Nutrition Research

The Effect of Ascorbic Acid on the Activity of Lipoprotein Lipase in the Baboon (*Papio ursinus*)

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SUMMARY

The intravenous administration of heparin-released lipoprotein lipase (LPL) into the circulatory system of the baboon (*Papio ursinus*) is described.

After a single heparin injection, a peak value of LPL activity appeared in the circulation within 5 minutes. At low doses of heparin (<100 units heparin/kg body mass), LPL disappeared from the circulation in an exponential fashion with a half-life of about 20 minutes.

An increase in the heparin dose increased the amount of LPL released into the circulation.

In baboons which were deficient in ascorbic acid, less LPL was released into the circulation after specific doses of heparin than in animals that were amply supplied with this vitamin (ascorbic acid 16 mg/kg body mass/day).

The separation of plasma LPL, released by heparin, on Sephadex G-150, revealed several distinct molecular species of LPL in the eluant from the columns.

In vitro studies indicated that ascorbic acid inhibited cardiac LPL strongly, whereas it had little effect on 'post-heparin plasma' LPL. Isomolar concentrations of another reducing agent, mercapto-ethanol, slightly stimulated cardiac LPL in baboons.


The enzyme lipoprotein lipase (LPL), which is also known as clearing factor, triglyceride lipase or triacylglycerol lipase, is not normally present in blood plasma, but is released into the circulatory system by the intravenous injection of heparin. This enzyme has also been found to be present in various tissues of animals and man, although the properties of the LPL present in the different tissues are not always identical.

Early studies by Korn showed that LPL prepared from rat heart muscle or liver responded differently to NaCl or protamine sulphate *in vitro*. He found that the heart muscle enzyme was strongly inhibited by both NaCl and protamine sulphate, whereas the liver enzyme was not measurably affected.

Heparin injection releases at least two types of LPL into the bloodstream. Sodium chloride and protamine sulphate inhibition studies characterised these two enzymes as the hepatic and the extrahepatic (adipose tissue) enzymes. Other researchers, however, have reported that heparin causes release of the hepatic or the extrahepatic enzyme in different species of animals.

Certain physiological stimuli, such as feeding, on the one hand, and fasting and exposure to the cold on the other, have inverse effects on the activities of adipose tissue and heart muscle LPL.

Feeding or fasting does not affect hepatic triglyceride lipase activity. These findings, therefore, suggest that at least three types of triglyceride lipase exist in animals, viz. hepatic, adipose and cardiac enzymes.

Our previous studies indicated that ascorbic acid inhibits cardiac lipoprotein lipase activated *in vivo* and probably also affects other lipoprotein lipases.

The present study was done to characterise the heparin-induced LPL activities of plasma and to elucidate the role of ascorbic acid in the control of heparin-induced cardiac LPL activities.

MATERIALS AND METHODS

Animals

Baboons (*Papio ursinus*) were housed and maintained in individual wire mesh cages. The animals were randomly divided into an experimental group whose members received ascorbic acid in their diet, and a control group whose members received no ascorbic acid. Both groups of animals were kept on their respective dietary regimens for at least 2 months before they were subjected to the experiments described.

The diet of the experimental and control groups consisted of precooked maize meal (280 g), a protein-vitamin-mineral mixture (30 g) and sunflower oil (8 ml). Ascorbic acid was added to the diet of the experimental group to provide an intake of 16 mg ascorbic acid per kg body mass. All ingredients were mixed into a stiff porridge, pressed into balls and fed to the animals twice daily.

Blood samples were obtained from the animals under Sernylan anaesthesia (1 mg/kg body mass).

Plasma LPL*

Lipoprotein lipase was released into the circulation of baboons which had been fasted overnight by the intravenous injection of heparin. Blood samples were obtained by venepuncture and plasma was prepared by the centrifugation of the blood at 4,000 g for 20 min at 5°C. LPL activity was determined directly in the plasma.

