Effect of protamine on lipoprotein lipase and hepatic lipase in rats

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The polycation protamine impedes the catabolism of triglyceride-rich lipoproteins and this has been suggested to be due to intravascular inactivation of lipoprotein lipase. We have made intravenous injections of protamine to rats and found that both lipoprotein lipase and hepatic lipase activities were released to plasma. The effect of protamine was more short-lived than that obtained by injection of heparin. The release of hepatic lipase by protamine was as effective as the release by heparin, while the amount of lipoprotein lipase released by protamine was only about one-tenth of that released by heparin. This was not due to inactivation of lipoprotein lipase, since injection of an excess of heparin 10 min after injection of protamine released as much lipoprotein lipase activity to plasma as in controls. The results *in vivo* differed from those obtained in model experiments *in vitro*. Protamine was able to almost quantitatively release both lipoprotein lipase and hepatic lipase from columns of heparin–agarose. The displacement was dependent on the total amount of protamine that had passed over the column, indicating that it was due to occupation by protamine of all available binding sites. Our results *in vivo* showed that the binding sites for lipoprotein lipase were not blocked as efficiently as those for hepatic lipase, indicating that the binding structures were not identical. It was concluded that the impaired turnover of lipoproteins by protamine probably was due to prevention of binding of the lipoproteins to endothelial cell surfaces rather than to impaired lipase function.

INTRODUCTION

Lipoprotein lipase (LPL) and hepatic lipase (HL) are related enzymes (Hide et al., 1992) which catalyse the hydrolysis of triglycerides and phospholipids in plasma lipoproteins (Deckelbaum et al., 1992). The enzymes are localized at the endothelium of blood vessels such that they are directly available for lipoproteins from blood (Olivecrona and Bengtsson-Olivecrona, 1993). The concentration of the lipases in circulating blood is low, but they are rapidly released into blood by heparin and other polyanions. This has led to speculation that the endothelial binding sites for the lipases are heparin-related polysaccharides, probably heparin sulphate. This hypothesis is in accord with model experiments carried out with purified lipases and polyanions *in vitro* (Bengtsson et al., 1980). For LPL the hypothesis has also received support from studies with cultured endothelial cells (Shimada et al., 1981; Stins et al., 1992).

Also lipoproteins have affinity for polyanions. This is due to apolipoprotein E and apolipoprotein B which contain heparin-binding segments (Chan, 1992; Shimano et al., 1992). This affinity for polyanions is probably important for the initial attraction which captures lipoproteins from the circulating blood and presents them for more specific interactions with lipases and receptors.

If the lipases are bound *in vivo* in the postulated manner it should be possible to displace them into the circulating blood by reducing the number of available binding sites by polycations. This has in fact been possible with the type of ampholytes used for isoelectric focusing (Bengtsson and Olivecrona, 1977). In the present study we have investigated the effects of protamine. This is a mixture of basic proteins with molecular sizes of 3–5 kDa and is produced from, for example, salmon sperm. Protamine is used in the clinic to neutralize heparin in anti-coagulant therapy.

It was previously reported that protamine causes a marked retardation of the metabolism of triglyceride-rich lipoproteins (Harwood et al., 1974). The interpretation was that protamine depleted the endothelium of active LPL. This was based on *in vivo* studies which showed that protamine inhibited LPL activity in post-heparin plasma. Our present data show that LPL but not HL remains on the endothelium after protamine injection and that both lipases are fully active. An alternative interpretation of the protamine effect is that it prevents binding of lipoproteins to endothelial heparin sulphate, an important event for their access to the lipases.

MATERIALS AND METHODS

Animal procedures

Male Sprague–Dawley rats (SPF) weighing 190–210 g from Moellegard Breeding Center (Ejby, Denmark) were allowed to acclimatize for at least 1 week before the start of the experiment. The rats were anaesthetized with an intramuscular injection of Hypnorm (Janssen Pharmaceutica, Bersee, Belgium) at 1 ml/kg body weight and Stesolid Novum at 1 ml/kg body weight (10 mg of diazepam/ml from Dumex, Copenhagen). Hypnorm is a combination of the narcotic analgesic fentanyl citrate (0.315 mg/ml) and the tranquilizer fluanisone (10 mg/ml). The food was withdrawn from the rat cages at noon the day before the experiment. The rats had access to water *ad libitum*. All animal procedures were approved by the animal ethics committee in Umeå.

For the injection studies jugular veins on both sides were exposed in an anaesthetized animal. An appropriate amount of protamine stock solution (10 mg/ml, Kabi Pharmacia Hospital Care, Stockholm), or heparin stock solution (5000 i.u./ml, Løvens kemiske Fabrik, Ballerup, Denmark), diluted in saline,

Abbreviations used: LPL, lipoprotein lipase; HL, hepatic lipase; HSPG, heparin sulphate proteoglycans.
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or saline alone was rapidly injected intravenously. Blood samples were withdrawn at indicated times from the opposite jugular vein into tubes with excess heparin (20 i.u. for 0.2 ml of blood). Plasma was separated by centrifugation at 4 °C and used for assay of lipase activities as detailed below.

Heart and liver perfusions

In anaesthetized rats, hearts were loaded with ^{125}I-LPL by intravenous injection 10 min before the hearts were removed. The isolated hearts were perfused retrogradely through the aorta at a rate of 10 ml/min. First, blood was washed out by a single pass of 10 ml of buffer A (Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 1 % BSA) at 37 °C. Then, 1 ml of buffer A with either protamine (0.0–10 mg/ml) or heparin (1.3 mg/ml) was perfused followed by 10 ml of buffer A to wash out all released lipase. The perfusates were collected on ice with an excess of heparin to balance out the potentially inactivating effect of protamine on LPL (Harwood et al., 1974).

Livers were perfused *in situ*. This method was selected in order to minimize the amount of labelled lipase sticking to the perfusion system tubing. The venae gastricae were ligated and the portal vein and the inferior cava vein were cannulated. The livers were flushed with 20 ml of buffer A through the portal vein at a flow rate of 15 ml/min using a syringe pump. Then protamine or heparin in 2 ml of buffer A was flushed through the liver to release the enzymes followed by an additional 20 ml of buffer A. The perfusate was collected through plastic tubing (150 mm × 1.5 mm i.d.) placed in the vena cava inferior into tubes standing on ice containing an excess of heparin. At the highest level of perfused protamine (3.3 mg/g of liver) the collected perfusate was turbid which was probably due to formation of complexes between protamine and heparin. To measure the release of LPL by protamine, livers were preloaded *in vivo* by a bolus injection of 300000 c.p.m. ^{125}I-LPL 10 min before the liver perfusion began.

