Characterisation of triacylglycerol hydrolase activities in human placenta

Ian J. Waterman a, Neil Emmison b, Asim K. Dutta-Roy a,*

a Rowett Research Institute, Aberdeen AB21 9SB, Scotland, UK
b The Robert Gordon University, School of Applied Sciences, Aberdeen, Scotland, UK

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Abstract

Triacylglycerol hydrolase activities were characterised in homogenates, cytosol, and microvillous membranes (MVM) of human placenta. Homogenates of placenta exhibited three distinct triacylglycerol hydrolase activities with pH optima 4.5, 6.0 and 8.0. On further fractionation, placental cytosol exhibited both acid cholesterol ester hydrolase (pH 4.5) and hormone sensitive lipase (pH 6.0) activities, whereas purified placental MVM exhibited two distinct triacylglycerol hydrolase activities; a minor activity at pH 8.0 and a second major activity at pH 6.0. Triacylglycerol hydrolase activity at pH 8.0 of MVM appeared to be lipoprotein lipase (consistent with criteria such as serum stimulation and salt inhibition), whereas at pH 6.0 the activity was unique in that it was almost abolished by serum, but was not affected by high NaCl concentrations. Our data, for the first time, demonstrate that human placental MVM, in addition to lipoprotein lipase, contain a newly identified triacylglycerol hydrolase activity at pH 6.0. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The developing fetus requires long chain polyunsaturated fatty acids (LCPUFA) for the formation of structural components of cell membranes, for participation in intracellular signalling, and in the provision of triacylglycerol stores necessary for postnatal life (see reviews [1,2]). During the third trimester of pregnancy, LCPUFA, mainly docosahexaenoic and arachidonic acids, are deposited in large amounts in the fetal brain and retina [1,2]. Placental transport of maternal plasma LCPUFA is in practice crucial for fetal growth and development because fetal synthesis of these fatty acids is considered to be very low [1,2]. The rate of deposition of LCPUFA in the developing fetus depends on their uptake by the placenta from maternal circulation and their subsequent transport to the fetus. We have recently shown that human placenta contains several membrane associated-, and cytosolic fatty acid-binding proteins and these proteins may be involved in placental uptake and subsequent transport of fatty acids to the fetus [3–5]. Of these binding proteins, a 40 kDa plasma membrane fatty acid-binding protein exclusively located in the microvillous membranes, is responsible for placental uptake of maternal LCPUFA [5–7].

Free fatty acids (FFA) in the maternal circulation
are the major source of fatty acids for transport across the placenta [1,2]. Recent data suggest that fatty acid profiles in the plasma FFA pool, triacylglycerol fatty acid composition as well as lipoprotein profiles change considerably as pregnancy continues in order to support fetal demands for LCPUFA [8–10]. FFA are generated either by intravascular lipolysis of lipoproteins or by the hydrolysis of triacylglycerols in the maternal adipose tissue. Since lipoprotein lipase is exclusively present on the maternal side of the placenta and its activity is increased several fold during the last stages of gestation [1,11,12], thus indicates that placental lipases may play an important role in fatty acid transfer between the mother and the fetus.

Previous studies have examined lipase activity at pH 4.0 for acid cholesterol hydrolase and/or at pH 8.0 for lipoprotein lipase in crude placental tissue homogenates [13,14]. Despite this fact, human placental lipases have not been well characterised. However, at least 50% of lipolytic activity of placental homogenates may be due to lipoprotein lipase [13]. Placental macrophages have been reported to have the most lipoprotein lipase activity of the placenta, whereas syncytiotrophoblasts have very little activity [15]. Important questions, therefore, still remain unanswered regarding not only the identity of the lipases, but also their location in placental cells. We have recently shown that once FFA are taken up by the placental trophoblasts, docosahexaenoic acid is preferentially incorporated into the triacylglycerol fraction [16,17], but the mechanisms involved in these processes are still not known. Placental lipases could play an important role in packaging and transporting of FFA once they are taken up by syncytiotrophoblasts.

In order to further characterise lipases and their cellular distribution in human placenta, we examined the presence of the triacylglycerol hydrolase activity in crude homogenates, cytosol and purified microvillous membranes of human placenta. We report in this paper, the presence of several triacylglycerol hydrolases, such as acid cholesterol hydrolase, hormone sensitive lipase and lipoprotein lipase in human placenta. In addition to lipoprotein lipase, human placental microvillous membranes contain another, major, triacylglycerol hydrolase activity with optimum activity at pH 6.0. By important diagnostic criteria, this enzyme activity is distinctly different from authentic lipoprotein lipase. At present, the structure and function of this newly identified enzyme activity at pH 6.0 is not known; however, this may play an important role in feto-placental lipid transport and metabolism.

