Interaction of size-fractionated heparins with lipoprotein lipase and hepatic lipase in the rat

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Heparin and heparin partially depolymerized by enzymatic digestion were separated into six size fractions. Hep 1 (tetrosaccharides), with a mean Mr of 1200, did not release significant amounts of either lipoprotein lipase (LPL) or hepatic lipase (HL) on intravenous injection into rats. Hep 2 (mainly octa- and deca-saccharides), with a mean Mr of 2400–3000, released both lipases. To evoke the same plasma activity of LPL and HL required about 10 times more by weight, or about 40 times more molecules, of this heparin than of hep 5 (mean Mr, 12000, similar to conventional heparin). Hep 5 impeded binding and degradation of 125I-labelled bovine LPL by perfused rat livers. In contrast, hep 2 had no detectable effect on these processes. This demonstrates a difference between the sites in the liver that mediate binding, uptake and degradation of LPL, and the extrahepatic sites that bind functional LPL, and the hepatic sites that bind functional HL. After injection of 3.25 mg of hep 5/kg body weight, plasma LPL activity rapidly rose and then remained high for at least 1 h. With hep 2, plasma LPL also rose rapidly, but then decreased to almost basal by 1 h. When a labelled triacylglycerol emulsion was injected 1 h after the heparins, the fractional catabolic rate was enhanced in the rats that had received conventional heparin, as expected from the high plasma LPL activity, but decreased compared with controls in rats that had received hep 2, indicating that available LPL had been depleted through enhanced transport to and uptake in the liver.

INTRODUCTION

Two lipases, lipoprotein lipase (LPL) of extrahepatic origin and hepatic lipase (HL) from liver, are released into plasma on intravenous injection of heparin (LaRosa et al., 1972; Assmann et al., 1973). The released fractions of the enzymes are those located at endothelial sites, where they act on plasma lipoproteins with different preferences (Olivecrona & Bengtsson-Olivecrona, 1990). LPL hydrolysates mostly triacylglycerols carried by chylomicrons and very-low-density lipoproteins, whereas HL may act on both triacylglycerols and phospholipids in chylomicron remnants, intermediate-density lipoproteins and high-density lipoproteins.

The ‘lipase-releasing’ effect of heparin is suggested to be through ionomic interaction of negative charges on heparin with positively charged amino acid residues in the lipases, thus displacing the lipases from their binding sites, forming lipase-heparin complexes in the circulation (Olivecrona & Bengtsson-Olivecrona, 1989). Even though LPL and HL belong to a lipase gene family (Kirchgesner et al., 1987), differences in their primary structure result in altered affinity in binding to immobilized heparin (Bengtsson et al., 1980). On release into plasma, the lipases soon disappear. For LPL this probably occurs by binding in the liver followed by internalization and degradation (Chajek-Shaul et al., 1988a,b). For HL it may occur by return to the endothelium in the liver (Peterson et al., 1986). Several other hepatic-like sulphated polysaccharides share this ‘lipase-releasing’ effect, e.g. heparan sulphate and dermatan sulphate (Bengtsson et al., 1980). This effect is related to size and sulphation as well as structure (Olivecrona & Bengtsson-Olivecrona, 1989; Lindblom et al., 1991). Several studies had shown that shorter heparins (low-Mr, heparin) result in lower plasma activities of both LPL and HL than conventional heparin (DeSwart et al., 1984; Merchant et al., 1986; Persson et al., 1987). Linhardt and colleagues reported that heparin fragments as small as tetrosaccharides released lipase activity in rabbit, but only decasaccharides released substantial amounts of LPL into the plasma (Merchant et al., 1986). In a previous study we compared the effects of a low-Mr heparin with those of conventional heparin by looking at their actions in releasing LPL from extrahepatic tissues and in impeding hepatic clearance of the enzyme (Liu et al., 1991). The lower LPL activity in plasma resulted from faster hepatic clearance instead of less release of the enzyme from extrahepatic sources. In that study both preparations were polydisperse. We have now prepared a series of heparin size fractions, and have studied their effects on plasma LPL and HL activity. For LPL, the effects on release and clearance were evaluated separately. The results confirm that short heparins release LPL from extrahepatic tissues but do not impede its uptake and degradation by the liver. This suggests that extrahepatic tissues might become depleted of endothelial LPL. To test this, the clearance of a labelled triacylglycerol emulsion was studied in rats that had received small heparins 1 h previously.