Heparin–agarose columns

Rat post-heparin plasma (2 ml) together with 200000 c.p.m. ^{125}I-LPL was applied on a 2 ml heparin–agarose column equilibrated with buffer B [0.15 M NaCl/1 mg/ml BSA/20 % glycerol/0.1 % Triton X-100/20 mM Tris-Cl (pH 8.5)]. The column was washed with 11 ml of the same buffer at a flow rate of 0.5 ml/min. It was then eluted by a 50 + 50 ml gradient of either 0–2 M NaCl or 0–1 mg of protamine/ml, all in buffer B. The flow-rate was 1 ml/min and fractions of 2 ml were collected. Fractions were subjected to measurements of ^{125}I-LPL, LPL and HL activity and the conductivity (only for the NaCl gradient).

In another set of experiments, ^{125}I-LPL was applied to an 0.5 ml heparin–agarose column. The column were first eluted with 10 ml of buffer C [20 % glycerol/0.1 % Triton X-100/20 mM Tris-Cl (pH 7.4)]. Then the column was eluted (1 ml/min) with either 40 ml of buffer C with 1 mg of protamine/ml or with 400 ml of buffer C with 0.1 mg of protamine/ml. In the first case 0.8 ml fractions were collected, and in the second case 2.5 ml were collected per fraction.

Assay of lipase activities

Plasma samples were analysed immediately or frozen at −20 °C for analysis at a later time. Control experiments indicated little or no loss of lipase activity by the freezing and storing. The assay systems have been described previously (Bengtsson-Olivecrona and Olivecrona, 1991a). The sample volumes were 15 μl or less. Both assays were run at 25 °C, for 30 to 120 min, depending on the expected activity. Control experiments with post-heparin plasma showed that both assays were linear with the amount of plasma sample and the time over the ranges used here. All determinations were run in triplicate. The activities were expressed in milliunits (mU), which corresponded to 1 nmol of fatty acid released/min.

The lipase activities in tissue homogenates were determined on 10 % or 5 % (w/v) homogenates of tissue samples. The tissues were rapidly excised from the animals, rinsed in cold water and blotted dry before weighing. The tissues were stored on ice before being homogenized in 9 or 19 vol. of buffer D [0.025 M NH_4Cl (pH 8.2)/5 mM EDTA/8 mg/ml Triton X-100/0.4 mg/ml SDS/33 μg/ml heparin/10 μg/ml leupeptin/1 μg/ml pepstatin/25000 i.u./ml Transylol] using a Polytron PT 3000 (Kinematica AG, Lucerne, Switzerland) at 25000 rev./min for 20 s at +4 °C. After a 3 min centrifugation at 10000 rev./min (5500 ***g***) in a Beckman Microfuge, the lipase activities were measured in the supernatant.

For assay of HL we used a sonicated emulsion of ^{3}H-labelled triolein in gum arabic, and included 1 M NaCl in the medium (Bengtsson-Olivecrona and Olivecrona, 1991a). This salt concentration is known to inhibit LPL activity. In accord with this, pre-incubation of the present post-heparin plasma samples with antibodies to LPL caused no significant reduction of the activity recorded in the HL assay. The final composition of the medium (excluding contributions from the sample itself) was: NaCl 1.0 M; Tris/HCl 0.1 M; triglycerides 4 mg/ml; gum arabic 6 mg/ml; heparin 10 μg/ml, albumin 60 mg/ml, pH 8.5.

For assay of LPL the substrate was prepared by sonication of ^{3}H-triolein into 10 % Intralipid (Bengtsson-Olivecrona and Olivecrona, 1991a). This emulsion was then mixed with a medium containing albumin and heat-inactivated rat serum as a source of apolipoprotein CII. The final composition of the assay mixture (excluding contributions from the sample) was: NaCl 0.1 M; Tris/HCl 0.1 M; triglycerides 4 mg/ml; phospholipids 0.24 mg/ml; heparin 10 μg/ml; albumin 60 mg/ml; serum 5 % (v/v). The pH was 8.5. Under these conditions HL was also active. To obtain a selective measurement of LPL, HL was first inhibited by incubation of the plasma samples for 2 h on ice with a rabbit IgG to rat HL (1:1, v/v).

The generated fatty acids were extracted using isopropanol/heptane/1 M H_2SO_4 and later alkaline ethanol (Bengtsson-Olivecrona and Olivecrona, 1991a).

Preparation of labelled LPL

Bovine LPL was purified from milk as described (Bengtsson-Olivecrona and Olivecrona, 1991b) and was labelled with iodine (Wallinder et al., 1984). The labelled LPL was purified from damaged protein and from free iodine by chromatography on heparin–agarose. The specific activity of the labelled LPL was approx. 10000 c.p.m./ng.

Other analyses

Measurement of plasma triglycerides was done using the enzymic kit 'Triglycerides without free glycerol' (Boehringer Mannheim, Stockholm).

Statistical analysis

Statistics were done using SPSS for Windows (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Displacement of lipases from heparin–agarose by protamine

We first tested if protamine could displace HL and LPL from heparin in a model system. For this we used small columns of heparin–agarose. When rat post-heparin plasma was applied to the column more than 91 % of the HL and LPL activities bound. Elution with a salt gradient resulted in two peaks of lipase activity (Figure 1a). The first peak represented HL, which gave activity in both assays. The second peak represented LPL, which gave activity only in the LPL assay. In the experiments shown in Figure 1 a trace amount of bovine ^{125}I-LPL was applied to the column together with the rat post-heparin plasma. The ^{125}I-LPL contained about 50 % active dimeric LPL, which eluted in peak 2, and about 50 % inactive monomeric LPL, which eluted at a similar position as HL, i.e. peak 1 (Liu et al., 1993).