2. Materials and methods

2.1. Materials

[1-14C]Oleic acid (55 mCi/mmol) and glycerol tri[9,10(n)-3H]oleate (14 Ci/mmol) were obtained from Amersham, UK. Trypsin (type I, from bovine pancreas), bovine serum albumin (fat free), and fetal calf serum were obtained from Sigma, Poole, UK. All other reagents used were of analytical grade.

2.2. Placental villous tissue homogenate preparation

Term human placentas delivered vaginally or by elective caesarean section from otherwise uncomplicated pregnancies were obtained within 1 h of delivery from Aberdeen Maternity Hospital, Aberdeen. Placental villous tissue homogenate was then prepared as described before [3,4]. Freshly prepared membranes were suspended in phosphate-buffered saline (PBS), pH 7.4, to give a membrane suspension of approximately 5 mg protein/ml and stored at −80°C for further use. Protein was determined by the Bradford method using bovine serum albumin as a standard [18].

2.3. Placental cytosol preparation

The tissue was homogenised in buffer containing 10 mM Tris-HCl, pH 7.4 and 250 mM sucrose. The supernatant from the 10000×g centrifugation was decanted and the resultant supernatant was again centrifuged for 1 h at 100000×g [5]. The resulting cytosolic supernatant was stored at −80°C for further use.

2.4. Human placental microvillous membrane preparation

Highly purified microvillous membranes (MVM)
were prepared from the placenta following a method described previously [4,5]. Following removal of chorionic and basal plates, the villous tissue was then excised in small pieces and washed in ice-cold PBS to remove excess blood. The villous tissue was then re-suspended in 5 vols. of ice cold PBS and gently stirred for 30 min. The suspension was then centrifuged for 300 g for 15 min and the supernatant decanted through a 250 μm pore size nylon mesh. The supernatant was then used as for the purification of MVM [4,5]. The purified MVM were then re-suspended in buffer containing 50 mM Tris-HCl, pH 7.4, 0.02% NaN₃, and 250 mM sucrose and stored at −80°C for further use.

2.5. Determination of triacylglycerol hydrolase activity assay

Triacylglycerol hydrolase activity of placental preparations was determined as described before [19], but with slight modifications. Substrate for the assay was prepared using 250 μM glycerol tri[9,10(n)-3H]oleate (5 μCi/μmol) in acetone, 10 μg phosphatidyl choline/phosphatidyl serine (1:1, w/w) in 10 mM Tris-HCl, pH 7.0, and 100 μg of bovine serum albumin in 10 mM Tris-HCl, pH 7.0. Under all assay conditions, the amount of acetone was maintained at 2.5% (v/v). Placental preparations were then added and incubated with the above mixture in a final volume of 200 μl. The reaction was stopped after 30 min by addition of 3.25 ml of methanol/chloroform/heptane (1.40:1.25:1.00 (v/v)) and 1.05 ml of 0.2 M borate-carbonate, pH 10.5. [1-14C]oleic acid (2500 dpm) was added to serve as an internal standard. The mixture was vortexed for 30 s and centrifuged at 750 × g for 20 min. A 0.5 ml of the upper aqueous phase containing [9,10(n)-3H]oleate and [1-14C]oleic acid was added to serve as an internal standard. The mixture was vortexed for 30 s and centrifuged at 750 × g for 20 min. A 0.5 ml of the upper aqueous phase containing [9,10(n)-3H]oleate and [1-14C]oleic acid was counted on dual channel Packard 1900CA Tri-Carb Liquid Scintillation Analyser (Packard, USA). Triacylglycerol hydrolase activity in all preparations was expressed in units (pmol of [3H]oleic acid released/min) per mg of protein.

pH dependence of triacylglycerol hydrolase activities of placental preparations were determined by incubating the placental preparations at different pH values from 3.5 to 9 using different buffer preparations, as described previously [19].

2.6. Effect of trypsin on triacylglycerol hydrolase activity of MVM

Effect of trypsin on triacylglycerol hydrolase activity of MVM was investigated. Briefly, 100 μg MVM was incubated with 316 BAEE (Nα-benzoyl-l-arginine ethyl ester) hydrolysing units of trypsin in 50 mM Tris-HCl, pH 8.0 containing 10 mM CaCl₂ for 3 h at 23°C in a total volume of 200 μl. After the incubation, membranes were washed and triacylglycerol hydrolase activity was determined as described above. Parallel experiments were performed under similar conditions with the exclusion of trypsin.

