Post-Heparin Triacylglycerol Lipases in Ovine Plasma

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Abstract

Lipoprotein lipase and hepatic lipase have been shown to be present in the post-heparin plasma of sheep. Intravenous injection of heparin into sheep produced a rapid increase in the free fatty acid concentration and lipolytic enzyme activity of the plasma, both peaking within 5-15 min and then falling to pre-heparin levels within 30-60 min. Lipolytic activity was not detected in plasma before heparin treatment. Two distinct lipolytic activities were separated from the plasma by chromatography on heparin-Sepharose 6B. Lipoprotein lipase was identified on the basis that the lipolytic activity was dependent upon the addition of plasma, inhibited by 1M NaCl, and inhibited by a specific antiserum against lipoprotein lipase. The second lipolytic activity of plasma was identified as hepatic lipase, as it was not dependent upon plasma for activity, nor was it inhibited by 1M NaCl or antiserum against lipoprotein lipase. Its properties were identical to the lipase extracted from the liver of sheep. Lipoprotein-lipase activity, but not hepatic-lipase activity, was dependent upon the nutritional state of the sheep at the time of heparin injection. However, hepatic lipase comprised a significant proportion of the total lipolytic activity.

Introduction

Intravenous infusion of heparin results in the rapid appearance of two readily distinguishable groups of triacylglycerol (TAG) lipases in the plasma. These lipolytic enzymes are released from the luminal surface of vascular endothelial cells of a variety of organs and peripheral tissues, where they are normally responsible for the hydrolysis of plasma lipoprotein TAG with subsequent uptake of free fatty acids (FFA) for utilization or storage. Lipoprotein lipase (EC 3.1.1.34; triacylglycerol-protein acylhydrolase) is released from a number of sites including skeletal and cardiac muscle, adipose and mammary tissues, lungs and aorta. The relative contribution of lipoprotein lipase from each site is dependent upon nutritional and physiological factors. Hepatic lipase and other lipases having similar characteristics to the liver enzyme, but from sites such as ovaries and adrenal glands, constitute the second group of heparin-releasable lipases (see Jansen and Hulsmann 1980). However, irrespective of the physiological state of the animal, hepatic lipase represents virtually all of this second group of lipases present in post-heparin plasma.

In the ruminant fed ad libitum, various peripheral tissues have been found to attain high levels of lipoprotein lipase activity (Haugebak et al. 1974; Tume et al. 1983) and have low plasma concentrations of very low-density lipoproteins (VLDL) and chylomicrons, the major transporters of TAG in plasma. This is indicative of a very efficient system, which results in the rapid uptake of TAG by peripheral tissues such as skeletal muscle and adipose tissue. Indeed, Bergman et al. (1971) have shown that plasma TAG-fatty acids are rapidly removed by peripheral tissues of sheep. On the other hand, the role of hepatic lipase in the metabolism of lipoproteins is unclear. It appears that hepatic lipase (and similar enzymes) might be involved in both the hydrolysis of high-density lipoprotein (HDL)-phospholipid and HDL-TAG and in the uptake of HDL-cholesterol (see Jansen and Hulsmann 1985).

Hepatic lipase has been reported to be either absent (Etienne et al. 1981), or present in...
low concentrations compared with lipoprotein lipase (Cordle and Clegg 1983), in the post-heparin plasma of bovine animals. To our knowledge, hepatic lipase has not been described in sheep nor has there been any report of the lipolytic enzymes released into plasma following the injection of heparin. The present work demonstrates the significant presence of both hepatic lipase and lipoprotein lipase in post-heparin plasma of sheep.

Materials and Methods

Experimental Animals
Border Leicester × Merino cross sheep, weighing 38-43 kg liveweight, were fed a diet of cereal-grain pellets ("Red Comb" cattle feed-lot pellets, 7230, Gillespie Bros. Pty Ltd, Wacol, Queensland) and lucerne chaff (1:1 w/w). This was fed either ad libitum or restricted to 200 g per day for 5 days.