In the experiment in Figure 1(b) the heparin–agarose column was eluted by a gradient of protamine (0–2 mg/ml). This resulted in elution of HL activity, LPL activity and labelled bovine LPL in a single peak. To ensure a selective measurement of the two lipases, all samples to be assayed in the LPL assay were first treated with antibodies to HL to inhibit this lipase. For HL the peak was distinct and almost symmetrical. For LPL there was a tendency of trailing, both for activity and for radioactivity. The recoveries were high: 93 % for HL activity, 77 % for LPL activity and 84 % for LPL radioactivity. These results showed that a gradient of protamine could displace both lipases from heparin–agarose, but the lipases were not separated into two peaks, as they were by salt gradients.

The effect of protamine on the release of lipases from the heparin–agarose might either result from competition because protamine at a certain concentration is able to compete with the lipases for available binding sites, or from displacement of the lipases because protamine occupied all binding sites. To test whether the release of ^{125}I-LPL was related to the total amount of protamine applied to the column, protamine was applied at two different concentrations, 0.1 or 1.0 mg/ml. These concentrations were chosen since in Figure 1 almost no radioactivity was released at a protamine concentration of 0.1 mg/ml while 1.0 mg/ml was more than enough to release the lipases. These two different ways of adding protamine to the column resulted in almost identical release of ^{125}I-LPL per mg of protamine passed over the column.

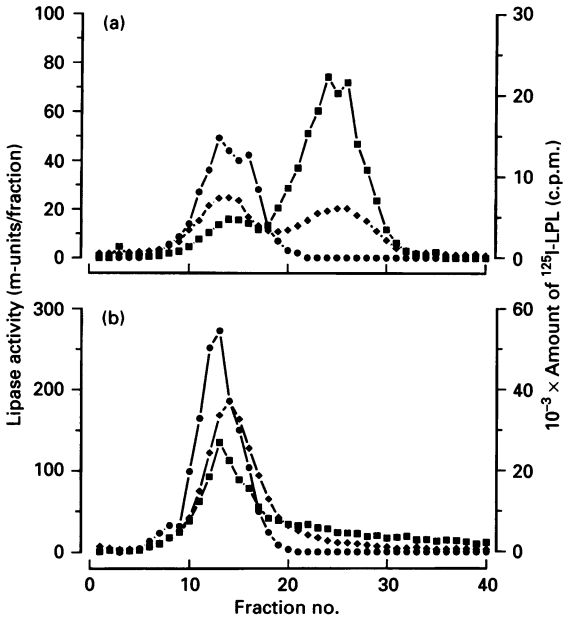

Figure 1 Release of plasma lipases from heparin–agarose

Plasma was collected from an anaesthetized rat 10 min after intravenous injection of 20 i.u. of heparin. Post-heparin plasma (2 ml) was applied to a heparin–agarose column together with a trace amount of ^{125}I-labelled bovine LPL (◆). Lipase activity was measured in an LPL assay (■) and in an HL assay (●). (**a**) The column was eluted by a gradient of sodium chloride (0–2 M). The first peak in the LPL assay corresponds to activity of HL and the second peak corresponds to LPL. (**b**) The column was eluted by a gradient of protamine (0–1 mg/ml). In this experiment the samples to be measured in the LPL assay were treated with antibodies to HL to suppress this lipase. Separate plasma samples were used for the two experiments.

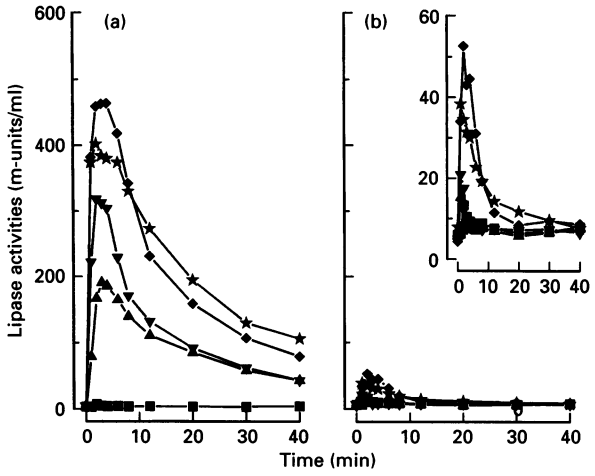

Figure 2 Release of the lipases by protamine *in vivo*

Rats were injected with increasing doses of protamine: 0.1 (■), 0.5 (▲), 1 (▼), 2 (◆) and 4 mg (★) per rat. Blood samples were collected in tubes containing heparin to stabilize the enzyme. The amount of heparin was always more than twice the amount required to neutralize the protamine in the sample. The Figure shows a typical experiment with one rat at each dose. Data for HL (**a**) and LPL (**b**) are from the same rat. The inset in (**b**) shows the same experiment but with a different ordinate scale.

Effects on HL *in vivo*

Injection of protamine led to rapid release of lipase activity to the circulating blood (Figure 2). After pre-incubation of the samples with antibodies to HL, the activity registered in the HL assay was virtually abolished, demonstrating that it was due to HL only. The maximal HL activity in blood after injection of 2 or 4 mg of protamine was similar to the activity obtained after injection of 0.13 mg (equivalent to 20 i.u.) of heparin. Previous studies have shown that this amount of heparin gives near maximal release of HL in corresponding rats (Liu et al., 1991). The HL activity soon declined so that it had dropped to about half by 10 min after the protamine injection and to less than one-third of maximal activity by 40 min (Figure 2). This was strikingly different from the response to 0.13 mg of heparin where the activity remained high for at least 60 min (Liu et al., 1991).