* Plasma LPL refers to LPL released into circulation by administration of heparin, unless stated otherwise.
Plasma LPL was precipitated by bringing the plasma to 60% ammonium sulphate saturation at 4°C and maintaining the pH at 7.4. The precipitate, containing about 90 ± 5% of the LPL, was collected by centrifugation at 20,000 g for 20 min at 4°C. The supernatant was decanted and the precipitate containing the LPL was resuspended in 30 times its volume of tris buffer (0.1M; pH 8.2), which diluted the ammonium sulphate concentration to less than 0.07M. The sample was recentrifuged as before to remove undissolved material.

Inhibition studies were performed on the redissolved LPL fraction in order to minimise inactivation by lengthy dialysis procedures.

Approximately 150 mg of the redissolved protein was applied to a Sephadex G-150 column (25 mm x 350 mm) that was previously equilibrated with tris buffer (0.1M; pH 8.2) and kept at 5°C. The column was eluted with the same buffer at a flow speed of 15 ml/h. 4-ml fractions were collected and the absorbancy of the fractions was determined at 280 nm. The void volume of the column was determined with blue dextran and was found to be 40 ml. The total volume of the column was 170 ml.

Heart Muscle LPL

Baboon heart muscle was suspended in Krebs-Ringer bicarbonate buffer (0.15M; pH 7.4) containing 50 units heparin/ml and was homogenised for 1 minute at 4°C by means of an Ultra Turrax homogeniser (Jancke and Kunkel, Stauffen, Breisgau, Federal Republic of Germany). The homogenate was centrifuged at 4000 g for 20 min at 4°C.

The supernatant fraction containing the LPL was decanted, precipitated with ammonium sulphate, centrifuged and redissolved in tris buffer as described for plasma LPL. LPL inhibition studies were done on the redissolved LPL fraction.

LPL Assay

LPL activity was determined according to the method of Krauss et al. and the liberated free fatty acids were extracted as described by Belfrage and Vaughan.

The substrate contained, in a total volume of 12 ml, 70 nmol glyceryl trioleate (38 mCi/mmol, Amersham/Searle Corp., Arlington Heights, Ill.); 113 μmol (100 mg) unlabelled triolein (Applied Science Laboratories, Inc., State College, Pa); 200 mg fatty acid free bovine serum albumin (Miles Laboratories, Epping, CP); 0.6 ml of a 1% Triton X-100 solution (Packard Instrument Co., Downer’s Grove, Ill.) and 0.194M, pH 8.2 tris-HCl buffer (I = 0.05) containing 0.15M NaCl. Human plasma, 1 ml, was added to the mixture. The final mixture was sonicated for 1 min at 4°C with a Branson Sonifier (Branson Sonic Power Co., Danbury, Conn.) at setting 5.0.

The substrate, 0.2 ml, and 0.2 ml of a solution containing the LPL enzyme were incubated in a Dubnoff shaking waterbath at 30°C for 30 min. The enzymatic reaction was stopped by the addition of 7.0 ml of a methanol-chloroform-heptane mixture (1:41:1.25:1 v/v) and immediately thereafter, 2.0 ml of a potassium carbonate buffer, pH 10.5, was added.

The test tubes (12 × 175 mm) were then stoppered and mixed vigorously for 20 seconds in a Vortex mixer. After separation of the phases by centrifugation (1000 rpm for 15 min at ambient temperature), 4.0 ml aliquots of the upper phase were transferred to counting vials, 10 ml Aquagel (Chemlab, Blairgowrie, TVl) was added, and the radioactivity was determined by means of a Packard liquid scintillation counter model 3375 (Packard Instrument Co., Downer’s Grove, Ill.). The quenching of the samples was corrected for by means of the predetermined AES ratios.

The assay yielded zero order kinetics with respect to substrate throughout the range of lipase activities studied.

Results were expressed as picomoles of fatty acids released per minute per millilitre of enzyme solution used. The lowest limit of LPL activity which may be detected is a release of about 100 pmol free fatty acids (FFA).

Protamine sulphate inhibition was achieved by incubating the LPL for 60 min at 27°C with 0.75 mg protamine sulphate/ml (Cal-biochem, Los Angeles, Calif.) before the LPL was assayed.

Protein and Ascorbic Acid Determination

Protein was determined according to the method of Layne. Ascorbic acid was determined in serum samples that were deproteinised with trichloro-acetic acid, according to the phenylhydrazine method of Roe and Kuether, as modified by Schaffert and Kingsley.