Preparation of Post-heparin Plasma
Sheep were injected in the jugular vein with heparin (100 U kg⁻¹) (Etienne et al. 1981), and blood samples (~10 ml) were collected in heparinized Vacutainer tubes (Becton Dickinson and Co., New Jersey) at T₀ (immediately prior to injection) and then at various times up to 75 min following the injection (see Fig. 1). Blood was immediately cooled to 2-4°C and plasma was prepared by centrifugation. Plasma was either used immediately or was stored at −20°C for up to 2 months without any discernable alteration in lipase activity

Separation of Hepatic and Lipoprotein Lipase by Affinity Chromatography
Plasma samples from individual sheep, obtained at 5, 10 and 15 min post-heparin injection, were pooled and mixed with an equal volume of 0-4 M NaCl, 0-01 M phosphate buffer (pH 7-0), containing 60% (v/v) glycerol; 20 ml of diluted plasma was loaded onto a heparin-Sepharose 6B column (130 × 10 mm) and the flow rate was adjusted to about 8 ml h⁻¹. Chromatography was done at 4°C. After loading the sample, the column was washed with 30 ml of a solution containing 0-4 M NaCl, 0-01 M phosphate buffer (pH 7-0), 30% (v/v) glycerol. The concentration of NaCl in the solution was increased first to 0-7 M and 30 ml taken to wash the column, then to 1-5 M for the final wash of the column. Aliquots from 2-5-ml fractions were assayed for hepatic and lipoprotein lipase activities.

Extraction of Lipase from Liver
Two grams of liver from sheep fed ad libitum were homogenized in 2 ml of 0-1 M Tris-HCl, pH 7-8. The lipid was extracted from the homogenate with successive washes of acetone, followed by ether, and the defatted pellet was dried and the powder extracted with 14 ml of 0-05 M NH₄OH–NH₄Cl buffer, pH 8-1, containing 0-5 mg heparin. The supernatant resulting from centrifugation (2000 g for 20 min) was assayed for hepatic lipase.

Assay of Lipoprotein Lipase and Hepatic Lipase
Total lipolytic activity of plasma, or column fractions, was measured at pH 8-1 in the presence of serum, but in the absence of added NaCl. The reaction mixture (0-2 ml) contained final concentrations of 67 mm Tris-HCl, pH 8-1, 5-65 mm [²H]triolein (c. 2000 dpm nmol⁻¹) as triolein-containing vesicles (Nilsson-Ehle and Schotz 1976), 1-5% (w/v) bovine serum albumin, and 8-5% (v/v) sheep serum; it was pre-incubated for 15 min at 24-26°C prior to adding the post-heparin plasma (0-03 ml) or the column fractions (0-01 ml). Hepatic lipase activity of plasma (0-03 ml) or column fractions (0-03 ml) was measured as described above, but at pH 8-8 in the presence of 1 M NaCl, and in the absence of added serum. In those experiments where rabbit antilipoprotein lipase serum was added, an amount of 'pre-immune' rabbit serum was added so that the total serum concentration remained the same. All assays were incubated at 37°C for 1-2 h and not more than 8% of the total triolein was hydrolysed during the incubation. Lipoprotein-lipase activity was calculated as the difference in activity between the total activity and the hepatic-lipase activity. All assays were performed in duplicate, and tubes containing additional buffer instead of plasma or column samples served as controls. The [²H]oleic acid present in the control tubes at the end of the incubation period amounted to 0-2–0-3% of the [²H]triolein added. The [²H]oleic acid released during the reaction was isolated by solvent partition (Belfrage and Vaughan 1969) as modified by Nilsson-Ehle and Schotz (1976), and the radioactivity in the upper phase was determined by liquid scintillation counting.

Measurement of Plasma FFA
FFA were extracted from plasma using the method of Dole (1956) and estimated by titration with tetrabutylammonium hydroxide (Kelley 1965).
Preparation of Antiserum to Ovine Milk Lipoprotein Lipase

Ovine milk lipoprotein lipase was prepared by heparin-Sepharose chromatography, as described by Bengtsson and Olivecrona (1977). Antiserum was produced in rabbits by injecting subdermally ~50 μg of purified milk lipoprotein lipase at 14-day intervals. Each rabbit received a total of three injections. For the first injection, lipoprotein lipase was mixed with Freund’s complete adjuvant, but Freund’s incomplete adjuvant was used for successive injections. Blood was collected and antiserum obtained 10 days after the last injection. Antiserum produced a single band against lipoprotein lipase on Ouchterlony plates, but serum from ‘pre-immune’ rabbits gave no reaction.

