Lipoprotein lipase and acid lipase activity in rabbit brain microvessels

Peter Brecher and Hui-Tseng Kuan
Departments of Medicine and Biochemistry, Boston University Medical Center, Boston, MA 02118

Abstract A preparation of cerebral microvessels was used to demonstrate the presence of lipoprotein lipase and acid lipase activity in the microvasculature of rabbit brain. Microvessels, consisting predominantly of capillaries, small arterioles, and venules, were isolated from rabbit brain. Homogenates were assayed for lipolytic activity using a glycerol-stabilized trioleoylglycerol-phospholipid emulsion as substrate. Lipoprotein lipase activity was characterized with this substrate by previously established criteria including an alkaline pH optimum, increased activity in the presence of heparin and heat-inactivated plasma, and reduced activity in the presence of NaCl and protamine sulfate. A different substrate, containing trioleoylglycerol incorporated into phospholipid vesicles, was used to reveal acid lipase activity that was not affected by heparin, plasma, NaCl, or protamine sulfate. Lipoprotein lipase did not show activity with the vesicle preparation as substrate. Intact microvessels, when incubated in the presence of heparin, released lipoprotein lipase into the incubation solution. In contrast, release of acid lipase activity from intact microvessels was not dependent on heparin. The data show the presence of both lipoprotein lipase and acid lipase in brain microvessels and suggest that lipoproteins are metabolized within the cerebral vasculature.

EXPERIMENTAL

Materials

Tri[9,10-3H]oleoylglycerol (397 mCi/mmol) was obtained from Amersham-Searle and tri[1-14C]oleoyl- glycerol (52 mCi/mmol) from New England Nuclear Corporation. Unlabeled trioleoylglycerol, protamine sulfate (salmine), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and bovine serum albumin (essentially fatty acid-free) were from Sigma Chemical Co. Glycerol (spectrophotometric grade) was purchased from J. T. Baker Chemical Co. Egg yolk lecithin was obtained from Lipid Products (Surrey, U.K.).

Preparation of microvessels

Microvessels from rabbit brain were prepared by our modification (15) of the method described origi-
nally by Brendel, Meezan, and Carlson (12). New Zealand white rabbits weighing 1.5–2.0 kg were used for all studies. Animals were killed by decapitation and the brain was removed and placed immediately into aerated HEPES buffer (28 mM, pH 7.4) containing 118 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgSO₄, 1 mM Na₂HPO₄, and 5.5 mM glucose. After removal of the pial membrane, the cerebrum was cleaned, homogenized, and subjected to subsequent filtration through nylon mesh and rehomogenization as described previously (15). The final preparation was collected onto a 53-µm nylon mesh.

For most experiments, the microvessels contained on the mesh were added directly to 2.0 ml of 0.1 M Tris buffer (pH 8.0) containing 1 U/ml of heparin and then were homogenized on ice in an all-glass, motor-driven homogenizing apparatus. After homogenization, the nylon mesh was free of adhering tissue and was removed from the homogenate. Samples of the homogenate were used directly for assays of lipolytic activity. Typically, microvessels obtained from one rabbit brain contained about 1 mg total protein as determined by the method of Lowry et al. (16).

Acetone–ether powders from microvessels were prepared using a modification of the method of Borensztajn, Otway, and Robinson (17). Microvessels contained on the 53-µm mesh after isolation were homogenized in 2.0 ml of 0.025 M NH₃-NH₄Cl (pH 8.1). Bovine serum albumin (100 mg) was added to the homogenate which was then added directly to 20 volumes of cold acetone and filtered slowly through Whatman #3 filter paper. The precipitate was washed three times with 20 ml of cold acetone and once with 20 ml of diethyl ether. The washed powder was stored in vacuo at −20°C for up to 2 days. Extraction of the powder was performed by homogenization at 4°C in 2.0 ml of 1.5 M NaCl, 0.005 M sodium barbital (pH 7.4) and centrifugation at 2300g for 10 min. The supernatant was dialyzed against 40 vol of 1 mM sodium barbital (pH 7.4) and the dialysate was centrifuged at 150,000 g for 15 min to remove small amounts of insoluble material. The resulting supernatant was used for enzyme assays.

Heat-inactivated plasma was prepared from rabbit plasma obtained from fasted animals and was heated at 60°C for 10 min. No lipolytic activity towards either the emulsion or the vesicle preparation was observed in the heat-inactivated plasma at pH 4.0 or 8.2.