MATERIALS AND METHODS

Materials

Low-Mr heparin (Logiparin) was produced by Novo Nordisk A/S, Bagsvaerd, Denmark, by enzymic depolymerization of pig intestinal mucosal sodium heparin by using heparinase from Flavobacterium heparinum. The low-Mr heparin was fractionated into five fractions (hep 1, hep 2, hep 3, hep 4 and hep 5) by ethanol precipitation followed by gel filtration (Nielsen & Østergaard, 1988). One high-Mr heparin fraction (hep 6) was prepared from pig intestinal mucosal sodium heparin by gel filtration. Heparin fractions prepared from enzymically depolymerized heparin have an unsaturated hexuronic acid residue at the non-reducing end, but, apart from that, have the same chemical structure as the parent mucosal heparin (Nielsen & Østergaard, 1988).

Abbreviations used: LPL, lipoprotein lipase; HL, hepatic lipase; hep, heparin fragment.
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Table 1. Characteristics found in vitro of the heparin fractions investigated in the study

<table>
<thead>
<tr>
<th>Heparin fraction</th>
<th>Peak $M_r$</th>
<th>Anti-(factor Xa) activity</th>
<th>Anti-(factor IIa) activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hep 1</td>
<td>1200</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hep 2</td>
<td>2400–3000</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Hep 3</td>
<td>4500</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>Hep 4</td>
<td>6300</td>
<td>93</td>
<td>46</td>
</tr>
<tr>
<td>Hep 5</td>
<td>12700</td>
<td>157</td>
<td>169</td>
</tr>
<tr>
<td>Hep 6</td>
<td>24900</td>
<td>130</td>
<td>153</td>
</tr>
</tbody>
</table>

The characteristics found in vitro of the fractions are shown in Table 1 and the $M_r$ distribution is depicted in Fig. 1. Comparison of the heparins was made on the basis of $M_r$. The conventional heparin used in the experiment in Fig. 6 was from AB Løvens, Malmö, Sweden.

LPL was purified from bovine milk (Bengtsson & Olivcrona, 1977), radiolabelled with $^{125}$I and repurified as previously described (Wallinder et al., 1984). An antiserum to rat HL was raised in rabbits (Peterson et al., 1985). Immunoglobulins were prepared by affinity purification on protein-G–Sepharose (Pharmacia, Stockholm, Sweden). BSA was from Sigma, St. Louis, MO, U.S.A. The labelled triacylglycerol emulsion was kindly provided by Novo Nordisk A/S, Copenhagen, Denmark. It contained 200 mg of soya-bean triacylglycerol, a trace amount of $^3$Holeic acid-labelled triolein and 12 mg of egg yolk phosphatidylycerine per ml.

The animals were anaesthetized with Hypnorm Vet (AB Leo, Helsingborg, Sweden; 10 mg/kg body weight) and Stesolid (Kabi-Pharmacia, Stockholm, Sweden; 2.5 mg/kg body weight). All experimental procedures were approved by the local Animal Ethics Committee.

Administration of heparins and withdrawal of blood in vivo was through exposed jugular veins on anaesthetized animals. Plasma was separated by centrifugation at 4 °C and incubated with an excess of anti-(hepatic lipase) immunoglobulins on ice for 2 h before assay of LPL activity. Supradiaphragmatic models were prepared as described by Bezman-Tarcher & Robinson (1965).

