Degradation of mono-oleoylglycerol, trioleoylglycerol and phosphatidylcholine in emulsions and lipoproteins by rat hepatic acylglycerol lipase

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The purpose of this study was to characterize the lipolytic activities released by heparin from rat livers. Heparin perfusates of rat livers degraded mono-oleoylglycerol, trioleoylglycerol and phosphatidylcholine in emulsions as well as in chylomicrons, chylomicron remnants, low-density lipoprotein/high-density lipoprotein-1 (LDL/HDL-1) and high-density lipoprotein-2 (HDL-2). The preferred substrate was mono-oleoylglycerol. Heparin perfusates were separated by chromatography on either heparin-Sepharose or *N*-desulphated, *N*-acetylated heparin-Sepharose into at least two related lipases which differed in their ability to hydrolyse HDL-2 phosphatidylcholine, but not in their ability to degrade mono-oleoylglycerol, trioleoylglycerol and phosphatidylcholine in emulsions. The sodium dodecyl sulphate (SDS)/polyacrylamide-gel-electrophoretic patterns of heparin perfusates purified on either normal or *N*-desulphated *N*-acetylated heparin-Sepharose were the same, despite differences in their ability to degrade HDL-2 phosphatidylcholine. There was a single band of M_r 56000 without 2-mercaptoethanol in the SDS disruption buffer and three major bands, of M_r 62000, 59000 and 56000, with 2-mercaptoethanol present. When mono-oleoylglycerol lipase was purified 161-fold, there was a concomitant enrichment of the M_r -56000 protein.

Perfusion of rat liver with heparin releases an enzyme capable of hydrolysing fatty acids from tri-, di- and mono-acylglycerols as well as from phosphatidylcholine (PtdCho) and phosphatidylethanolamine (Assmann *et al.*, 1973; Waite & Sisson, 1973*a,b*; Ehnholm *et al.*, 1975; El-Magh-rabi *et al.*, 1978; Hulsmann *et al.*, 1980; Waite *et al.*, 1978). There is evidence that one enzyme has triacylglycerol lipase, monoacylglycerol lipase and phospholipase activities with artificial substrates (Kuusi *et al.*, 1979*b*; Jensen *et al.*, 1982; Twu *et al.*, 1984).

Abbreviations used: PtdCho, phosphatidylcholine; HAL, hepatic acylglycerol lipase; LDL, low-density lipoprotein; HDL-1, high-density lipoprotein-1; HDL-2, high-density lipoprotein-2; SDS, sodium dodecyl sulphate.

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The enzyme has been called a number of names, such as hepatic triglyceride lipase, hepatic phospholipase A and monoacylglycerol acyltransferase. In this study, however, the enzyme will be called 'hepatic acylglycerol lipase' (HAL), since the enzyme has a broad specificity. Because of its broad substrate specificity, the physiological role of HAL is unknown. It is assumed to play a role in the metabolism of plasma lipoproteins. Here we report on the ability of partially purified HAL to degrade mono-oleoylglycerol, trioleoylglycerol and PtdCho in emulsions as well as in chylomicrons, chylomicron remnants, LDL/HDL-1 and HDL-2.

Experimental

Preparation of chylomicrons

Intestinal lymph chylomicrons were obtained from male Sprague-Dawley rats (300–350g) as described by Warshaw (1972). At 3h before surgery the rats were given 1.0ml of corn oil by gastric intubation. In addition to the intestinal lymph cannula, a PE-50 cannula was inserted into

the duodenum 1 cm proximal to the entry of the common bile duct, advanced 2.5 cm distally, and secured with a purse-string closure. At 3 h after the operation, the rats were given a bolus infusion through the duodenal cannula of 1.0 ml of a radiolabel/Intralipid mixture (described under 'Preparation of radiolabelled chylomicrons'). Lymph was collected on ice for 16 h in the presence of EDTA and 5,5'-dithiobis-(2-nitrobenzoic acid) (final concns. 0.01% and 0.004% respectively).

In order to isolate the chylomicrons, the lymph was layered under a $d = 1.006$ solution (0.9% NaCl containing 0.001% EDTA, pH 7.4), centrifuged for 60 min at $72000g_{av}$ at 15°C , and the chylomicrons at the top of the tube were removed with a tube slicer. The chylomicrons were then washed under the same conditions of isolation.