Reagents

Anhydrous emulsions of [3H]triolein with glycerol and phosphatidylcholine were prepared as described by Nilsson-Ehle and Schotz (1976) and stored at 4°C until used. Tri[9,10-3H]oleoyl-glycerol was purchased from Amersham Australia Pty Ltd. Unlabelled triolein was purchased from Calbiochem-Behring Corp., California. The bovine serum albumin used (Fraction V, Sigma Chemical Co., Missouri) was essentially free of lipase activity. Heparin (5000 i.u. ml⁻¹), used for injection, was purchased from Weddel Pharmaceuticals, New South Wales. Heparin (176 USP units mg⁻¹) used for affinity chromatography was purchased from Sigma Chemical Co., Missouri, U.S.A. Sepharose 6B was purchased from Pharmacia, Uppsala, Sweden. Heparin was coupled to Sepharose 6B using the method of March et al. (1974).

Results and Discussion

The plasma FFA concentration of fed sheep (0.41 ± 0.06 μmol ml⁻¹; mean ± S.E.M. of seven sheep) prior to heparin treatment was similar to those values previously reported (Annison 1963; Aulie et al. 1971; Jarrett et al. 1976) and accounts for about 6% of the total plasma lipids (Nelson 1973). Ruminants have significantly higher plasma FFA concentrations than have humans, and it has been suggested that this might reflect a different intestinal absorption pathway favouring FFA rather than TAG (Simmonds 1972). Upon injection of heparin, the plasma FFA concentrations of fed sheep increased rapidly (from ±0.4 to 1.2 μmol ml⁻¹), returning to near pre-treatment levels within 60 min (Fig. 1). Restricted feeding of sheep resulted in a much higher basal level of plasma FFA (~1.7 μmol ml⁻¹), which is comparable to that observed in fasted sheep by others (Jarrett et al. 1976). Treatment of the diet-restricted sheep with heparin also produced a sharp increase in plasma FFA, of magnitude similar to that of sheep fed ad libitum, but the FFA concentration fell rapidly to near pre-injection level within 20 min.

Intravenous injection of heparin into sheep fed ad libitum resulted in the rapid release of lipolytic enzymes into the plasma. The appearance of lipoprotein-lipase activity in the plasma peaked within 10-15 min and was rapidly removed during the next 60 min (Fig. 2a). Hepatic-lipase activity was greatest when first assayed 5 min post-heparin, but was removed at a slower rate than was lipoprotein lipase. Pre-heparin plasma contained insignificant lipolytic activity.

Sheep whose dietary intake was restricted for 5 days prior to heparin injection showed similar time responses in plasma lipase activity (Fig. 2b). However, the overall lipoprotein-lipase activity...
was significantly reduced, while hepatic-lipase activity was similar to that observed in the sheep fed *ad libitum*. By determining the area under the various time curves for lipoprotein lipase and hepatic lipase, the results for all animals (*n* = 3) yielded the following data for lipoprotein lipase as a percentage of the total lipolytic activity: *ad libitum* fed sheep 67%, diet-restricted sheep 40%. In other experiments (Tume, Thornton, Johnson, unpublished data) using lactating sheep, lipoprotein lipase accounted for more than 80% of the total post-heparin plasma lipolytic activity. In previous work (Tume *et al.* 1983), a restricted dietary regime, as used here, reduced lipoprotein-lipase activity of the major adipose sites to about 4–10% of its activity in *ad libitum* fed sheep. It is likely therefore that the lipoprotein-lipase activity present in the plasma of these diet-restricted sheep represents the contribution from sites other than adipose tissue, such as skeletal and cardiac muscle, in which lipoprotein-lipase activity is known to remain high (Tan *et al.* 1977).
Passage of post-heparin plasma through heparin-Sepharose 6B resulted in the complete binding of lipolytic activity to the column. Using a conventional stepped gradient (Etienne et al. 1981), two peaks of lipolytic activity were separated; one eluted with 0.7 M NaCl and the other with 1.5 M NaCl (Fig 3). The first peak eluted (0.7 M NaCl) was insensitive to 1 M NaCl and 8.5% (v/v) serum in the assay medium, whereas the second peak (1.5 M NaCl) was sensitive to NaCl and dependent upon the presence of serum. Figs. 3a and 3b show the relative amounts of lipoprotein-lipase and hepatic-lipase activities in the plasma of fed and diet-restricted sheep. Clearly, the restricted diet markedly reduced the lipoprotein-lipase fraction.