Liver perfusion

Livers were perfused with Eagle’s minimal essential medium supplemented with 3% BSA, 1 mg of glucose/ml and 1 mm-glutamine at 37 °C (medium A). The medium was passed through an artificial ‘lung’ (Hamilton et al., 1974) for oxygenation. The procedures were as previously described (Liu et al., 1991) but with bile drainage through a cannula inserted in the common bile duct to avoid contamination of the perfusion medium with bile. After $^{125}$I-labelled bovine LPL had been added, perfusate samples were taken at the indicated times and trichloroacetic-acid-precipitable and -soluble radioactivity was determined (Wallinder et al., 1984).

Determination of lipase activities

Conditions for the assays were as described elsewhere (Peterson et al., 1985). For assay of LPL activity the substrate emulsion was Intralipid (Kabi-Pharmacia, Stockholm, Sweden) into which tri[9,10-$^3$H]oleylglycerol had been incorporated by sonication. The incubation medium contained 5% such emulsion, 5% heat-inactivated rat serum as a source of apolipoprotein cII for activation of the lipase, 6% BSA and 15 I.U. of heparin/ml, pH 8.5. For the assay of HL, the substrate was an emulsion of tri[9,10-$^3$H]oleylglycerol in gum arabic prepared by sonication. This medium contained no serum and the concentration of NaCl was 1 m to suppress LPL activity. The incubation for both enzymes was carried out for 30 min at 25 °C in a water-bath. The reaction was stopped and the fatty acids were extracted and counted for radioactivity in an LKB Rack $\beta$-counter as detailed by Peterson et al. (1985). One millilitre of lipase activity is expressed as 1 nmol of fatty acids released/min.

Statistics

Student’s $t$ test was used. The data are expressed as means ± S.E.M.

RESULTS

In the first experiment the effect of the six heparin size fractions on plasma LPL and HL activities was studied in intact rats. The dose (3.25 mg/kg body weight) was the same as used in a previous study (Liu et al., 1991), and would result in near-maximal plasma activity of the enzymes for conventional heparin. Injection of hep 2–6 resulted in the rapid appearance of substantial activities of both enzymes (Figs. 2a and 2b). In contrast, hep 1 gave only a very slight increase in plasma lipase activities, less than 1% of the increase caused by hep 5 and 6. The peak values of LPL activity in Fig. 1(a) increased stepwise from hep 2 to hep 5, whereas with hep 6 the activity was similar to that with hep 5. With hep 2–4 the activities started to decline immediately so that the 10 min values were lower than the 5 min values. With hep 5 and hep 6 the activities increased from 5 to 10 min and remained unchanged until the end of the experiment (60 min).

The profiles of HL activity induced by these heparins resembled
Low-\(M\)\textsubscript{r} heparin, lipoprotein lipase and hepatic lipase

Fig. 2. Effect of size-fractionated heparins on plasma LPL and HL activities in rats

At zero time, 3.25 mg of the heparins/kg was injected intravenously to anaesthetized rats. Blood samples were taken before and 1, 5, 10, 30 and 60 min after the injection. (a) LPL activities; (b) HL activities. The data are means \(\pm\) S.E.M. for three rats for each heparin. ○, Hep 1; ●, hep 2; ▲, hep 3; ▼, hep 4; ■, hep 5; ♦, hep 6.

those of LPL activity in many respects, but there were some differences. Hep 2 produced relatively higher HL than LPL activity, compared with the maximum evoked by hep 5 and hep 6 the activity was above 60\% of maximum for HL but less than 30\% of maximum for LPL. Furthermore, the peak value of HL activity with hep 4 was close to those reached with hep 5 and 6, whereas for LPL there was a substantial increase from hep 4 to hep 5 and 6. As observed with LPL, the plasma HL activity evoked by hep 2–4 soon started to decline, whereas with hep 5 and 6 the activity remained high throughout the experiment.