Preparation of radiolabelled lipids

[*palmitoyl*- ^3H]PtdCho {1-[9,10- ^3H (n)]*palmitoyl*-2-acyl-*sn*-glycero-3-phosphocholine} was prepared from egg lecithin (Sigma Chemical Co., St. Louis, MO, U.S.A.) by removing the *sn*-1-acyl group with phospholipase A_1 as described by Slotboom *et al.* (1970) and reacylating the resulting 1-lyso-2-acyl-*sn*-glycero-3-phosphocholine with [9,10- ^3H (n)]*palmitate* by using rat liver microsomal fraction (Newkirk & Waite, 1973). The [*palmitoyl*- ^3H]-PtdCho was purified from the other lipids by silicic acid column chromatography and t.l.c. The efficiency of incorporation of [^3H]palmitic acid into PtdCho was approx. 77%.

Ptd[^{14}C]Cho (1,2-diacyl-*sn*-glycero-3-phospho-[*N*-methyl- ^{14}C]choline) was synthesized from egg lecithin (Sigma) by demethylating the egg PtdCho to dimethylphosphatidylethanolamine and re-methylating with [^{14}CH]methyl iodide by the procedure of Stoffel (1975).

Radiolabelled 1-mono-oleoylglycerol was kindly prepared by Dr. Craig Miller of this Department by an adaptation of the method of Renoll & Newman (1955), with [2- ^3H]glycerol as radiolabel.

Preparation of radiolabelled chylomicrons

The radiolabelled lipids were prepared for infusion into the duodenum by adding 1 mCi of tri[9,10- ^3H]oleoylglycerol and 68 μCi of Ptd-[^{14}C]Cho to the same test tube and evaporating the chloroform under N_2 . After the material had been dried, 1.0 ml of a soya-bean triacylglycerol and egg-yolk lecithin emulsion, Intralipid (Cutter Laboratories, Berkeley, CA, U.S.A.), was added. The emulsion was vortex-mixed for 1 min and sonicated for 10 min repeatedly until all of the radiolabelled lipid was emulsified, as determined by liquid-scintillation counting. Approx. 80% of the infused ^3H and 10% of the infused ^{14}C radiolabel were recovered in the isolated chylomicrons.

Of the recovered radiolabel, 90% of the ^3H was contained in triacylglycerol, and over 99% of the ^{14}C was in PtdCho. Of the chylomicron molar mass, 88% was in triacylglycerol, 9% was in phospholipid and 3% was in cholesterol and cholesterol ester.

Preparation of radiolabelled lipoproteins

Radiolabelled lipoproteins were prepared for studies *in vitro* with HAL. Functionally hepatectomized male Sprague-Dawley rats (300–370 g) anaesthetized with pentobarbital were prepared by ligating and clamping the hepatic artery and portal vein. Each rat was injected with 20 μmol of chylomicron acylglycerol. The rats were exsanguinated 12.5–15 min after injection. Radiolabelled blood from six rats was pooled in 0.02% EDTA and 0.008% 5,5'-dithiobis-(2-nitrobenzoic acid). The total radiolabelled lipoprotein fraction was isolated from plasma by centrifugation at $d = 1.225$, followed by separation of the lipoprotein concentrate on a Bio-Gel A-5m (6% agarose) 200–400 mesh column (1.0 cm $\times$ 90 cm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) maintained at 4°C as described by Rudel *et al.* (1974). The column fractions were pooled into a chylomicron-remnant fraction, an LDL/HDL-1 fraction and an HDL-2 fraction. The three pooled lipoprotein fractions were then concentrated by dialysis for 8 h against 25% poly(ethylene glycol) (approx. M_r 20000) (Sigma). The three concentrated lipoprotein fractions were dialysed overnight in 0.15 M NaCl/0.01% NaN_3 before use. The three fractions were pure as judged by agarose electrophoresis and co-migrated with lipoproteins isolated by classical density centrifugation.

Purification of HAL by liver perfusion

Liver perfusion was carried out as described by Kuusi *et al.* (1979b). Male Sprague-Dawley rats (250–275 g) were anaesthetized with sodium pentobarbital (25 mg/kg body wt.) and the abdominal wall was opened to expose the liver. The portal vein was cannulated, and the inferior vena cava was cut at the level of the renal veins. The liver was washed free of blood with 250 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) (Krebs & Henseleit, 1932) maintained at 37°C and saturated with O_2/CO_2 (19:1). The flow rate was 17 ml/min. Immediately upon start of the liver washout, the chest was opened and the superior vena cava was cannulated just above the liver. At this point, the inferior vena cava was ligated below the liver, and the washout was completed by using this portal vein-liver-superior-vena-cava system. To release HAL into the perfusate, the medium was changed to 50–150 ml of Krebs-Ringer bicarbonate con-

taining 40 i.u. of heparin/ml (Sigma, grade II, from pig intestinal mucosa), and allowed to recirculate for 3 min. The same medium was used for consecutive perfusions of six to eight rat livers.

