pH 7.4 (15 ml/25 g of moist small intestine). 40-ml Batches were homogenized for 2 min at high speed in a Virtis 45 homogenizer with ice-water as coolant. The homogenate was sonicated in batches of 6 ml in a plastic tube with a Branson Sonifier with microtip for 2 min at 60 W. The combined sonicates were centrifuged (12,000 × g for 30 min at 4°C), and the supernatant was again centrifuged (100,000 × g for 30 min at 4°C, International B-60 centrifuge with SB-283 rotor). The supernatant was dialyzed in Visking tubing against the homogenizing buffer at 4°C for 8 hr. A continuous stream of nitrogen bubbles provided vigorous stirring.

**Incubation of RS-Heparin with Intestinal Depolymerase.** A three-stage depolymerization of RS heparin, in which the enzyme to substrate ratio was kept constant, was performed as follows: RS heparin (9.0 mg) was incubated for 16 hr at 37°C with a depolymerase preparation from six rats, and heparin product A was recovered. Two-thirds of product A was incubated with a depolymerase preparation from six rats, and product B was recovered. Half of product B was incubated with a depolymerase preparation from three rats, and product C was recovered. In each experiment, after incubation solid NaCl was added to a concentration of 0.5 M, followed by 3 volumes of ethanol. After they remained overnight at 4°C, the solids were recovered by centrifugation, washed with ethanol, and dried under reduced pressure. The product was suspended in 1.2 M NaCl, and heparin was precipitated as the cetylpyridinium complex by addition of excess cetylpyridinium chloride (1 g/100 ml of 1.2 M NaCl) (14). The insoluble complex was recovered by centrifugal filtration and extracted with 2 M NaCl. Heparin was precipitated with ethanol by procedures described for rat-skin heparin (7).

**Gel Filtration of Heparin with Agarose Gels.** Samples (each 2.5 mg) of depolymerized RS heparin products A, B, and C were dissolved in 0.5 M NaCl (1 ml) and applied to a column of Bio-Gel A 1.5 m (60 × 1.5 cm, volume 103 ml) equilibrated with 0.5 M NaCl. PM heparin (2.0 mg) was run through the column. The effluent was monitored for activity of preparations of rat heparin in vivo.

**Fig. 2.** Gel filtration on a column of Bio-Gel A 1.5 m agarose gel granules. Δ, C, and •, depolymerized RS heparin products A, B, and C, respectively; ◊, PM heparin. V₀ = void volume, determined with tobacco mosaic virus. Vₜ = total volume of the column.
same column. In each case, 3-ml fractions were collected by drop counter, and the uronic acid content was determined by the carbazole method (15).

**RESULTS**

The recoveries of depolymerized RS heparin products A, B, and C by precipitation with cetylpyridinium chloride were 97%, 93%, and 98%, respectively. As controls, intestinal depolymerase was prepared from 4 rats, and the sediments removed from this preparation by centrifugation were combined and digested with Pronase. These preparations were precipitated with ethanol and treated with cetylpyridinium chloride in 1.2 M NaCl. No uronic acid-containing material was recovered, i.e., no endogenous heparin was found in rat small intestine.

Fig. 1 shows the electrophoresis of products A, B, and C in agarose gel at pH 3.0. The presence of two components, both with higher mobilities than undegraded RS heparin, with the proportion of the faster-moving component increasing with each depolymerization, is analogous to the way in which ascorbic acid breaks down RS heparin (7).

Fig. 2 shows the gel filtration elution profiles of the three depolymerization products. These show a decrease in the proportion of high molecular weight material, excluded from the gel, and a corresponding increase in the proportion of lower molecular weight material, included in the gel, after each treatment with intestinal depolymerase. Although the yield of the smaller product increased with successive incubations, the overall molecular weight distribution of this fraction did not change. The average molecular size of the smaller product was greater than that of PM heparin, run through the same column for comparison, but the molecular size ranges of the two overlapped. Arbitrary cuts into four fractions were made as indicated in Fig. 2, giving products A-1, 2, 3, and 4, B-1, etc. The heparin in these fractions was precipitated with ethanol. Fractions used for LPL activation tests were dissolved in 0.15 M NaCl. Heparin concentrations were calculated on the assumption that their uronic acid content was 31%, as in RS heparin (7).