We chose hep 5 as a fully effective and hep 2 as a marginally effective preparation for further studies. There was no size overlap between the two fractions (Fig. 1). In a wide range of 10-fold increases in dose neither heparin evoked substantial plasma lipase activities at doses below 30 \(\mu\)g/kg body weight (Fig. 3a). The activities of LPL and HL then increased in a similar manner up to the highest dose (3.25 mg/kg body weight). The dose range of 50–800 \(\mu\)g/kg body weight was then selected for a more detailed study. For hep 2 both lipase activities increased almost linearly with dose over this range (Fig. 3b). For hep 5 there was a linear rise of LPL and HL activities up to 200 \(\mu\)g/kg body weight, and a slower increase thereafter. From the linear ranges it can be estimated that 70–100 \(\mu\)g of hep 5 was needed to evoke the same lipase activities as 800 \(\mu\)g of hep 2, i.e. hep 5 was about 10 times more potent. This estimate was similar for HL and LPL activities.

To explore the release of LPL separate from hepatic uptake of the enzyme, the supradiaphragmatic rat was used as a simple and suitable model. As it was for intact animals, hep 1 had virtually no effect in this model either (Fig. 4). Compared with intact animals, hep 2 caused higher plasma LPL activity in the supradiaphragmatic rats. The LPL activity released by hep 3 was lower

Fig. 3. Dose–response curves for hep 2- and hep 5-evoked plasma LPL and HL activities

In (a) the heparins were given in doses that increased by a factor of 10, from 0.325 \(\mu\)g/kg to 3.25 mg/kg. Note that the x-axis is logarithmic. From this, the dose range of 50–800 \(\mu\)g/kg was selected for the experiment in (b). Note that here the x-axis is linear. Blood samples were taken 5 min after i.v. injection of the heparins. Data are means for two rats at each dose. ○, Hep 2; ■, hep 5; ——, LPL; ———, HL.

Fig. 4. Release of LPL activity in the supradiaphragmatic model by heparins

Heparins were administered intravenously within 5 min after the superior vena cava and the abdominal aorta below the diaphragm had been tied off. Blood samples were taken immediately before and 1, 5, 10, 30 and 60 min after injection of heparins. Data are means for two rats for each heparin. Symbols are the same as in Fig. 2 for each heparin.
Isolated rat livers were perfused for 30 min in a recirculating mode after a 10 min wash by single-pass perfusion. $^{125}$I-labelled LPL (about 50 ng, $10^6$ c.p.m.) was then added. When present, stock heparin solution was added 2 min before LPL to give a final concentration in the medium of 130 $\mu$g/ml. Non-liver perfusions were run for each of the three conditions: heparin-free, hep 2 and hep 5. Values of trichloroacetate-precipitable radioactivity (----) remaining in the medium are expressed as percentage of the values for each non-liver control at the corresponding time-interval. Trichloroacetate-soluble radioactivity (-----) was expressed as the percentage of 5 min values of $^{125}$I-labelled bovine LPL of trichloroacetate-precipitable radioactivity. Perfusion was without heparin (O), and with hep 2 (●), hep 5 (■). Data points for no heparin and hep 2 fall close together at most times and are therefore not resolved in the graph.

than that released by hep 4, whereas there was little difference between hep 4 and 5. The most marked difference compared with intact animals in Fig. 2 was that the disappearance of lipase activity from plasma was much slower.

In the next experiment we compared the effect of hep 2 and 5 on hepatic uptake of $^{125}$I-labelled bovine LPL by perfused rat livers. Substantial amounts of labelled LPL disappeared from the perfusion medium when this was circulated through the system without a liver. This reflects the surface activity of the lipase, which gives it a propensity to bind to the walls of tubing and containers (Olivecrona & Bengtsson-Olivecrona, 1987). The extent of this ‘non-specific binding’ was decreased by heparin, and this differed between the heparin preparations. Most of the non-specific binding occurred within the first 5 min, but there was a slow loss of label from the medium during continued circulation through the system. It was therefore necessary to have separate non-liver controls for perfusions without heparin, with hep 2 and hep 5, and to correct the data accordingly. In perfusions without heparin a large fraction of the labelled LPL was rapidly extracted by the liver, and there was then a slower continued extraction (Fig. 5). Hep 5 markedly decreased the hepatic extraction, whereas hep 2 had no effect. After 15 min, trichloroacetate-soluble radioactivity appeared in the medium, indicating that some of the lipase had been degraded by the liver. No trichloroacetate-soluble material was generated during perfusion without liver. After 1 h, about 40% of the labelled LPL had been converted into trichloroacetate-soluble products in perfusions without heparin. This value was the same with hep 2, and only slightly lower with hep 5.