Preparation of normal and N-desulphated N-acetylated heparin-Sepharose

Normal heparin-Sepharose was prepared by covalent linkage of crude heparin (Inolex Corp., Forest South, IL, U.S.A.) to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) (Iverius, 1971). *N*-Desulphated, *N*-acetylated heparin-Sepharose was prepared as described by Bengtsson *et al.* (1980). *N*-Desulphated heparin was prepared by dissolving 500 mg of heparin in 10 ml of 0.4 M-HCl and heating to 50°C for 16 h. The reaction was stopped by raising the pH to 7.0 with 10 M-NaOH. The reaction mixture was then freeze-dried to a dry powder. *N*-Desulphated heparin was then coupled to CNBr-activated Sepharose 4B as above (Iverius, 1971). The *N*-desulphated heparin-Sepharose was acetylated by suspending 75 g of the gel in 50 ml of aq. 10% (v/v) methanol. Then 250 mg of Na₂CO₃ was added and the mixture cooled to 0°C, and 1.0 ml of acetic anhydride was added dropwise over a period of 30 min (0.1 ml every 3 min). The pH was maintained at 7.0–7.5 by intermittent neutralization with additional Na₂CO₃. After 30 min the gel was washed with deionized water on a sintered-glass funnel.

Heparin-Sepharose chromatography

All further purification steps were carried out at 0–4°C. Perfusate from six to eight rat livers was applied to a 3.8 cm-diameter column containing a 75 ml bed volume of heparin-Sepharose or *N*-desulphated *N*-acetylated heparin-Sepharose equilibrated with 0.15 M-NaCl/5 mM-Veronal (pH 7.4)/20% (v/v) glycerol as previously described (Kuusi *et al.*, 1979b). After perfusate application, the column was washed with 200 ml of 0.4 M-NaCl/5 mM-Veronal/20% glycerol, followed by 200 ml of the same buffer containing 0.1% Triton X-100. (For purification on *N*-desulphated *N*-acetylated heparin-Sepharose, the NaCl concentration was 0.3 M instead of 0.4 M, in view of the observation that HAL is eluted from that column at 0.6 M-NaCl, whereas it is eluted from normal heparin-Sepharose at 0.7 M-NaCl.) HAL was eluted with a 200 ml linear 0.4–1.0 M-NaCl gradient (0.3–1.0 M-NaCl for *N*-desulphated *N*-acetylated heparin-Sepharose) containing 5 mM-Veronal, pH 7.4, 20% glycerol and 0.1% Triton X-100. Fractions with activity on mono-oleoylglycerol were pooled and stored at –70°C until further use.

Concentration of pooled heparin-Sepharose fractions

The thawed heparin-Sepharose fractions were dialysed at 4°C for 2–3 h in 2 litres of 0.15 M-NaCl/5 mM-Veronal/20% glycerol. After dialysis the pooled heparin-Sepharose fractions were applied to a 1.0 cm × 10 cm column containing a 2 ml bed volume of heparin-Sepharose. The gel was washed with 5 column vol. (10 ml) of 50 mM-Tris/HCl, pH 7.2, containing 30% glycerol. HAL was eluted with a single step of 1.0 M-NaCl/50 mM-Tris (pH 7.2)/30% glycerol. This concentration technique resulted in a 40–50-fold concentration of HAL with over 95% recovery of activity on mono-oleoylglycerol from the pooled heparin-Sepharose fractions. In addition, this provided a convenient method to remove Triton X-100 from the enzyme.

Purification on DEAE-Sephacel

Concentrated HAL was further purified on DEAE-Sephacel (Pharmacia) as described by Jensen & Bensadoun (1981). Concentrated HAL was dialysed for 4 h in 2 litres of 50 mM-Tris/HCl (pH 7.2)/30% glycerol. The dialysis residue containing HAL was then applied to a 1.6 cm × 12 cm column of DEAE-Sephacel equilibrated with the same buffer. The column was then washed with 60 ml of the equilibration buffer at a flow rate of 20 ml/h. This was followed by 100 ml of 50 mM Tris/HCl, pH 7.2, at a flow rate of 33 ml/h. HAL was eluted with a single step of 80 mM-NaCl/0.2% Triton X-100/100 mM-methyl α -D-mannoside (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.)/50 mM-Tris/HCl, pH 7.2. The peak fractions were pooled and applied directly, without dialysis, on to 2 ml of heparin-Sepharose and concentrated exactly as described above. The enzyme could be stored at –70°C for weeks with no loss of activity.