To further study the relation between release of HL by protamine and by heparin the experiment in Figure 3 was carried out. First, 2 mg of protamine was injected. After 10 min the HL activity had peaked and declined again to about 300 m-units/ml (compare with Figure 2). At this time 1.3 mg of heparin was injected. This was the dose estimated to neutralize the injected amount of protamine. Harwood et al. (1974) have reported that

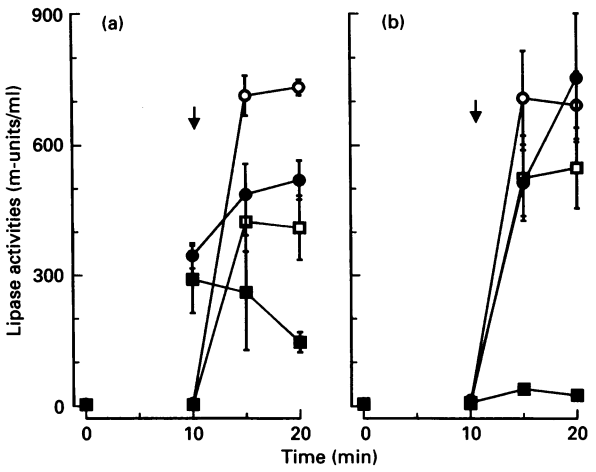

Figure 3 Effect of protamine on the heparin-releasable pool of HL and LPL

Rats received saline (○, □) or 2 mg of protamine (●, ■) at time 0. Heparin was injected 10 min later (arrow), either 0.13 mg (□, ■) or 1.3 mg (○, ●). For HL (**a**), the activities 5 min after heparin were not significantly different in rats who had received protamine or not. For LPL (**b**), the activity was significantly lower in the rats who received 0.13 mg of heparin after 2 mg of protamine, compared with the rats who received this heparin dose after saline. Data are means ± S.E.M. for groups of 3 rats.

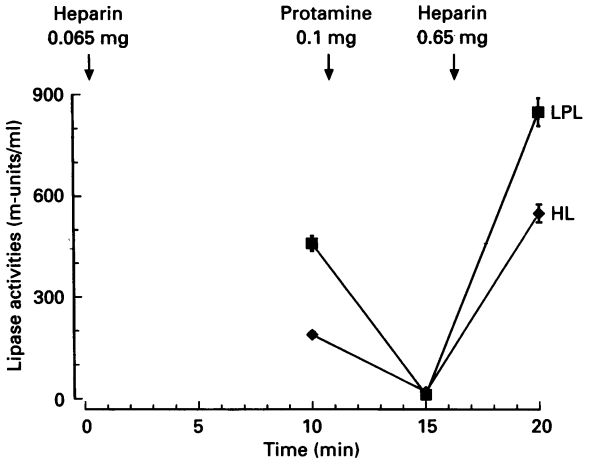

Figure 4 Protamine neutralizes the lipase-releasing effect of heparin, but does not inactivate the lipases *in vivo*

Anaesthetized rats were given heparin (0.065 mg). After 10 min a blood sample was taken. Protamine (0.1 mg) was given 30 s later to neutralize the circulating heparin. A blood sample was taken 5 min later and followed after 30 s by a large dose of heparin (0.65 mg). A final blood sample was taken 5 min later. All blood samples were assayed for LPL (■) and HL (◆) activities. Data are means ± S.E.M. ($n = 5$).

Table 1 Effect of protamine on lipase activities in plasma, liver and adrenals

Saline alone (0.2 ml), or containing 2 mg of protamine or 0.65 mg of heparin was injected into a tail vein of unanaesthetized rats. The rats were killed 10 min later by decapitation. Homogenates were prepared and the lipase activities were assayed later the same day. ANOVA was used for statistics and differences between groups were tested for with Neuman–Keuls test ($\alpha = 0.05$). SH, SP and PH denotes a significant difference between saline and heparin, saline and protamine, and protamine and heparin, respectively. Data represent mean ± S.E.M. ($n = 5$).

Lipase	Tissue	Saline	Protamine	Heparin	
HL	Plasma m-units/g	8 ± 3	517 ± 38	770 ± 59	SH, SP, PH
	Liver m-units/g	2305 ± 469	703 ± 78	789 ± 83	SH, SP
	Adrenal m-units/g	172 ± 18	152 ± 25	85 ± 13	SH, PH
LPL	Plasma m-units/ml	8.7 ± 2.0	15.2 ± 1.1	600 ± 36	SH, SP, PH
	Liver m-units/g	171 ± 30	127 ± 12	614 ± 100	SH, PH
	Adrenal m-units/g	322 ± 44	333 ± 65	368 ± 20	

only about 10% of the injected protamine is still in plasma 10 min after injection. Hence, we expected that some, but not all, of the heparin would be neutralized. After the heparin injection the HL activity increased again to about 500 m-units/ml. In rats which had not received protamine the same dose of heparin (1.3 mg) resulted in HL activities of around 700 m-units/ml. When 0.13 mg of heparin was injected, the amount estimated to be equivalent to the protamine remaining in blood, the HL activity did not rise, but continued to decline.

HL is present in liver and adrenals, but not in other tissues in male rats (Doolittle et al., 1987). After injection of heparin the HL activity, as measured in tissue homogenates, decreased by 70% in liver and by 50% in adrenal (Table 1), demonstrating that the lipase had been released from both tissues. After injection of protamine the activity decreased in liver to a similar extent as after heparin (65%), but did not change significantly in the adrenals.

Effects on LPL *in vivo*

LPL was released to the circulating blood after injection of protamine, but in relatively lower amounts than those of HL (Figure 2). The heparin-releasable LPL activity in corresponding rats was about 600 m-units/ml (0.65 mg of heparin/kg) (Liu et al., 1991). Protamine (2 or 4 mg) released less than one-tenth of this amount, about 50 m-units/ml. Maximal activity was reached in 1 min. The activity then rapidly declined and was only about 15 m-units/ml by 10 min. The decline was more rapid than that seen after injection of heparin (see Figure 1 in Liu et al., 1991), and was also somewhat more rapid than the decrease of HL activity after protamine injection (compare Figures 2a and 2b).

It has been reported that protamine makes LPL unstable (Harwood et al., 1974). To minimize the risk of inactivation during collection and analysis of the samples, an excess of heparin over protamine was added to all tubes used for blood sampling. Control experiments showed that there was little or no loss of LPL activity during storage of these samples on ice for up to 3 h. We concluded that the LPL activities, as measured here, accurately reflect the LPL activity in circulating plasma at the time of sampling. To evaluate the possibility that LPL might be inactivated in the circulating blood, plasma samples to which no heparin was added were incubated at 37 °C before assay. Under these conditions the lipase activity decreased by about 50% in 10 min. Thus, loss of catalytic activity in blood could contribute to, but not fully explain, the rapid decrease of LPL activity seen in Figure 2.

Rat livers contain low but significant LPL activity (Peterson et al., 1985). As there is no synthesis of LPL in the adult rat liver (Vilaró et al., 1988a), this activity must derive from uptake of LPL from blood. In accord with this, the activity in liver increased by 360% after heparin injection (Table 1). In contrast, the activity in liver tended to decrease after protamine injection, but this was not statistically significant.