These data suggest that hep 2 releases substantial amounts of LPL but does not prevent hepatic uptake of the enzyme. This implies that administration of hep 2 may deplete the capacity for hydrolysis of lipoprotein triacylglycerol. A triacylglycerol clearance experiment was designed to test this hypothesis. Rats were injected with 3.25 mg of conventional heparin or hep 2/kg. After 1 h a bolus dose of labelled triacylglycerol emulsion was injected. As this time plasma LPL activity was high (211 munits/ml) in the rats given conventional heparin, but had returned towards basal values in the rats given hep 2 (7.8 munits/ml compared with 4.3 in controls). Fig. 6(a) shows that in controls the labelled triacylglycerol disappeared after an exponential decay with a fractional catabolic rate of $0.195 \pm 0.023$/min, corresponding to a half-life of 3.5 min. In the rats given conventional heparin the labelled triacylglycerol disappeared more rapidly, with an initial fractional catabolic rate of $0.935 \pm 0.038$. In contrast, the emulsion was cleared less rapidly in rats given hep 2. The initial fractional catabolic rate was $0.132 \pm 0.023$. The differences in plasma triacylglycerol radioactivity were statistically significant ($P < 0.05$) at all times between 1 and 16 min. For instance, 10 min after the injection, plasma radioactivity was 12.5%, 0.63% and 31.3% of the injected dose in the rats given saline, conventional heparin and hep 2 respectively.

To test further the ability to clear triacylglycerol, rats were given a large bolus of the emulsion, 64 mg of triacylglycerol (Fig. 6b). This resulted in slower clearing, and the data cannot be accurately fitted to either zero- or first-order kinetics. It is clear, however, that the clearance was slower in rats that had received hep 2 h before, than in controls. For instance, at 10 min, 56.6% of the injected dose remained in plasma in rats that had received hep 2 compared with 39.7% in the controls ($P < 0.005$).
DISCUSSION

This study establishes a difference between the effects of heparin on binding of LPL in extrahepatic tissues, and in the liver. This difference has already been indicated in a previous study (Liu et al., 1991), where we found that a low-Mr heparin had a decreased effect on binding of LPL in the liver compared with conventional heparin. The low-Mr preparation had a mean Mr of 4400, similar to that of heparin used here, but was polydisperse, with about 10% of the molecules larger than 10000. In the present study, heparin, with a mean Mr of 2400–3000 and virtually no molecules larger than 5000 (i.e. mainly octa- and deca-saccharides) had no detectable effect on binding or degradation of 125I-labelled LPL in the perfused liver, but did release the enzyme from extrahepatic tissues. Furthermore, heparin 2 released HL, demonstrating a difference between the binding of this enzyme and that of LPL in the liver.