Preparation of substrates

The triacylglycerol emulsion stabilized with glycerol was prepared essentially as described by Nilsson-Ehle and Schotz (18) using a Polytron PT 10 OD equipped with a PT 10 ST generator. Homogenization was performed for four 1-min intervals, at a power setting of 4. The optically clear emulsion contained egg yolk lecithin (3.6 mg/ml) and triolein (60 mg/ml, 100 µCi tri[9,103H]oleylglycerol/ml), and was stored at 4°C.

Triacylglycerol–phospholipid vesicles were prepared by sonication using a procedure similar to that described previously (19). A mixture of 60 mg of egg yolk lecithin, 12.5 mg of triolein, and 8.0 µCi of tri-[1–14C]oleylglycerol was evaporated under nitrogen, the sample was lyophilized, and the lipids were resuspended in 6.0 ml of 0.1 M NaCl, 0.02% sodium azide, and 0.01 M Tris, pH 7.4. This suspension was sonicated at 20–25°C for 8–10 min using a W-350 sonifier (Branson Instruments) fitted with a standard 0.5-in horn at a power setting of about 130 watts. The resulting suspension was centrifuged at 150,000 g for 30 min and the slightly opaque supernatant, which contained virtually all the original lipid, was stored at 4°C and used within 10 days of preparation.

Enzyme assays

Lipoprotein lipase activity was determined using the triolein–phospholipid emulsion as substrate. Prior to assay, the glycerol-stabilized emulsion was diluted with 20 volumes of 0.2 M Tris-HCl buffer (pH 8.0) containing 2% (w/v) bovine serum albumin and with 0.8 volume of heat-inactivated plasma. Aliquots (0.15 ml) of this diluted substrate were added to a series of incubation tubes (15 x 100 mm disposable test tubes) and the reaction was initiated by adding 0.15 ml of tissue homogenate or other enzyme source. Under standardized conditions, each assay tube contained 1.48 mM triolein, 0.6 µCi of labeled triolein, 0.11 mM lecithin, 1% bovine serum albumin (w/v), 1.7% heat-inactivated plasma, 2.2% glycerol, 0.15 units of heparin, and 0.15 M Tris-HCl buffer at pH 8.0. Incubations were carried out at 37°C for 90 min in a Dubnoff metabolic incubator with moderate shaking.

The reaction was terminated by the addition of 3 ml of benzene–chloroform–methanol 1:0.5:1.2 (v/v/v) and the free fatty acid formed by hydrolysis was determined by solvent partition using a previously described radioassay (19). The upper phase obtained from blank assay tubes incubated in the absence of tissue contained less than 0.15% of the total radioactivity present in the assay tube. All assays were performed in duplicate and the data were normally expressed as nmol fatty acid formed/hr per mg tissue protein, after correcting for the partition of oleic acid into the upper phase (93% in upper phase).

Lipolytic activity measured using the triglyceride–phospholipid vesicles was determined essentially as described previously (19). Assays were performed in a total volume of 0.3 ml and each incubation tube
contained 75 μl of the vesicle preparation and 50–100 μl of enzyme solution in 0.15 M sodium acetate buffer, pH 4.0. The final concentrations of triolein and lecithin were 0.6 mM and 3.0 mM, respectively, and each assay tube contained 0.10 μCi of [1-14C]triolein. Incubations were conducted at 37°C for 30 min and the labeled free fatty acid formed was measured following solvent partition exactly as described above. Assay blanks contained less than 0.1% of the total radioactivity added in the upper phase.

For the studies utilizing intact microvessels, described in Fig. 5, microvessels were isolated and collected into a 53-μm nylon mesh, the mesh was bisected with a fine scissors, and each portion was placed directly into an incubation tube containing 3.0 ml of the 28 mM HEPES buffer employed for the isolation procedure. Incubation tubes also contained heat-inactivated plasma (4% v/v), and heparin (5U/ml) was added to the designated incubation tube. The microvessels were then preincubated at 37°C for 120 min. At selected time intervals, samples of the preincubation solution were removed, filtered through a 0.2-μm Millipore filter (SXHA, 13 mm), and immediately assayed for lipolytic activity using the emulsion or vesicle preparation as substrate. Where required, heparin was added to assay tubes for LPL activity so that the final amount of heparin present was 0.75 U/assay tube.

**Sepharose chromatography**

A Sepharose 4B column (2.6 x 24 cm) pre-equilibrated with 0.1 M NaCl, 0.01 M Tris HCl (pH 7.4), and 0.02% NaN₃ was used to characterize the substrate preparations. Sample aliquots were applied to the column and eluted by ascending chromatography with the pre-equilibration buffer (flow rate 20 ml/hr). Eluted fractions were analyzed directly for radioactivity.