Analytical procedures

Lipids were extracted with methanol/chloroform (2:1, v/v) by the method of Bligh & Dyer (1959). Lipid classes were separated in either a neutral lipid (I) or a phospholipid (II) t.l.c. system on silica-gel H; system I consisted of heptane/diethyl ether/formic acid (20:10:1, by vol.); system II consisted of chloroform/methanol/acetic acid/water (150:96:25:9, by vol). The silicic acid containing these compounds was scraped from the plate and counted for radioactivity in Budget-Solve (Research Products International, Elk Grove Village, IL, U.S.A.).

Neutral acylglycerol was measured by the method of Sardesai & Manning (1968). Phospholipid phosphorus was measured by the method of Chalvardjian (1970). Cholesterol was determined by g.l.c., with stigmasterol (Applied Science

Laboratories, State College, PA, U.S.A.) as an internal standard (Ishikawa *et al.*, 1974).

Lipolytic assay

All lipolytic assays were performed in 0.1M-Tris/HCl at the specified pH in a total assay volume of 0.5 ml at 37°C in a shaking water bath. The reactions were stopped by the addition of 1.5 ml of methanol/chloroform (2:1, v/v), and the lipids were extracted into the lower chloroform phase while free glycerol remained in the upper aqueous phase (Bligh & Dyer, 1959). Optimal substrate, CaCl₂, albumin and NaCl concentrations and optimal pH and incubation time were determined for each assay used. The results are expressed in units; 1 unit equals 1 µmol of substrate transformed/min. It should be noted that Triton X-100 was removed from the enzyme during the concentration step, so that all lipolytic assays could be run in the absence of Triton X-100.

Mono-oleoylglycerol assays

Activity towards mono-oleoyl[2-³H]glycerol (sp. radioactivity 1.87 Ci/mol) was determined by counting the radioactivity in a sample of the upper aqueous phase of the lipid extract (Bligh & Dyer, 1959). The presence of radioactivity in this phase represented the production of [2-³H]glycerol. The mono-oleoylglycerol substrate was prepared by drying the chloroform solution of mono-oleoylglycerol in a test tube and adding 1.0 ml of water/5.0 µmol of mono-oleoylglycerol. This was vortex-mixed for 15 s and then sonicated for approx. 3 min in a sonic water bath (Heat Systems Ultrasonic, Plainview, NY, U.S.A.) until the solution was clear. The optimal amount of substrate routinely used was 1 mM and, unless specified differently, assays were for 10 min.

On the pH-activity curve, activity towards mono-oleoylglycerol increased linearly 6-fold between pH 6.1 and pH 9.5. Although activity at pH 9.5 was 10% higher than at pH 9.0, pH 9.0 was employed in all assays in order to minimize any non-specific hydrolysis. NaCl (0–1.0 M) and CaCl₂ (0–10 mM) had no effect on the enzyme activity toward mono-oleoylglycerol and so were not used.

PtdCho vesicle assays

The 1-[palmitoyl-³H]PtdCho was diluted with egg PtdCho (Sigma) to a specific radioactivity of 166 mCi/mol. Hydrolysis with snake-venom phospholipase showed that 96% of the ³H was in the *sn*-1 position (Waite & van Deenen, 1967). The [³H]PtdCho was prepared for assay by drying a chloroform solution of [³H]PtdCho in a test tube and adding 1.0 ml of water per 1.0 µmol of PtdCho up to 3 ml total volume. This was vortex-mixed for 15 s and sonicated in a sonic water bath for 10 min.

Optimal conditions for PtdCho hydrolysis were pH 8.8, 1.0 M-NaCl, 5 mM-CaCl₂, and a substrate concentration of 200–600 µM. The reaction products in the chloroform layer were separated by t.l.c. in chromatographic system I, and the [³H]palmitate in the PtdCho and fatty acid fractions was measured by liquid-scintillation counting.

Assay of trioleoylglycerol emulsions

Trioleoylglycerol was assayed as described by Jensen & Bensadoun (1981). Tri[9,10(n)-³H]-oleoylglycerol (sp. radioactivity 5.0 mCi/mol) was emulsified in the presence of 5% (w/v) gum arabic (Sigma). The assay mixture contained the following, in a total volume of 0.5 ml: 1.25 µmol of [³H]trioleoylglycerol, 2.5 mg of gum arabic and 5 mg of charcoal-defatted bovine serum albumin (Chen, 1967). In addition, the assay was run in 0.1 M-Tris/HCl, pH 9.0, in 1.0 M-NaCl and 10 mM-CaCl₂. Mono-, di- and tri-oleoylglycerol and fatty acid were separated in chromatographic system I, and the [³H]oleate in these fractions was measured by liquid-scintillation counting.