RESULTS

Effects of Dose of Heparin on LPL Release

In the first experiment, the effect of the heparin dose level on the release of LPL into the circulatory system of the baboon and the subsequent removal rate of the enzyme were investigated.

Heparin was injected into anaesthetised baboons that were on the ascorbic acid diet. The heparin doses were 4, 20, 100, 250, 500 and 1250 U/kg body mass. Blood samples were drawn at the time intervals indicated in Fig. 1. All the results presented are the average values obtained from at least 2 animals.

![Fig. 1. The plasma LPL activity in baboons after the intravenous injection of different heparin doses. Each point in the figure represents the average value of two separate determinations.](image-url)

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The intravenous injection of a single dose of heparin into a baboon invariably resulted in the appearance of peak LPL activities in the circulatory system in about 5 min. As the heparin dose increased, the activity per 1 ml plasma, measured 5 min. after the injections, increased steadily. When doses of heparin above 250 U/kg body mass were given, the LPL peak values were not observed immediately after the heparin injection, but the activity in the circulatory system kept rising, and the times for the maximum values to be reached were delayed. When heparin 1 250 U/kg body mass was given, the maximum LPL value was attained about 65 min after the heparin injection.

At low doses of heparin (<100 units heparin/kg body mass), the T$_1$ value for the removal of LPL from the circulation was about 20 min. Above 500 units heparin/kg body mass, the circulating LPL activity did not decrease during the first 60 min after heparin injection, but increased steadily after the first 5 min. In order to determine whether an increase in the heparin dose did not release different LPL species in sequence, post-heparin plasma, obtained from baboons which were injected with different doses of heparin and from which blood samples were taken at various times after the heparin injection, was incubated with protamine sulphate as described by Krauss et al., to differentiate between hepatic and extrahepatic LPL.

The results obtained showed no change in the relative proportions of the two lipases either with time, after the heparin injection, or simultaneously with the heparin dose. Incubation of plasma with protamine sulphate inhibited the lipolytic activity to about 40% of the control values. This finding indicates that the plasma LPL contained approximately 40% hepatic and 60% extrahepatic LPL activity.

**Effect of Ascorbic Acid on LPL Release by Heparin**

In the next experiment, the effect of dietary ascorbic acid on the maximum values that can be released by specific doses of heparin was investigated. The animals that received dietary ascorbic acid had mean serum ascorbic acid values of 1.45 ± 0.14 mg/dl, whereas the control animals had mean serum ascorbic acid values of 0.32 ± 0.09 mg/dl. The mean ascorbic acid values of the 2 groups of animals were significantly different (P<0.01). No clinical signs of scurvy were visible in the animals which received no ascorbic acid.

The results are depicted in Fig. 2. Each point in the figure represents the average value of at least 3 animals. Statistical comparison of the corresponding LPL values on the two curves in Fig. 2 showed that the plasma LPL values of the baboons that received the dietary ascorbic acid were significantly higher (P<0.05) than those of the animals which received no ascorbic acid.

**Gel Filtration of Plasma LPL**

Plasma LPL was next separated on a Sephadex G-150 column in order to establish whether more than one molecular LPL species was present (Fig. 3). The redissolved ammonium sulphate precipitate of the plasma (about 150 mg protein) was applied to the Sephadex G-150 column and 4 protein peaks were eluted. The protein peaks appeared in fractions 23, 31, 69 and 92, respectively. Determinations of the LPL activity of the fractions eluted from the column revealed the presence of 6 LPL activity peaks (Fig. 3). The first activity peak appeared in the void volume ahead of the major protein peak and was most probably associated with a macromolecular lipid fraction. Sephadex G-150 excludes proteins of a molecular mass greater than 400 000 daltons, which suggests that the molecular mass of the first LPL activity peak was greater than 400 000.
tamine sulphate strongly inhibited the LPL activities of peaks I, II, III and V, whereas peaks IV and VI were not significantly inhibited. These findings indicate that baboon post-heparin plasma contains lipolytic enzymes, the characteristics of which resemble those of hepatic or adipose tissue LPL described as being present in rat and human post-heparin plasma.2,3

**Effect of Ascorbic Acid on Cardiac LPL**

Studies on the redissolved LPL which was precipitated by 60% ammonium sulphate saturation confirmed previous observations2,3 and indicated that ascorbic acid inhibits cardiac LPL over a wide concentration range (1.13 x 10⁻⁸ to 1.13 x 10⁻⁴ M) (Table I).