**RESULTS**

Two different substrate preparations were used to characterize the lipase activity in brain microvessels. Both preparations contained triolein and lecithin, but differed in the ratio of these components, the mode of dispersion, and the physical state. Differences between the two preparations with respect to particle size are shown in Fig. 1 where the radioactive elution profiles of both preparations on a Sepharose 4B column are compared. The tritium-labeled triolein in the emulsion eluted at the void volume of the column, indicating that the triolein was associated with large particles that were completely excluded by the column. The 14C-labeled triolein contained in the vesicle preparation was included within the column and eluted at a position consistent with that of unilamellar phospholipid vesicles. The emulsion preparation was shown previously to be suitable for the assay of lipoprotein lipase (18) and triolein-containing phospholipid vesicles were shown to be an effective substrate for an aortic acid lipase (19).

The effect of pH on hydrolysis of the emulsified fatty acid-labeled triglyceride by aliquots of the microvessel homogenate is shown in Fig. 2.

In the absence of heat-inactivated rabbit plasma, lipolytic activity was most pronounced in the acid pH range with a pH optimum near 6. Triglyceride hydrolysis did occur between pH 7 and 9, but to a lesser extent than at acid pH. When heat-inactivated plasma was included in the incubation mixture, lipolytic activity was increased at pH values between 7.2 and 8.5, whereas plasma addition had no effect on the acid lipolytic activity. Background values for the substrate preparation incubated in the absence of tissue protein were constant over the entire pH range studied and represented less than 0.15% of the radioactivity present in the incubation tube. Background values were not changed by the presence of heat-inactivated plasma.

The lipolytic activity in microvessel homogenates, which was stimulated by heat-inactivated plasma, was further characterized at pH 8.0 using the labeled triglyceride emulsion as substrate (Fig. 3). Triglyceride hydrolysis was directly proportional to incubation time for up to 150 min (Fig. 3A) and to the amount of microvessel protein added to the incubation mixture (Fig. 3B). Saturation kinetics were obtained when the substrate concentration exceeded 1 mM (Fig. 3C), and maximal activation of lipolysis by heat-inactivated plasma occurred when the plasma constituted 2% (v/v) of the assay solution (Fig. 3D).

The experiments summarized by Fig. 3 were performed in the presence of 1% albumin, and heparin (1.0 U/ml) was present in the microvessel homogenates. Heparin was required for maximal hydrolysis. Using assay conditions standardized so that hydrolysis was proportional to incubation time and protein concentration, and independent of substrate concentration, the effect of heparin was studied by preparing a microvessel homogenate in the absence of heparin, and subsequently adding increasing amounts of heparin to homogenate aliquots prior to incubation. Addition of 0.15 or 0.3 units of heparin per assay tube caused a 2.5-fold increase in activity (39.1 nmol·hr⁻¹·mg⁻¹ without heparin vs. 100.4 and 101.2 nmol·hr⁻¹·mg⁻¹ with 0.15 and 0.3 units of heparin, respectively). Addition of 0.5 and 1.0 units of heparin to assay tubes also stimulated activity, but to a lesser extent (87.5 and
Fig. 1. Comparison of the elution profile of labeled triolein contained in the emulsion and in the vesicle preparation using Sepharose 4B chromatography. The vesicle preparation (200 μl) or the emulsion preparation (10 μl) was diluted with the elution buffer to 0.5 ml and applied to the column. Aliquots of the eluted fractions were analyzed for radioactivity by liquid scintillation counting.

71.8 nmol·hr⁻¹·mg⁻¹, respectively. Omission of albumin from the incubation mixture resulted in approximately 50% reduction of activity, where incubations containing 0.5, 1.0, or 2.0% albumin gave optimal hydrolysis.

Table 1 shows the effects of heat-inactivated plasma, NaCl, and protamine on the lipolytic activity present in microvessel homogenates using standardized incubation conditions. Lipolytic activity averaged 90.4 nmol·hr⁻¹·mg⁻¹ for seven separate determinations, the individual values ranging from 51 to 127 nmol·hr⁻¹·mg⁻¹. Omission of heat-inactivated plasma caused a marked reduction in activity. Addition of 1 M NaCl or 2% protamine sulfate reduced enzymatic activity to levels slightly lower than those observed in the absence of plasma activation. The lipolytic activity observed at acid pH and using the emulsion as substrate was not affected by heat inactivated plasma, as was shown in Fig. 2, and in separate experiments the effects of heparin, 1 M NaCl, protamine sulfate, and albumin were shown to have little or no influence on triglyceride hydrolysis when using the emulsion as substrate and performing the assay at pH 6.0.