Assay of chylomicrons and chylomicron remnants

Radiolabelled chylomicrons and chylomicron-remnant preparations contained tri[9,10(n)-³H]-oleoylglycerol, with a specific radioactivity of 3.67×10^6 d.p.m./µmol in chylomicrons and 2.96×10^6 d.p.m./µmol in chylomicron remnants, and Ptd[¹⁴C]Cho, with a specific radioactivity of 1.02×10^6 d.p.m./µmol in chylomicrons. The specific radioactivity of the Ptd[¹⁴C]Cho was too low for analysis of hydrolysis in remnants. The optimal conditions for assay of both chylomicron trioleoylglycerol and PtdCho were pH 8.3, 1.0 M-NaCl, 10 mM-CaCl₂ and 2 mg of defatted bovine serum albumin, with an acylglycerol concentration of 0.2–1.0 mM. The same conditions were used to assay chylomicron remnants. The lipids were separated by t.l.c. in chromatographic systems I and II.

LDL/HDL-1 and HDL-2 assays

The activity of HAL towards Ptd[¹⁴C]Cho in these lipoproteins was measured by using the conditions for PtdCho vesicles. The specific radioactivity of Ptd[¹⁴C]Cho was 1.09×10^5 d.p.m./µmol in LDL/HDL-1 and 8.70×10^4 d.p.m./µmol in HDL-2. That of [³H]trioleoylglycerol was too low to be measured. The lipids were separated by t.l.c. in chromatographic system II.

Other methods

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970), and protein was determined by a modified Lowry assay (Bensadoun & Weinstein, 1976).

Materials

$^{14}\text{CH}_3\text{I}$ and $[9,10\text{-}^3\text{H}(\text{n})]$ palmitate (sp. radioactivities 16.5 Ci/mol and 12.1 Ci/mmol respectively) were purchased from New England Nuclear, Boston, MA, U.S.A. Glycerol tri $[9,10(\text{n})\text{-}^3\text{H}]$ oleate (sp. radioactivity 500 Ci/mol) and $[2\text{-}^3\text{H}]$ glycerol (sp. radioactivity 500 Ci/mol) were purchased from Amersham/Searle Co., Arlington Heights, IL, U.S.A.

Results

Liver perfusion with heparin

The first step in the isolation of rat HAL was the perfusion of rat livers with heparin. It can be calculated from Table 1 that heparin perfusion of rat liver released approx. 0.754, 0.432 and 0.018 unit of activity towards mono-oleoylglycerol, trioleoylglycerol/gum arabic emulsion and PtdCho vesicles respectively per g of liver (assuming 10.5 g as the average weight of liver in 250–275 g rats). Heparin perfusates degraded mono-oleoylglycerol, trioleoylglycerol and PtdCho in emulsions as well as in chylomicrons, chylomicron remnants, LDL/HDL-1 and HDL-2. Degradation of mono-oleoylglycerol, the preferred substrate, was 1.7 times greater than that of trioleoylglycerol emulsions, and 50 times greater than that of PtdCho vesicles.

Heparin–Sephacel chromatography

HAL was further purified by affinity chromatography on heparin–Sephacel (Table 1). Our purification of HAL on heparin–Sephacel was improved 5-fold by increasing the concentration of NaCl in the initial wash steps from 0.15 to 0.4 M. The enzyme was eluted from the column with a linear salt gradient of 0.4–1.0 M-NaCl in the presence of 0.1% Triton X-100. HAL was eluted at 0.7 M-NaCl from heparin–Sephacel, as measured by activity on mono-oleoylglycerol. The inclusion of Triton X-100 increased the yield of enzyme by 20%. Moreover, the detergent- and non-detergent-eluted enzymes were identical, as judged by SDS/polyacrylamide-slab-gel electrophoresis (results not shown).

The purification of HAL on heparin–Sephacel is summarized in the appropriate column of Table 1. Activity towards mono-oleoylglycerol and PtdCho vesicles was purified 108–127-fold compared with heparin perfusates. On the other hand, activity towards trioleoylglycerol in chylomicrons, chylomicron remnants and emulsion, and activity on PtdCho in chylomicrons, chylomicron remnants, LDL/HDL-1 and HDL-2 was only purified 26–50-fold. This suggests that the lipolytic activities present in heparin perfusates of rat liver are heterogeneous in nature. The proportions of

activities towards different substrates in the perfusate remained constant during storage at 4°C.