**TABLE I. EFFECT OF ASCORBIC ACID ON BABOON PLASMA POST-HEPARIN LIPASE ACTIVITY AND HEART MUSCLE LPL ACTIVITY**

<table>
<thead>
<tr>
<th>Ascorbic acid concentration (molarity)</th>
<th>Plasma post-heparin lipase activity</th>
<th>Heart muscle lipoprotein lipase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1.13 x 10⁻⁸</td>
<td>105.6</td>
<td>42.2</td>
</tr>
<tr>
<td>1.13 x 10⁻⁷</td>
<td>97.2</td>
<td>37.4</td>
</tr>
<tr>
<td>1.13 x 10⁻⁶</td>
<td>90.9</td>
<td>36.1</td>
</tr>
<tr>
<td>1.13 x 10⁻⁵</td>
<td>95.0</td>
<td>31.2</td>
</tr>
<tr>
<td>1.13 x 10⁻⁴</td>
<td>92.7</td>
<td>26.5</td>
</tr>
<tr>
<td>1.13 x 10⁻³</td>
<td>—</td>
<td>14.3</td>
</tr>
</tbody>
</table>

* As percentage of the control value.

At an ascorbic acid concentration of 1.13 x 10⁻⁴ M, baboon cardiac LPL was already inhibited to 42.2% of the control value, and at 1.13 x 10⁻³ M the enzyme revealed only 14.3% of the control activity. In contrast to this, the post-heparin lipase, treated in the same way, showed little if any inhibition.

**Comparison of Ascorbic Acid and Mercapto-ethanol**

To establish whether the inhibitory effect of ascorbic acid on cardiac LPL was owing to the reducing properties of the compound, an experiment was done to compare ascorbic acid with another reducing agent, mercapto-ethanol, at equimolar concentrations (Table II). Ascorbic acid has a reducing potential of E° = 0.062 volts against the –0.32 volts for the much stronger reducing agent, mercapto-ethanol. At similar concentrations ascorbic acid strongly inhibited baboon heart muscle LPL, whereas mercapto-ethanol actually showed a slight stimulation of the enzyme (36%).

**DISCUSSION**

Heparin releases LPL into the circulatory systems of all mammalian species tested. If only a single intravenous injection is administered, LPL will rise sharply and reach a peak value in about 5 min and will subsequently disappear from the circulation in an exponential fashion.5,6 We found that the T½ value for the removal rate of LPL from the circulation of the baboon was 20 min at low doses of heparin (<100 units heparin/kg body mass), as compared with the 25.4 min in man, 18.6 min in dogs and 7.3 min in rabbits.26 On the other hand, Whayne et al.7 reported the T½ values of the hepatic removal of LPL in dogs to be 4.5 min.

Increased doses of heparin apparently blocked the removal of LPL from the circulatory system of baboons. Naito and Felts27 made a similar observation in the rat. Above 500 units heparin/kg body mass, the LPL activity did not decrease perceptibly during the initial 60 min after the administration of heparin.

Chajek et al.28 provided evidence that the plasma lipoprotein lipase, which was released into the circulation of rats, originated from two separate pools in the body. The first pool consisted of a preformed activated enzyme which could be directly released by heparin. The second pool had to be transported to the site of the first pool before it could be released. This implies that the two pools have more or less fixed sizes. Our results, however, did not suggest that two fixed LPL pools exist in the baboon. Somehow, the increased doses of heparin managed to release increasing LPL activities within the first few minutes after the administration of heparin, with a subsequent slow release of LPL from another pool. The LPL which was released slowly with the large heparin doses did not appear to be different from the LPL release within the first few minutes after heparin injection, because protamine sulphate inhibition studies revealed a constant proportion of hepatic and extrahepatic LPL during the two phases of LPL release.