Table 2 Effect of protamine on post-heparin lipase activities

Unanaesthetized rats were given saline alone (controls) or 2 mg of protamine in saline in a tail vein. After 40 min the rats were anaesthetized and 20 min later they were given heparin (1.3 mg) in the left jugular vein. Blood samples were taken from the right jugular vein 1 min before and 10 min after heparin injection. Statistics were done using two-tailed Student's *t*-test. Data represent mean ± S.E.M. ($n = 5$).

	Control	Protamine	*P*
Triglycerides before heparin (mmol/l)	1.30 ± 0.09	2.38 ± 0.34	$P < 0.001$
HL preheparin (m-units/ml)	5.5 ± 0.4	52.6 ± 9.8	$P < 0.01$
HL post-heparin (m-units/ml)	480 ± 70	200 ± 41	$P < 0.05$
LPL preheparin (m-units/ml)	6.0 ± 0.7	10.2 ± 0.6	$P < 0.01$
LPL post-heparin (m-units/ml)	991 ± 95	591 ± 111	$P < 0.05$

To further evaluate the relation between release of LPL activity by heparin and by protamine the experiment in Figure 3 was carried out. Ten minutes after injection of 2 mg of protamine, heparin was injected. A large dose of heparin (1.3 mg) caused release of similar amounts of LPL as when the same amount of heparin was injected with no prior injection of protamine. Hence, protamine had not significantly decreased the amount of releasable LPL. When 0.13 mg of heparin was injected only a small release of LPL ensued. When no protamine had been injected this amount of heparin caused almost the same release as the larger dose. This showed that protamine was able to neutralize the effect of heparin on lipase release.

In the experiment in Figure 4, a low dose of heparin was first injected. This caused a release of both LPL and HL to plasma. When an amount of protamine calculated to neutralize the heparin was given 10 min after the heparin, the circulating lipase activities returned towards basal values within 5 min (Figure 4). A second (larger) heparin dose 5 min after the protamine injection, released more lipase activity (both LPL and HL) to plasma than the first heparin injection. These amounts of lipases were similar to those released by heparin in rats not given protamine (compare with Figure 3). This shows that when protamine neutralized the effect of the first heparin injection, both lipases returned to sites from which they could be released by heparin.

To study the effect of protamine at longer times the experiment in Table 2 was carried out. One hour after injection of 2 mg of protamine, plasma triglycerides had almost doubled. This was in accord with previous reports (Harwood et al., 1974). Both HL and LPL activities in blood were significantly elevated over control values, indicating a persisting effect of protamine injection. A large dose of heparin, in excess of that needed to neutralize all injected protamine, caused release of both HL and LPL activities, but the amounts released were significantly lower (42% and 60% of control for HL and LPL, respectively) than those released in controls. This suggested that protamine had partially depleted the stores of releasable lipases.

The above results showed that protamine released some LPL into blood and that the released lipase rapidly disappeared from the blood. To study the effect of protamine on the clearance of LPL from blood, labelled bovine LPL was injected. Protamine did not impede the removal of labelled lipase (results not shown). During the first few minutes the amount of labelled lipase in blood was actually somewhat lower in rats given protamine. Nine minutes after the injection of labelled lipase about 10% of the radioactivity remained in the blood. When heparin (1.35 mg) was injected at this time, lipase radioactivity in blood rapidly rose to about 30% of the initial dose regardless of whether the rats first had been given saline or protamine (results not shown). In rats first given saline and then given protamine no significant amounts of labelled lipase were released in the blood (results not shown). Hence heparin but not protamine could release the injected, labelled LPL back into the circulating blood.

The major effect of protamine on the tissue distribution of the labelled LPL was that less was taken up in the liver than in control rats (Table 3). This was opposite to the effect of heparin, which did not decrease the uptake of LPL by the liver. Protamine

Table 3 Effect of protamine on tissue distribution of ^{125}I-LPL

Fasted rats were anaesthetized and given saline alone (controls) or 2 mg of protamine (0.2 ml). ^{125}I-LPL was injected 2 min later intravenously. The rats were killed 10 min later by exsanguination and the tissues dissected out, rinsed, weighed and radioactivity determined. Blood volume remaining in the tissues had previously been determined in corresponding rats given ^{51}Cr-labelled red blood cells (Hultin et al., 1992). These data were used to calculate the amount of ^{125}I-LPL contributed by blood in the tissue samples and this was subtracted from the values shown. All values are expressed as a percentage of injected dose per organ, except for diaphragm and epididymal fat in which case the data are expressed as a percentage of injected dose per gram of tissue. Statistics were done using two-tailed Student's *t*-test. Data represent mean ± S.E.M. ($n = 5$).

Tissue	Control	Protamine	*P*
Blood	5.55 ± 0.34	3.86 ± 0.10	$P < 0.001$
Liver	47.6 ± 2.71	28.5 ± 1.12	$P < 0.001$
Spleen	4.44 ± 0.51	1.30 ± 0.10	$P < 0.001$
Lung	7.07 ± 0.58	11.85 ± 1.08	$P < 0.01$
Kidney	0.91 ± 0.05	0.40 ± 0.01	$P < 0.001$
Heart	3.79 ± 0.20	1.42 ± 0.11	$P < 0.001$
Diaphragm	0.94 ± 0.09	0.69 ± 0.04	$P < 0.05$
Epididymal fat	0.54 ± 0.03	0.78 ± 0.04	$P < 0.001$

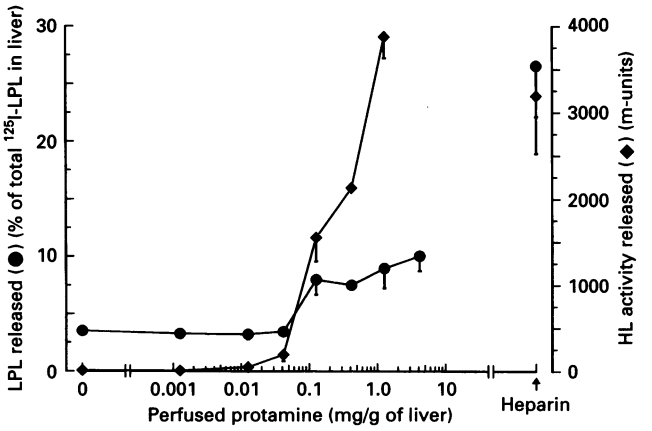

Figure 5 Dose–response curves for the release of LPL and HL by protamine from perfused rat livers