In order to rule out any contamination of HAL with extrahepatic lipoprotein lipase, we assayed HAL activity on chylomicron trioleoylglycerol and PtdCho with increasing concentrations of NaCl (results not shown). Optimal activity occurred at 1.0 M-NaCl. The classical way to differentiate extrahepatic lipoprotein lipase from HAL has been with NaCl (Fielding, 1972). Lipoprotein lipase is almost completely inhibited at and above 0.5 M-NaCl. Therefore our HAL preparations appear to contain no lipoprotein lipase. In addition, goat anti-(rat lipoprotein lipase) antiserum, which was generously supplied by Dr. Michael Schotz, Wadsworth Veterans Hospital, Los Angeles, CA, U.S.A., did not inhibit any of our preparations of HAL (results not shown).

DEAE–Sephacel chromatography

HAL was further purified on DEAE–Sephacel as described by Jensen & Bensadoun (1981). DEAE–Sephacel chromatography of HAL (Table 1) increased the purity of all enzyme activities tested except the hydrolysis of PtdCho in lipoprotein substrates. Overall, compared with heparin perfusates, the specific activity of HAL on mono-oleoylglycerol and PtdCho vesicles was increased 153–161-fold and the specific activity of HAL towards trioleoylglycerol in chylomicrons, chylomicron remnants and emulsion was increased 52–67-fold. The activity towards PtdCho in the lipoproteins increased in the two steps only 20–40-fold and showed no significant increase in the ion-exchange step. In general, the pure lipid emulsion substrates were degraded by HAL to a greater extent than were the lipoprotein substrates.

N-Desulphated N-acetylated heparin–Sephacel chromatography

In some preparations of HAL, *N*-desulphated *N*-acetylated heparin–Sephacel was substituted for normal heparin–Sephacel. The modified heparin has an advantage over normal heparin in that it does not bind antithrombin, a major contaminant of lipases purified from post-heparin plasma (Bengtsson *et al.*, 1980). HAL purified on *N*-desulphated *N*-acetylated heparin–Sephacel was similar to that purified on normal heparin–Sephacel. Activity on mono-oleoylglycerol and PtdCho vesicles was purified 119–136-fold, whereas activity on trioleoylglycerol was purified 34–83-fold (results not shown). The only difference seen between the two preparations was hydrolysis of HDL-2 PtdCho. Measurement of activity towards HDL-2 PtdCho as a function of that towards mono-oleoylglycerol (Fig. 1) revealed that the heparin perfusate and HAL purified on normal

Table 1. *Purification of rat HAL activity on different substrates*

Lipolytic measurements were carried out in the absence of Triton X-100 under optimal conditions described in the Experimental section with $n=2$; for protein determinations, $n=1$; all assays were performed on the same enzyme preparation on the same day. Results are means $\pm$ S.D. Abbreviation used: TG, trioleoylglycerol. A unit of activity is $1 \mu\text{mol}$ of substrate transformed/min.

Substrate	Step . . .			Heparin-Sephadex			DEAE-Sephadex		
	Total protein from 24 rats (mg) . . .	Perfusate		60 $\times$ Specific activity (units/mg)	Recovery (%)	Purification (fold)	60 $\times$ Specific activity (units/mg)	Recovery (%)	Purification (fold)
	71.0								0.094
Mono-oleoylglycerol*	160.0 $\pm$ 1.0	100		17344.0 $\pm$ 3344.0	51.3	108	25794.0 $\pm$ 397.0	21.4	161
Chylomicron TG†	47.7 $\pm$ 1.8	100		1266.0 $\pm$ 198.0	12.6	27	2529.0 $\pm$ 267.0	7.0	53
Remnant TG‡	27.9 $\pm$ 1.5	100		1317.0 $\pm$ 0.0	22.4	47	1455.0 $\pm$ 135.0	6.9	52
Gum arabic/TG emulsion§	96.6 $\pm$ 2.1	100		3762.0 $\pm$ 36.0	18.4	39	6510.0 $\pm$ 2640.0	8.9	67
PtdCho vesicles	3.3 $\pm$ 0.0	100		420.0 $\pm$ 20.0	60.3	127	504.0 $\pm$ 47.0	20.3	153
Chylomicron PtdCho¶	4.5 $\pm$ 0.1	100		224.0 $\pm$ 15.0	23.5	50	219.0 $\pm$ 32.0	6.5	49
LDL/HDL-1 PtdCho**	0.81 $\pm$ 0.06	100		31.1 $\pm$ 3.9	18.2	38	26.2 $\pm$ 1.7	4.3	32
HDL-2 PtdCho**	0.45	100		11.7 $\pm$ 1.4	12.3	26	8.6 $\pm$ 2.6	2.5	19

* 1.0 mM-mono-oleoylglycerol in assay; assay time = 0.5 min.