In recent literature there are controversial reports on the presence of two different molecular species of post-heparin lipase in blood. Krauss et al.29 found both hepatic and extrahepatic enzymes in the plasma of rats and humans. Greten et al.30 could only detect hepatic LPL in the post-heparin plasma of dogs, whereas Zieve and Zieve31 detected practically only extrahepatic LPL in the post-heparin plasma of rats, since hepatectomy did not affect the enzyme activity. Ehnholm et al.32 found that the LPL of human post-heparin was markedly activated by high NaCl concentrations, which resembled the characteristics of the hepatic enzyme. In contrast, the post-heparin lipolytic activity in dogs and swine was markedly inhibited at 0.5M to 1.0M NaCl concentrations, indicating that dog and swine post-heparin lipase resembles adipose tissue LPL.

**TABLE II. COMPARISON BETWEEN THE EFFECTS OF EQUIMOLAR CONCENTRATIONS OF TWO REDUCING AGENTS, ASCORBIC ACID AND MERCAPTO-ETHANOL, ON BABOON HEART MUSCLE LPL ACTIVITY**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (molarity)</th>
<th>LPL activity (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>1.13 x 10⁻⁸</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>1.13 x 10⁻⁷</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>1.13 x 10⁻⁶</td>
<td>92</td>
</tr>
<tr>
<td>Mercapto-ethanol</td>
<td>1.13 x 10⁻⁸</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>1.13 x 10⁻⁷</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>1.13 x 10⁻⁶</td>
<td>280</td>
</tr>
</tbody>
</table>
To find out more about hepatic and extrahepatic plasma LPL, we separated the enzymes on a Sephadex G-150 column. Our study revealed 6 enzyme peaks with lipolytic activity, 4 of which had the characteristics of adipose tissue LPL and 2 of which resembled hepatic LPL.

One basic difference between our results and those of Ehnholm et al. is that we continued the elution until about 440 ml had passed through the column, instead of the 140 ml they used. We also observed 2 LPL activity peaks in the 2 major protein peaks, but subsequent elution produced 3 more LPL-activity peaks in a region where only 2 very small protein peaks were observed.

The pre-incubation of human post-heparin LPL apparently changed the elution pattern, but only one LPL peak was observed by Ehnholm et al., whereas the baboon plasma LPL, which was treated with ammonium sulphate, revealed 6 different peaks.

The present study, which verifies our previous findings, shows that ascorbic acid inhibits cardiac LPL over a wide range of concentrations (1.13 × 10^{-3}M to 1.13 × 10^{-4}M). In the body, ascorbic acid concentrations can range from 10^{-3}M in blood to 10^{-4}M in adrenals. This implies that at physiological concentrations ascorbic acid strongly inhibits cardiac LPL. In contrast to this, the post-heparin lipases were not inhibited by ascorbic acid.

The inhibitory effect of ascorbic acid did not seem to be owing to the oxidation-reduction properties of the compound. Ascorbic acid also inhibited hormone-sensitive lipase and myrosinase, but, as in the case of LPL, the inhibitory effect of the compound could not be ascribed to its oxidation-reduction properties.

From the data presented, it is evident that ascorbic acid exerts at least part of its biological function in lipid metabolism by controlling the activity of cardiac LPL.

CONCLUSIONS

In baboons, heparin released both hepatic and extrahepatic LPL into the circulation. The proportion of the two species of LPL is not dependent on the dose of heparin.

By a mechanism as yet unknown, ascorbic acid facilitates an increase in the release of LPL into the circulatory system of the baboon at specific doses of heparin, compared with the activities released into the circulation of animals receiving no ascorbic acid.

The separation of post-heparin plasma on Sephadex G-150 and the inhibition studies of the separated fractions by protamine sulphate present supporting evidence of the presence of both hepatic and extrahepatic LPL in baboon post-heparin plasma.

In vitro studies showed that ascorbic acid inhibits cardiac LPL, while it has little effect on plasma LPL. The inhibitory effect of ascorbic acid in LPL cannot be ascribed to its properties as a reducing agent.

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REFERENCES