In previous studies we have shown that phospholipid vesicles containing triglyceride or cholesteryl ester were suitable substrates for acid lipase activity in aortic tissue (19). Experiments were performed to assess the acid lipase activity in microvessel preparations using the vesicles as substrate. Fig. 4A shows the hydrolysis by the microvessel homogenate of labeled triolein associated with phospholipid vesicles as a function of pH. Maximum activity was measured between pH 3 and 3.5 and a second smaller peak between pH 4 and 4.5. No hydrolysis occurred at pH values above 7.0 when using the sonicated vesicle preparation in contrast to that observed with the triglyceride emulsion as substrate. In separate experiments it was found that the addition of heat-inactivated plasma had no effect

Fig. 2. Effect of pH on the hydrolysis of triolein contained in the emulsion preparation by aliquots of the microvessel homogenate. Each assay tube contained tissue homogenate (52 μg protein), triolein (1.48 mM), lecithin (0.11 mM), albumin (1%), and heparin (0.15 units). Heat-inactivated plasma (1.7%) was added where indicated. pH was adjusted with 0.15 M Tris-maleate buffer between pH 5.0 and 9.0, and 0.15 M acetate at pH 4.0. All tubes were incubated at 37°C for 90 min and hydrolysis was measured as described in the experimental section.
on hydrolysis at pH 3.5 or 4.5, nor did plasma activate hydrolytic activity at pH values between 7 and 9. Addition of protamine sulfate or heparin also did not influence the activity at acid pH.

Using a resuspension of an acetone-ether powder prepared from the microvessel homogenate, lipolytic activity was determined using both the triglyceride emulsion and the sonicated vesicle preparations (Fig. 4B). Using the sonicated vesicle preparation as substrate, acid lipase activity was evident, and the pH--activity profile was similar to that observed in the original homogenate. When the trioleylglycerol emulsion was incubated with the acetone powder resuspension, hydrolysis at acid pH was not pronounced, in contrast to the results obtained using the microvessel homogenate (Fig. 2). A well-defined peak of activity at pH 8.0 was observed, providing heat-inactivated plasma was included in the incubation mixture. This activity at pH 8.0 was dependent on the addition of plasma and was inhibited by NaCl and protamine sulfate in a manner almost quantitatively similar to the data shown in Table 1 for using the homogenate preparation. Because albumin was used as a "carrier" for the acetone powder preparation, the data in Fig. 4B are not expressed on the basis of tissue protein. No hydrolysis with either substrate was observed when using an acetone powder extract obtained from albumin alone, in the absence of tissue.

The experiments described, using broken-cell preparations, suggested that at least two separate lipases with different substrate specificities were present in microvessels. Fig. 5 shows results from a representative experiment where intact microvessels contained on a nylon mesh were preincubated in the presence or

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**TABLE 1. Effect of plasma, NaCl, and protamine sulfate on lipoprotein lipase activity of microvessel homogenates**

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Activity (nmol·hr⁻¹·mg⁻¹)</th>
<th>Percent of Complete System</th>
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<tbody>
<tr>
<td>Complete system (7)</td>
<td>90.4 ± 11.9</td>
<td>100</td>
</tr>
<tr>
<td>Without plasma (3)</td>
<td>16.5 ± 5.7</td>
<td>20</td>
</tr>
<tr>
<td>With NaCl (1M) (3)</td>
<td>9.5 ± 1.6</td>
<td>11</td>
</tr>
<tr>
<td>With protamine sulfate (2% w/v) (3)</td>
<td>14.6 ± 1.3</td>
<td>16</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. Numbers in parentheses refer to the number of separate determinations. All assays were performed in duplicate under standardized incubation conditions.
absence of heparin to determine whether heparin could cause the release of lipolytic activity into the incubation solution. Subsequent assay of the incubation solution for LPL activity, using the emulsion, or acid lipase activity, using the vesicle preparation, indicated no LPL activity if heparin was omitted during preincubation; but activity clearly was present when the microvessels were preincubated with heparin. Acid lipase activity as measured with the vesicle preparation was released both in the presence and absence of heparin pretreatment, and to approximately the same extent.

DISCUSSION

This is the first study to document lipase activity in the cerebral microvasculature. We have shown that rabbit cerebral microvessels contain acid lipase activity and a lipase with characteristics typical of lipoprotein lipase. The use of two different substrate preparations facilitated the distinction between the different lipolytic activities.