Rats were injected with ^{125}I-LPL (300000 c.p.m., 30 ng) 10 min before the *in situ* liver perfusion was started. The blood was first washed out of the liver with 20 ml of perfusate A. Then, 2 ml of protamine buffer was perfused through the liver followed by 20 ml of perfusate A to wash out all released lipase. Collection of perfusate began at the same time as the protamine buffer was perfused. The collected perfusate was assayed for HL activity (◆) and ^{125}I-LPL radioactivity (●). For comparison, other livers were perfused with heparin-containing buffer (2.6 mg of heparin in 2 ml) instead of protamine buffer. In the experiment with ^{125}I-LPL, samples of liver tissue were counted. This plus the released LPL radioactivity was taken as total in liver at time 0, and the released ^{125}I-LPL was expressed as a percentage of this value. It was not possible to measure the LPL activity at the highest concentration of protamine (3.3 mg/g of liver) due to the turbid appearance (heparin–protamine complexes) of the collected perfusate. Data are means ± S.D. ($n = 2$–3, total 26 rats).

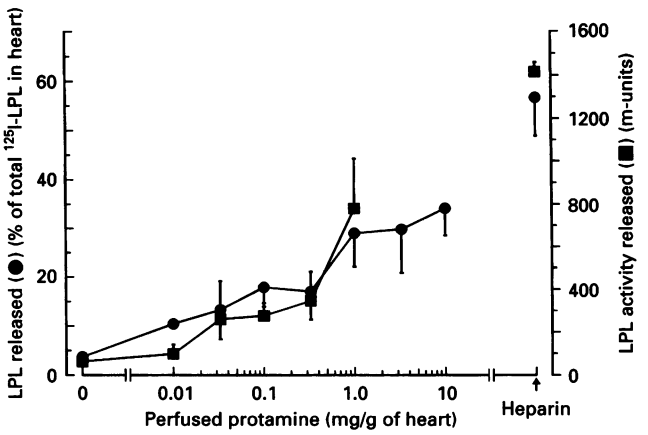

Figure 6 Dose–response curve for release of LPL by protamine in perfused rat hearts

Rats were injected with ^{125}I-LPL (400000 c.p.m., 40 ng) 10 min before the heart was prepared for single pass perfusion. The blood was first washed out of the heart with 10 ml of perfusate A. Then, 1 ml of protamine buffer was perfused through the heart followed by 10 ml of perfusate A to wash out all released lipase. Collection of perfusate began at the same time as the protamine buffer was perfused. The collected perfusate was assayed for LPL activity (■) and ^{125}I-LPL radioactivity (●). For comparison, other hearts were perfused with heparin-containing buffer (1.3 mg of heparin) instead of protamine buffer. In the experiments with ^{125}I-LPL, the radioactivity in samples of heart tissue were counted. This plus the released LPL radioactivity was taken as the total in the heart at time 0, and the released ^{125}I-LPL was expressed as a percentage of this value. It was not possible to measure the LPL activity at the highest concentration of protamine (10 mg/g of heart) due to the turbid appearance (heparin–protamine complexes) of the collected perfusate. Values are mean ± S.D. ($n = 3$, total 27 rats).

also resulted in a lower uptake of radioactive lipase in the spleen, the kidney, the heart and the diaphragm, while uptake in the lung and the epididymal adipose tissue increased.

Liver perfusions

In the next set of experiments we studied the ability of protamine to release lipases from isolated organs. HL was released from the liver by protamine in a dose-dependent manner (Figure 5). Approx. 0.1 mg of protamine/g of liver increased the release of HL from 6.6 ± 2.2 m-units in 22 ml of perfusate to 3900 ± 400 m-units (mean ± S.D., $n = 2$–3); 1 mg of protamine/g of liver released as much HL as a large amount of heparin (2.6 mg).

Since the amount of LPL in the liver is low, we preloaded the liver *in vivo* with ^{125}I-labelled bovine LPL to study release of LPL by protamine. We injected labelled lipase into the intact rat and then waited 10 min before initiating the liver perfusion. In this manner we hoped to minimize non-physiological binding of labelled lipase to tubing and glassware. Under the conditions used, a large dose of heparin released about 25% of the ^{125}I-LPL bound by the liver. The rest had apparently bound to heparin-insensitive sites or had been internalized (Vilaró et al., 1988b). Protamine also released LPL from the liver. A substantial release of ^{125}I-LPL required about 0.1 mg of protamine/g of liver, i.e. the same dose as for release of HL. Higher doses of protamine increased the release somewhat, but even at very high doses of protamine the release was only about one-third of that seen with heparin, or about 10% of the ^{125}I-LPL taken up by the liver.

Heart perfusions

Heart is one of the tissues with high endogenous LPL activity. Of this, about 30% (in our experiments 1400 m-units per heart) can be released by heparin (Chajek-Shaul et al., 1988). In the present experiment the hearts were preloaded with exogenous ^{125}I-LPL *in vivo* by the same procedure as that used for the liver perfusions. The hearts were perfused with a single bolus of Krebs buffer containing either protamine or heparin. Of the ^{125}I-LPL bound by the heart approx. 60% was released by heparin (Figure 6). Protamine also released LPL from the heart and the amount released increased gradually with the protamine dose. At 1 mg of protamine/g of heart, the release was about 35% of the labelled LPL in the heart, i.e. only a little over one-half of the release caused by heparin. Activity measurements revealed that a similar proportion of the endogenous LPL was released. The maximal release of LPL activity was 800 m-units by protamine (1 mg) and 1400 m-units by heparin.

DISCUSSION

It has generally been assumed that LPL and HL at vascular surfaces are anchored to heparan sulphate proteoglycans (HSPG) (Olivecrona and Bengtsson-Olivecrona, 1993). In theory, such binding should be competed out by other polycations, and this presumption was confirmed by model experiments. *In vivo*, HL but not LPL was released from its binding sites by protamine. This poses the questions of what additional components are involved in LPL binding, and of the mechanism(s) by which protamine impedes catabolism of triglyceride-rich lipoproteins.