Fig. 3 shows a semilogarithmic plot of the relative activities of several heparins in the in vitro assay of activity of rat-heart LPL. The mean activity of heparin-free controls was 0.40 ± 0.12 μmol FFA per ml of assay mixture per hr (number of observations, n = 12). This control activity was arbitrarily set at 100 and all other activities were expressed relative to this value. The biphasic response to PM heparin, with an initial peak with 1.25 μg added to the assay mixture followed by further activation at higher heparin concentrations, was seen consistently in these experiments. RS heparin, C-1, and C-2 did not stimulate LPL activity. On the contrary, each showed a concentration-dependent inhibition, most marked with RS heparin and least with C-2, i.e., the degree of inhibition was directly proportional to molecular size. C-3 neither inhibited nor activated. Only C-4, the smallest depolymerization product, activated LPL in this assay system. The activity of C-4 relative to PM heparin was lower at all amounts tested. The highest relative activity, with 10 μg of C-4 added to the assay system, was 62% (i.e., 6.2 μg of PM heparin would activate LPL to the same degree).

Fig. 4 shows the in vivo effect of PM heparin on the activity of LPL in plasma at three dosages. These values are the maximum activities observed. In 54% of the tests, the highest activity was obtained in the 1-min samples; in the rest it was obtained with samples taken after 3 min. This linear response to low doses of PM heparin was used to estimate the relative activities of preparations of rat heparin. Doses of the latter were chosen to give 'peak responses' in the same range as those plotted in Fig. 4.

Fig. 5 shows the time course of the in vivo activity of PM heparin at two dosages. The peak response was attained within 1–3 min, followed by a rapid decline. Fig. 6 illustrates similar experiments with RS heparin and depolymerized rat heparin C-4. The time course for C-4 was essentially similar to that of PM heparin. For RS heparin, LPL activity appeared in the circulation relatively slowly, with maximum activity at 7 min. Also, the peak response with RS heparin was lower, although the dosage was 4-times greater.
mode of action of ascorbic acid (7) has been noted, and requires further study. If the reactions are similar, fractions 3 and 4 are analogous to the heparin chains and fractions 1 contain the core material referred to in the work with ascorbate.

Depolymerization products that activate LPL are of two types. The fraction 4 type is active in vitro and in vivo. This is the smallest product, in the same molecular size range as PM heparin. The fraction 3 type of product is inactive in vitro, but as active as fraction 4 in vivo. This fraction is, on average, of greater molecular size than PM heparin. (The polydisperse nature of all heparins precludes a more precise description of size relationships.) It is possible that further depolymerization of fraction 3 occurs in vivo, but many negatively charged macromolecules show lipemia-clearing activity in vivo (3, 16).

In contrast, heparin is the only naturally occurring mammalian glycosaminoglycan known to stimulate the release of LPL from a rat heart acetone-powder preparation in vitro (17). The present work shows that there is a quite sharply defined upper limit to the molecular size of rat heparin that is active in this assay system.

The nature of the depolymerase reaction is shown in this work, but a different approach is required to demonstrate the site of action. Reaction within mast cells, leading to the release of biologically active heparin, is an attractive hypothesis. However, in the present work no endogenous heparin was found in the intestine, suggesting that no mast cells were present in this tissue.

Work on purification of the depolymerase has progressed slowly, because the enzyme is extremely labile. For example, it is inactivated by ammonium sulfate and by lyophilization. In this study, the depolymerase preparations were dialyzed to show that small molecules such as ascorbic acid were not responsible for the activity observed. In fact, the activity is of high molecular weight, being excluded from Bio-Gel A 1.5 m (Horner & Young, unpublished work).

The gel filtration patterns of products A, B, and C indicate that successive treatments with depolymerase increased the yield of fractions 3 and 4, but did not alter their molecular size distribution. The depolymerase appears to act at a limited number of sites on the macromolecule. A similarity to the

FIG. 5. Time course of LPL activity in rat plasma after injection of PM heparin at dosage levels of 62.5 μg/kg (●) and 125 μg/kg (○). Values are mean values (±SD, represented by vertical bars), and n = 5 in both cases.

FIG. 6. Time course of LPL activity in rat plasma after injection of macromolecular RS heparin, 500 μg/kg, n = 5 (●) and depolymerized rat skin heparin fraction C-4, 125 μg/kg, n = 6 (○). Values are mean values (±SD, represented by vertical bars).
The main conclusion from this study is that the depolymerase acts on macromolecular heparin to produce a series of products ranging from potent inhibitors to potent activators of LPL. Since the activity of the depolymerase can control the relative proportions of these products, it may also indirectly control the activity of LPL. This is only one aspect of the metabolic control of LPL, for both the synthesis and activity of LPL are probably under hormonal control (18). However, the present work introduces a new factor that must be considered in elucidating the physiological role of this important enzyme.

I acknowledge the skilled technical assistance of Mrs. H. Weigand. This work was supported by the Ontario Heart Foundation.