A heparin-binding segment in LPL has been identified by ligand blotting to a segment in the C-terminal half of the enzyme (Bengtsson-Olivecrona et al., 1986). The amino acid sequence shows there are 16 arginine or lysine residues but only three glutamate or aspartate in a stretch of 60 residues from 261 to 321 (Senda et al., 1987). In pancreatic lipase the corresponding segment is located in a surface crease between the two folding units, on the side opposite to the entrance of the active-site pocket (Winkler et al., 1990; Persson et al., 1991). An octa- or deca-saccharide could probably engage most of the positively charged residues in this putative 'heparin-binding site'. Heparin 1 contained mainly tetrasaccharides, and about 15% hexasaccharides. It had virtually no effect in any of the present systems. Heparin 2, which released both lipases, contained a mixture of mainly octa- and deca-saccharides. Thus, the tetrasaccharides must have been effective. Whether octa- and perhaps hexa-saccharides, have some effects must be tested with more monodisperse fractions. In a previous study Merchant et al. (1986) found that deca-saccharides were the smallest oligosaccharides that resulted in substantial lipase release in rabbits. In a study in vitro, Bengtsson et al. (1980) found that octasaccharides were the smallest heparin fragments that could dissociate LPL from immobilized conventional heparin. Heparin 5 with a mean Mr of 12700 was about 10 times more effective per weight in evoking plasma LPL activity than heparin 2. This translates to a more than 40 times higher effect per molecule. Clarke et al. (1983) have presented evidence that heparins up to Mr about 5000 form 2:1 complexes with LPL dimers, whereas larger heparins form 1:1 complexes with higher affinity, presumably by simultaneously binding to both subunits. In terms of releasing the lipases from extrahepatic sites, as evaluated in the supradiaphragmatic model, heparins as small as deca- and octasaccharides were effective. Hence the releasing molecule does not have to bridge both subunits. It is of course still possible that attachment to the endothelium involves interaction of both LPL subunits to heparan sulphate, either by one chain bridging both subunits, or by interaction with two chains from a proteoglycan. Also it is not possible to draw any firm conclusion about whether the higher efficiency of longer heparins, per molecule, was because they could bridge both subunits, or because the probability of optimal charge distribution is higher in longer heparin molecules.

HL binds to immobilized heparin, but is eluted at lower salt concentration than LPL, indicating that fewer charges are involved (Bengtsson et al., 1980). In the segment corresponding to the implied heparin-binding site in LPL, HL lacks some of the arginine or lysine residues (Komaromy & Schotz, 1987). It has been suggested that another cluster of positively charged residues near the C-terminus of HL are involved in its binding to heparin (Stanhke et al., 1987). The different tissue distribution of HL and LPL in vivo indicates differences in how the two enzymes recognize vascular binding sites. Despite these differences, the present study indicates that the minimal size of heparin for release of the two lipases from their binding sites in vivo is similar. This is in concert with previous model studies with natural and modified heparin-like polysaccharides (Bengtsson et al., 1980).

Available evidence suggests that hepatic lipase is turned over relatively slowly; a half-life of 4.6 h has been estimated by Schoonderwoerd et al. (1981). Virtually all of this lipase resides in the liver, and there is little or no hepatic lipase in most of the extrahepatic tissues where endothelial LPL resides (Jansen & Hüllmann, 1980). Injected HL binds mainly in the liver, and to some extent in ovaries and presumably adrenals (Hixenaugh et al., 1989). This pattern suggests that the injected lipase binds to its functional sites. When heparin was injected to mice 1 h after human HL, almost all the injected enzyme returned to the circulating blood (Peterson et al., 1985). Hence, the exogenous HL remained at exposed sites in the liver, and was not rapidly degraded, in contrast with LPL. The present considerations suggest that it is probably more appropriate to compare the effect on HL in the intact rats with the effect on LPL in the supradiaphragmatic rather than the intact rats. Compared in this manner, the relative potency was similar with respect to release of the two lipases. Heparin 1 had virtually no effect on either, and heparin 2 at the dose given released more than half-maximal amounts of both lipases. Hence we did not find a heparin preparation that selectively released one but not the other lipase.

An implication of the present results is that short heparins might cause an accelerated flow of LPL from extrahepatic sites to the liver with depletion of overall available lipase activity. We did in fact find that the clearance of a labelled triacylglycerol emulsion was slower 1 h after injection of heparin than in controls (Fig. 6). Persson et al. (1985) infused Intralipid at a steady rate into volunteers. Simultaneous administration of low-Mr heparin initially caused a decrease in plasma triacylglycerol concentration, presumably because LPL released to the circulation enhanced triacylglycerol clearance, as observed here for the rats that received conventional heparin. Later during the infusion, the triacylglycerol concentrations increased in the individuals given low-Mr heparin. This may have been due to a depletion of LPL, as observed here. Implications of these findings for clinical use of different heparins need to be considered. In a broader sense, the present findings underscore that functional LPL is governed not only by LPL delivery to the endothelium but also by the rates of lipase transport to and degradation in the liver.

REFERENCES


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