In agreement with previous studies our data show that the release of HL and LPL to plasma by heparin can be reversed by protamine. Brown observed this in rat experiments already in 1952 (Brown, 1952). More recently, Harenberg et al. (1989) have shown the same in humans given low-molecular-weight heparin preparations. The likely mechanism is that protamine binds to the circulating heparin molecules and that the lipases, when deprived of heparin, return to endothelial/hepatic binding sites. To what extent these sites are the same as those originally occupied by the enzymes is not clear. A similarity in the binding is that a large dose of heparin could again bring the lipases back into the plasma. The experiments with ^{125}I-labelled LPL showed, however, that the tissue distribution of the labelled lipase was different after protamine.

Our data furthermore show that protamine itself can release the lipases into blood, but the effect is more short-lived than that of heparin. This is in accord with previous data that injected protamine is rapidly cleared from plasma (Harwood et al., 1974). Ten minutes after protamine injection the HL activity had peaked and decreased again to less that half of peak values. When heparin was injected at this time the full amount of HL activity was again brought into plasma. Hence, the HL which had been released by protamine and then disappeared from plasma had not been irreversibly lost, but had returned to binding sites where it was available for heparin release.

All our data are consistent with the hypothesis that the main binding sites for HL are polyanions, perhaps heparan sulphate proteoglycans. Protamine releases HL effectively both in liver perfusions and in the intact animal, just as it does in model experiments with heparin–agarose. Injection of heparin after protamine gives little or no further release of HL. Hence, protamine and heparin appear to release the same pool of the enzyme. HL is synthesized only in the liver (Semenkovich et al., 1989), but there is HL also in the adrenals (Berg et al., 1990) and in the ovaries (Hixenbaugh et al., 1989). How this specific localization is regulated is not known. One possibility is that HL binds to a subgroup of HSPG present only in liver, adrenals and ovaries. A precedent for this is the binding of anti-thrombin III to heparin/heparan sulphate, which requires a specific penta-

saccharide sequence (Lindahl, 1994). If there is such a specific polysaccharide structure for binding to HL, this structure must be present in heparin, which releases the enzyme effectively. Commercial heparin is usually prepared from lung or from intestinal mucosa (Lindahl, 1994). Another possibility is that additional molecules are involved at the binding sites, at least in adrenals and ovaries. It is of interest to note that HL was not released from the adrenals by protamine. The mechanism behind the tissue specificity of HL binding is a challenging question which is not resolved with the type of experiments made in our study.

The amount of LPL that appeared in plasma after injection of protamine was less than the amount of HL. This raised the question of whether the low release of LPL was an artifact, because we missed an early peak. It is unlikely that the small release of LPL activity *in vivo* was due to a large release of LPL combined with a fast inactivation since 10 min after injection of protamine a heparin injection could recruit as much LPL as in control animals. Also, if a large amount of LPL was released by protamine and then was quickly cleared from plasma, increased LPL activity in the liver would be expected since the liver is the primary site for clearance of LPL. Protamine did not increase the activity of LPL in the liver. In fact, the uptake of ^{125}I-LPL in the liver was decreased by protamine. This makes it unlikely that rapid clearance by the liver kept plasma LPL low after protamine. Nor can the low LPL activities be explained by a high rate of inactivation. Addition of protamine to plasma enhanced inactivation of LPL, but the half-life was longer than 10 min.

In model experiments LPL was displaced from heparin–agarose by protamine. The hypothesis that the enzyme is bound to HSPGs *in vivo* therefore predicts that it should be released by protamine. However, only a small amount of LPL was released. How can this be explained? First, the doses used *in vivo* might have been too small. In perfusion studies, doses of up to 1 mg of protamine/g of tissue were used. This would correspond to ~ 200 mg of protamine *in vivo* and we only used 2 mg. However, 2 mg of protamine is sufficient to have large effects on lipid metabolism and this dose released HL almost quantitatively. Secondly, it is possible that the LPL released by protamine was a large part of the functional pool of LPL. It has been suggested that the functional pool of LPL is only a fraction of the heparin-releasable pool (Borensztajn, 1987). Liu and Olivecrona (1992) have reported that heparin released LPL from perfused hearts in at least three phases. During the first minutes of perfusion, heparin released LPL which was immediately accessible at the endothelium. Then there was a shoulder of LPL release (from 2 to 20 min), which might largely represent LPL at other extracellular sites in the tissue. In guinea pigs this fraction corresponded to twice the amount of LPL released during the first 2 min. The third component was newly synthesized LPL. A possible interpretation of the low release of LPL by protamine might be that protamine released only LPL directly exposed to blood, but did not recruit LPL from subendothelial stores. In fact, in our experiments the release of LPL by heparin *in vivo* was somewhat delayed in protamine-treated rats compared with controls (Figure 3). A third possibility is that binding of LPL to peripheral tissues is not directly affected by protamine. This assumes another component in the binding of LPL to endothelium in addition to HSPG or instead of HSPG. Sivaram et al. (1992) have reported a 116 kDa endothelial protein (116hrp) that binds to both LPL and HSPG and they have suggested that this protein participates in the binding of LPL to its endothelial sites. The complex LPL–116hrp–HSPG is dissociated by heparin but it is possible that the complex is resistant to protamine. This could explain why protamine released only a small fraction of the heparin-releasable LPL, but it does not explain why protamine decreases the lipolysis of chylomicrons and very low density lipoproteins.

Data on binding of LPL in the liver are complex. Previous experiments have shown that there is both a heparin-sensitive and a heparin-insensitive component (Vilaró et al., 1988b). It has been proposed that LPL binds first to the heparin-sensitive sites (HSPG or some other polycation) and then transfers to another receptor for internalization/degradation. In the present liver perfusions a dose of protamine which gave maximal release of HL released only about one-third of the heparin-releasable LPL. This suggests that there are at least two types of heparin-sensitive sites for binding of LPL in the liver. One likely candidate is the putative polyanion sites which bind HL. What other sites are involved is presently not known. One possible catabolic site in the liver from which LPL is released by heparin, but perhaps not by protamine, is the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (Beisiegel et al., 1991; Nykjær et al., 1993).