† 2.0 mM neutral acylglycerol in assay; assay time = 10 min.

‡ 200 μM neutral acylglycerol in assay; assay time = 10 min.

§ 2.5 mM-trioleoylglycerol in assay; assay time = 10 min.

|| 434 μM -PtdCho in assay; assay time = 5 min.

¶ 172 μM -chylomicron phospholipid in assay; assay time = 10 min.

** 100 μM -phospholipid in assay; assay time = 10 min.

Fig. 1. Hydrolysis of HDL-2 PtdCho by heparin perfusate, normal heparin-Sepharose fractions and N-desulphated N-acetylated heparin-Sepharose fractions as a function of activity on mono-oleoylglycerol

Values are means $\pm$ S.D. of two determinations. Abbreviations: perf, heparin perfusate; N, heparin perfusates purified on normal heparin-Sepharose; A, heparin perfusates purified on N-desulphated N-acetylated heparin-Sepharose.

Fig. 2. Slab-gel electrophoresis in 0.1% SDS and 10% polyacrylamide of HAL prepared with 2-mercaptoethanol in the disruption buffer

Lanes 1 and 8 are standards with the indicated M_r values: phosphorylase *b* (94000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (30000) and soya-bean trypsin inhibitor (20000); lane 2, heparin perfusate (19 μ g); lane 3, N-desulphated N-acetylated heparin-Sepharose pool (5 μ g); lane 4, N-desulphated N-acetylated heparin-Sepharose pool further purified on DEAE-Sephacel (5 μ g); lane 5, normal heparin-Sepharose pool further purified on DEAE-Sephacel (5 μ g); lane 6, normal heparin-Sepharose pool (5 μ g); lane 7, heparin perfusate (15 μ g). The arrows indicate the M_r values of the three major bands in lanes 3-6.

heparin-Sepharose had 5-6 times more activity on HDL-2 PtdCho than did HAL purified on the modified heparin-Sepharose. The ratio of activity towards mono-oleoylglycerol to activity towards HDL-2 PtdCho was approx. 1000:1 in heparin perfusates and perfusates purified on normal heparin-Sepharose. Hydrolytic activity towards HDL-2 PtdCho by heparin perfusates was stable for weeks at 4°C; however, that by heparin perfusates purified on normal heparin-Sepharose was not as stable and decreased with time. With this one exception the ratios of activities between the two types of preparation on the various substrates were reproducible.

SDS/polyacrylamide-gel electrophoresis of HAL

Fig. 2 shows the results of slab-gel electrophoresis of HAL (plus 2-mercaptoethanol in the disruption buffer) in 0.1% SDS and 10% polyacrylamide at the three stages of purification. Purification on heparin-Sepharose and DEAE-Sephacel produced three major bands with apparent M_r 62000, 59000 and 56000; the major band had M_r 56000. The band with M_r 59000 appears to be a doublet. There were no detectable differences between HAL purified on either N-desulphated N-acetylated or normal heparin-Sepharose. The heparin perfusate gave three major bands with apparent M_r 65000, 62000 and 59000, but only a faint trace of a band with M_r 56000. The band with M_r 65000 comigrated with albumin. The results suggest that the M_r -56000 protein was the major protein purified.

Fig. 3 shows heparin perfusates that were purified on DEAE-Sephacel in conjunction with either N-desulphated N-acetylated heparin-

-Sepharose or normal heparin-Sepharose. Both preparations of HAL and the M_r standards were boiled without 2-mercaptoethanol in the SDS disruption buffer and run on slab-gel electrophoresis in 0.1% SDS and 10% polyacrylamide. Both preparations of HAL migrated as a diffuse band with apparent M_r 56000. Owing to the differences, we cannot say with absolute certainty that it is a single band.

Discussion

The purpose of the present study was to characterize the substrate specificity of the heparin-releasable HAL. For this purpose, we used mono-oleoylglycerol, trioleoylglycerol and PtdCho in emulsions as well as in chylomicrons, chylomicron remnants, LDL/HDL-1 and HDL-2. The enzyme degraded every acylglycerol substrate tested, and for this reason we propose that the enzyme be named 'hepatic acylglycerol lipase' (HAL). Mono-oleoylglycerol was the preferred substrate. Heparin perfusates had at least a 1.7-fold higher specific activity towards mono-oleoylglycerol than towards trioleoylglycerol. This is in contrast with results of others (Kuusi *et al.*, 1979b;

Fig. 3. Slab-gel electrophoresis in 0.1% SDS and 10% polyacrylamide of HAL prepared without 2-mercaptoethanol in the disruption buffer

Lane 1 is HAL purified on *N*-desulphated *N*-acetylated heparin-Sepharose followed by DEAE-Sephacel (5 μ g); lane 2 is HAL purified on normal heparin-Sepharose followed by DEAE-Sephacel (5 μ g); and lane 3 contains standards (same as in Fig. 2).