LPL activity in the microvessels was demonstrated using a radioactive trioleoylglycerol emulsion which previously was shown to be applicable to LPL assays using adipose tissue (18) or plasma and aortic tissue (20) as enzyme sources. Our studies showed that hydrolysis of the triolein emulsion occurred over a broad pH range, suggesting the presence of more than one lipolytic enzyme, but only the activity of an alkaline pH was increased by addition of plasma and inhibited by NaCl and protamine sulfate. These properties are typical of LPL obtained from other tissue sources. LPL activity was found both in homogenates and in acetone–ether powders of the isolated microvessels. Corey and Zilversmit (20) utilized a triolein emulsion to assay for lipolytic activity in a supernatant fraction obtained from rabbit aortic tissue homogenates and demonstrated both acid lipase and LPL activity in that vascular tissue. In a subsequent study they showed that both lipolytic activities in rabbit aortic tissue were linearly related to the progressive development of atherosclerotic lesions induced by cholesterol feeding (21).

Labeled triolein associated with phospholipid vesicles also was hydrolyzed by microvessel homogenates and resuspensions of the acetone–ether powder. In contrast to the triolein emulsion, hydrolysis occurred only at acid pH and no activity was observed above pH 7, indicating that triolein in vesicles was not hydrolyzed by LPL. In a previous study (19) we showed that phospholipid vesicles containing labeled triolein were an effective substrate for an acid lipase from rabbit aortic tissue. This aortic enzyme was subsequently shown to have broad specificity toward different cholesteryl esters and triolein when these lipids were associated with lecithin vesicles (22). In the current study the molar ratio of lecithin to triolein was 5:1, in contrast to a molar ratio of 70:1 used in our previous studies. Effective sonication at the higher molar ratio used for these studies resulted in the formation of unilamellar vesicles and permitted a higher concentration of triolein to be used for the assay system.

The acid lipase and LPL activities present in the cell-free fractions from microvessels differed with respect
to pH optima, sensitivity to heat-inactivated plasma, NaCl, and protamine sulfate, and specificity toward two different substrate preparations. In addition to these biochemical characteristics of the assay systems, a striking difference was observed after preincubation of intact microvessels with heparin. Heparin-dependent release of LPL has been shown to occur in several experimental systems including the perfused rat heart (25), adipose tissue (24), cultured rat adipose cells (25), and cultured cardiac mesenchymal cells (26). Furthermore, it is generally agreed that the postheparin lipolytic activity found in plasma is due to the release of LPL from vascular endothelial cells by the administered heparin. The lack of any detectable LPL activity in the incubation medium from microvessels incubated in the absence of heparin suggests the enzyme is strongly associated with the intact tissue preparation.

Acid lipase activity also was released into the incubation medium following preincubation of intact microvessels, but heparin did not influence acid lipase release. It is likely that the acid lipase measured in these studies is of lysosomal origin. Lysosomal lipase activity toward both cholesteryl esters and triglycerides has been found in several tissues and a deficiency in this activity is characteristic of Wolman's disease and cholesteryl ester storage disease (27). A lysosomal localization for an aortic acid lipase was demonstrated using sophisticated fractionation techniques (28). Release and reuptake of a lysosomal acid lipase from cultured fibroblasts was observed (29), and it has been proposed that intercellular exchange of lysosomal enzymes by secretion and reuptake mechanisms is a normal physiological event (30). Recent studies on lipoprotein metabolism in cultured cells have shown that an important function of the lysosomal acid lipase is to hydrolyze lipoprotein-associated cholesteryl esters that are internalized by endocytosis and subsequently incorporated into lysosomes (31).

Since plasma lipoproteins are the physiological substrates for both LPL and the lysosomal acid lipase, it is of interest to establish whether these enzymes have an important physiological role in the regulation of cerebral lipid metabolism. The microvessel preparation is enriched in endothelial cells but also contains vascular smooth muscle cells and fragments of glial cells attached to capillary basement membranes. Therefore, we cannot determine precisely the anatomical localization or site of synthesis for either of the lipases examined. The LPL activity measured in the microvessel preparation is much greater than that observed in plasma or whole brain homogenates.

Therefore trace contamination by neural cells or plasma cannot be a major source of enzymatic activity. If LPL is localized on cerebrovascular endothelial cells, the enzyme may contribute to the influx of free fatty acids into the brain through its action on circulating chylomicrons and VLDL particles. If lipoproteins are internalized by cells of the microvasculature, intralysosomal hydrolysis by an acid lipase would produce an increase in intracellular fatty acids and cholesterol. This study has shown that enzymes involved in lipoprotein metabolism are present in the cerebral microvasculature. Additional studies are required to determine if lipoproteins actually are degraded by the cerebral microcirculation and whether the subsequent metabolism of the products of lipase action is restricted to the vasculature or can occur in the underlying nervous tissue.

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