Earlier studies by Harwood et al. (1974) and Hirata et al. (1987) have shown that protamine decreases the clearance of triglyceride-rich lipoproteins *in vivo*. Our data show that after protamine the amount of heparin-releasable LPL remains virtually unchanged, and that the enzyme retains full catalytic activity. This implies that there is still LPL in an active form at the vascular endothelium, which is in accord with the observation by Harwood et al. (1974) that injection of protamine does not decrease the amount of LPL activity measurable in adipose tissue homogenates. It is difficult to explain the decreased clearance of triglyceride-rich lipoproteins as a result of LPL inhibition/depletion. It is more likely that protamine impedes binding of the lipoproteins to endothelial lipolysis sites. This binding probably involves interaction of apolipoproteins E and B with cell surface HSPGs since it is known that these apolipoproteins have polyanion binding sites (Chan, 1992; Shimano et al., 1992). Protamine may deny the lipoproteins access to these sites. If so, the explanation for the delayed lipoprotein catabolism would be that the lipoproteins circulate in blood without the possibility of coming into contact with the endothelial-bound lipase.

This work was supported by grants from the Swedish Medical Research Council (03X-727 and 03P-10158-01; fellowship to M.H.), the Swedish Medical Society, the Swedish Nutrition Foundation and the Bank of Sweden Tercentenary Foundation.

REFERENCES

Beisiegel, U., Weber, W. and Bengtsson-Olivecrona, G. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8342–8346

Bengtsson, G. and Olivecrona, T. (1977) in Electrofocusing and Isotachophoresis (Radola, B. J. and Graesslin, D., eds.), pp. 189–195, de Gruyter, Berlin

Bengtsson, G., Olivecrona, T., Höök, M., Riesenfeld, J. and Lindahl, U. (1980) Biochem. J. **189**, 625–633

Bengtsson-Olivecrona, G. and Olivecrona, T. (1991a) in Lipoprotein Analysis (Converse, C. and Skinner, E. R., eds.), Oxford University Press, Oxford

Bengtsson-Olivecrona, G. and Olivecrona, T. (1991b) Methods Enzymol. **197**, 345–356

Berg, A. L., Hansson, P. and Nilsson-Ehle, P. (1990) J. Intern. Med. **228**, 257–260

Borensztajn, J. (1987) in Lipoprotein Lipase (Borensztajn, J., ed.), pp. 133–148, Evener, Chicago, IL

Brown, W. D. (1952) Q. J. Exp. Physiol. **37**, 75–84

Chajek-Shaul, T., Bengtsson-Olivecrona, G., Peterson, J. and Olivecrona, T. (1988) Am. J. Physiol. **255**, E247–E254

Chan, L. (1992) J. Biol. Chem. **267**, 25621–25624

Deckelbaum, R. J., Ramakrishnan, R., Eisenberg, S., Olivecrona, T. and Bengtsson-Olivecrona, G. (1992) Biochemistry **31**, 8544–8551

Doolittle, M. H., Wong, H., Davies, R. C. and Schotz, M. C. (1987) J. Lipid Res. **28**, 1326–1334

Harenberg, J., Stehle, G., Dempfle, C. E., von Hodenberg, E. and Heene, D. L. (1989) Folia Haematol. (Leipzig) **116**, 967–980

Harwood, J. L., Riley, S. E. and Robinson, D. S. (1974) Biochim. Biophys. Acta **337**, 225–238
Hide, W. A., Chan, L. and Li, W.-H. (1992) J. Lipid Res. **33**, 167–178
Hirata, M. H., Oliveira, H. C., Quintao, E. C., Redgrave, T. G. and Maranhao, R. C. (1987) Biochim. Biophys. Acta **917**, 344–346
Hixenbaugh, E. A., Sullivan, T. R. J., Strauss, J. F., Laposata, E. A., Komaromy, M. C. and Paavola, L. G. (1989) J. Biol. Chem. **264**, 4222–4230
Hultin, M., Bengtsson-Olivecrona, G. and Olivecrona, T. (1992) Biochim. Biophys. Acta **1125**, 97–103
Lindahl, U. (1994) Nord. Med. **109**, 4–8
Liu, G. and Olivecrona, T. (1992) Am. J. Physiol. **263**, H438–H446
Liu, G., Bengtsson-Olivecrona, G., Östergaard, P. and Olivecrona, T. (1991) Biochem. J. **273**, 747–752
Liu, G., Bengtsson-Olivecrona, G. and Olivecrona, T. (1993) Biochem. J. **292**, 277–282
Nykjær, A., Bengtsson-Olivecrona, G., Lookene, A., Moestrup, S. K., Petersen, C. M., Weber, W., Beisiegel, U. and Gliemann, J. (1993) J. Biol. Chem. **268**, 15048–15055
Olivecrona, T. and Bengtsson-Olivecrona, G. (1993) Curr. Opin. Lipidol. **4**, 187–196

Received 22 March 1994/20 June 1994; accepted 4 July 1994

Peterson, J., Olivecrona, T. and Bengtsson-Olivecrona, G. (1985) Biochim. Biophys. Acta **837**, 262–270
Semenkovich, C. F., Chen, S. H., Wims, M., Luo, C. C., Li, W. H. and Chan, L. (1989) J. Lipid Res. **30**, 423–431
Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H. and Fanburg, B. L. (1981) J. Clin. Invest. **68**, 995–1002
Shimano, H., Yamada, N., Katsuki, M., Yamamoto, K., Gotoda, T., Harada, K., Shimada, M. and Yazaki, Y. (1992) J. Clin. Invest. **90**, 2084–2091
Sivaram, P., Klein, M. G. and Goldberg, I. J. (1992) J. Biol. Chem. **267**, 16517–16522
Stins, M. F., Maxfield, F. R. and Goldberg, I. J. (1992) Arterioscler. Thromb. **12**, 1437–1446
Vilaró, S., Llobera, M., Bengtsson-Olivecrona, G. and Olivecrona, T. (1988a) Biochem. J. **249**, 549–556
Vilaró, S., Llobera, M., Bengtsson-Olivecrona, G. and Olivecrona, T. (1988b) Am. J. Physiol. **254**, G711–G722
Wallinder, L., Peterson, J., Olivecrona, T. and Bengtsson-Olivecrona, G. (1984) Biochim. Biophys. Acta **795**, 513–524