The centres of the hepatic and lipoprotein lipase peaks eluted from the column were collected and pooled, respectively, to give a stock of partially purified lipase. These peaks were then assayed and their identity was confirmed by the following experiments. Concentrations of NaCl up to 1.0 M did not alter the activity of the lipase present in the first peak (hepatic lipase) but strongly inhibited that of peak 2 (lipoprotein lipase) (Fig. 4a). The addition of serum (source of apolipoprotein CII) had little effect on hepatic-lipase activity; however, serum was an absolute requirement for lipoprotein-lipase activity (Fig. 4b). Lipoprotein lipase was completely inhibited by rabbit antiserum to ovine lipoprotein lipase, but hepatic lipase was not significantly affected (Fig. 4c).

The work presented clearly shows the presence of at least two lipolytic activities in post-heparin plasma, the relative amounts of each enzyme depending upon the nutritional state.
of the sheep. The salt-resistant enzyme was confirmed to be of hepatic origin. Extracts of liver tissue had a salt-resistant activity of 133 ± 22 nmol FFA min⁻¹ g⁻¹ tissue (mean ± s.e.m. of five sheep). The presence of hepatic lipase in post-heparin serum has been found in most species studied and it constitutes a significant but variable percentage of the total lipase activity. In human post-heparin plasma, hepatic-lipase activity is generally two to three times greater than lipoprotein-lipase activity (Huttunen et al. 1975; Musliner et al. 1979; Gamlen and Muller 1980; Ehnholm et al. 1982; Mirani-Oostdijk et al. 1985), whereas in mice and rats the ratio has been quite variable, hepatic-lipase activity being 0.5 to 2 times lipoprotein-lipase activity (Kuusi et al. 1980, Skottova et al. 1983; Peterson et al. 1986). Hepatic lipase has been reported to be absent from post-heparin plasma and liver tissue extracts in bovine animals (Etienne et al. 1981), or present in small quantities amounting to only 5% of the lipoprotein lipase in post-heparin plasma (Cordle and Clegg 1983). However, Cordle and Clegg (1983) did demonstrate the presence of neutral lipase in bovine liver homogenates which had properties similar to the salt-resistant enzyme found in post-heparin plasma. The appearance of these two lipolytic enzymes, the activities of which are directed towards plasma lipoproteins, is of particular interest in view of the overall lipoprotein composition of sheep plasma. The ruminant, and in particular sheep, unlike many other species, has extremely low concentrations of plasma VLDL and chylomicrons. Most of the lipoprotein mass (>80%) is present as HDL (Nelson 1973; Cross et al. 1983) in a form equivalent to human HDL₃ which has a small diameter (Fortet al. 1983) and is devoid of apolipoprotein E (apoE) (Cordle et al. 1985). More than 66% of the total lipid, mainly cholesterol esters and phospholipids, is carried by the HDL fraction (Nelson 1973). In human plasma, HDL₃ has been implicated as a participant in the overall lipoprotein-lipase reaction as an acceptor for apoC and apoE from VLDL (Kinnunen 1984). Therefore, with very high concentrations of an equivalent lipoprotein in ruminants, it is likely that the lipoprotein-lipase reaction will proceed at maximum capacity. The lipoprotein products of the lipoprotein-lipase reaction, the apoE-containing and high- and intermediate-density lipoproteins, are then acted upon by hepatic lipase which hydrolyses the excess phospholipids and TAG associated with the lipoproteins (Kinnunen 1984). Therefore, the presence of these two tissue-bound lipases ensures the rapid removal of the chylomicron or VLDL-TAGs from sheep plasma for tissue utilization or storage.

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References


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