Jensen *et al.*, 1982), who found that heparin perfusates had a 2.5-fold higher specific activity towards trioleoylglycerol relative to mono-oleoylglycerol. This discrepancy is partially explained by the difference in the way activity on monoacylglycerol was determined. HAL degrades monoacylglycerols by two pathways, hydrolysis and transacylation (Waite & Sisson, 1973b, 1974, 1976). The hydrolysis reaction (monoacylglycerol + $H_2O \rightarrow$ glycerol + fatty acid) produces glycerol and fatty acid. The transacylation reaction (two monoacylglycerols $\rightarrow$ glycerol + diacylglycerol) produces glycerol and diacylglycerol. Under optimal assay conditions where the monoacylglycerol concentration is not rate-limiting, transacylation is the favoured reaction (Waite & Sisson, 1973b). Therefore measurement of the production of non-esterified fatty acids would measure hydrolysis but not transacylation activity, whereas measurement of glycerol production would measure both hydrolysis and transacylation activity by HAL. Other groups (Kuusi *et al.*, 1979b; Jensen *et al.*, 1982) may have underestimated the activity of HAL towards monoacylglycerol by measuring only hydrolysis. In addition, the presence of detergents in the assay may have given suboptimal measurement of HAL activity.

Another reason for our lower activity on triacylglycerol could be due to proteolysis. The same workers (Kuusi *et al.*, 1979a; Jensen *et al.*, 1982) have reported that incubation of HAL with proteinases selectively decreases the triacylglycerol lipase activity of the enzyme with no effect on monoacylglycerol or phospholipid hydrolase activity. However, it is difficult to see any differences between our liver perfusion techniques and that reported by others (Kuusi *et al.*, 1979b; Jensen & Bensadoun, 1981) that would account for any increase in proteolysis. It is clear that some alteration (perhaps proteolysis) or selection is occurring during the purification, since the ratio of activity towards triacylglycerol and PtdCho in lipoproteins decreases during the purification steps.

Another type of enzyme heterogeneity was obtained by purifying heparin perfusates on either heparin-Sepharose or *N*-desulphated, *N*-acetylated heparin-Sepharose (Fig. 1). Heparin perfusates purified on normal heparin-Sepharose had 5–6 times more activity towards HDL-2 PtdCho than had perfusates purified on the modified heparin-Sepharose. The specific activity of heparin perfusates purified on the modified heparin-Sepharose (results not shown) was the same as heparin perfusates purified on normal heparin-Sepharose on all substrates tested except HDL-2 PtdCho. In addition, structural differences were not detected by SDS/polyacrylamide-gel electro-

phoresis (Figs. 2 and 3); therefore, it is possible that the loss of the ability to degrade HDL-2 PtdCho was due to a conformational change in the lipase. Because both forms of HAL had equal activity towards PtdCho vesicles (results not shown), the difference in activity towards HDL-2 PtdCho may have been in the ability of the two forms to bind to HDL-2. Indeed, Bengtsson & Olivecrona (1980) demonstrated that HAL binds to HDL that has been coupled to Sepharose.

Our preparation of enzyme in the presence of 2-mercaptoethanol showed three major bands of M_r 62000, 59000 and 56000 (Fig. 2). Two other laboratories (Kuusi *et al.*, 1979b; Jensen & Bensadoun, 1981) reported a major band at M_r 62000, whereas the third group reported an M_r of 53000 (Twu *et al.*, 1984). In addition, our preparation migrated as a single band of M_r 56000 in the absence of 2-mercaptoethanol, suggesting that the M_r -62000, -59000 and -56000 proteins may be related in structure. In the absence of 2-mercaptoethanol the enzyme could possibly retain some secondary structure which masks differences in primary structure by maintaining shape or preventing release of proteolytic fragments.

It is possible that there is more than one heparin-releasable lipase in liver and that HAL exists in several M_r forms which have slightly different substrate specificities. Clearly, more study is needed to resolve this long-standing confusion in the literature. However, despite this question of enzyme heterogeneity, it is apparent that HAL can degrade a wide range of both neutral lipids and phosphoacylglycerols, if not all lipoprotein particles. The function of HAL may simply be to assist the liver in the clearance of not one specific lipoprotein particle, but all lipoprotein particles.

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