2.7. Western blot analysis of hormone-sensitive lipase of human placental cytosol

Monospecific polyclonal antisera against purified hormone sensitive lipase raised in rabbits were kindly supplied by Prof. S.J. Yeaman, Department of Biochemistry and Genetics, University of Newcastle upon Tyne, UK. Western blot analyses of placental homogenates, cytosol and pure MVM were carried out as described before [4]. Polyacrylamide gel electrophoresis of cytosolic proteins in the presence of sodium dodecyl sulphate (SDS) was carried out under reducing conditions on SDS-PAGE gel homogenous 20 (Phast System, Pharmacia, UK). After electrophoresis, proteins were transferred onto a nitrocellulose membrane by diffusion blotting at 70°C for 1 h. The membranes were probed for the presence of hormone sensitive lipase by incubating with polyclonal antiserum to this protein. Antibody–antigen complexes were then detected with HRP-anti-IgG fraction of donkey polyclonal antiserum (Scottish Antibody Production Unit).

3. Results

3.1. Identification of triacylglycerol hydrolase activities in human placental crude homogenates and cytosol

Firstly, triacylglycerol hydrolase activities were investigated in crude placental homogenates. Fig. 1 shows the effect of pH on placental triacylglycerol hydrolase activity. Three distinctive peaks of triacyl-
glycerol hydrolase activity were detected at pH 4.5, 8.0, and a much broader peak in the pH range 6.0–7.0, suggesting that two or more enzymatic activities might be present in the crude preparation.

To establish whether the broader peak of enzymatic activity in the pH range 6.0–7.0 consisted of more than one triacylglycerol hydrolase activity, homogenates of placental villi tissue were centrifuged at 100,000×g for 60 min to remove subcellular organelles and membrane components. The supernatant was subsequently screened for triacylglycerol hydrolase as shown in Fig. 2. One distinctive peak at pH 5.0 was detected and a broader peak between pH 6.5 and 7.0 (Fig. 2). Western blot analysis show that the peak between pH 6.5 and 7.0 has immunoreactivity with antibodies raised against human hormone sensitive lipase (84 kDa) (Fig. 3).

3.2. Triacylglycerol hydrolase activities in placental microvillous membranes

Macrophages and trophoblasts are two main parenchymal components of the human placenta [20]. Macrophages comprise most of the interstitial cells of the human placental villi and include about 50% of isolated placental parenchymal cells. Placental macrophages have been reported to contain the majority of lipoprotein lipase activity of the placenta compared with syncytiotrophoblasts [15]. Therefore, placental microvillous membranes were used to screen the triacylglycerol hydrolase activities, using a range of pH values (Fig. 4). Two clearly distinguishable peaks of triacylglycerol hydrolase activities were identified with pH 6.0 and 8.0. Compared with the activity at pH 8.0, the activity of pH 6.0 was 4–5-fold higher (P < 0.001, t-test). Western blot analysis indicated that pure syncytiotrophoblast membranes did not contain hormone sensitive lipase (Fig. 3) suggesting that the activity at pH 6.0 in purified MVM was not due to hormone sensitive lipase activity.

3.3. Differentiation of the triacylglycerol hydrolase activities in human placental MVM

To confirm the presence of previously reported lipoprotein lipase in the placental MVM and also to establish whether the triacylglycerol hydrolase activity at pH 6.0 is either unique or related to lipoprotein lipase in terms of its important diagnostic criteria, we investigated the triacylglycerol hydrolase activities in
human placental MVM at pH 8.0 and pH 6.0 in the presence of serum and high salt concentrations, and the heparin releasability of triacylglycerol hydrolase activity of MVM.

Heparin releasability of MVM triacylglycerol hydrolase activity was examined by incubating the membranes with heparin for 60 min at 37°C, followed by centrifugation at 100,000 g. The relative triacylglycerol hydrolase activities (at pH 6 and 8) in the pellet and supernatant were then determined. Triacylglycerol hydrolase activity at pH 8.0 was almost 84 ± 7.3% released (24.19 ± 0.26 pmol oleic acid/mg protein/min) from the membranes (initial activity 27.19 ± 4.27 pmol oleic acid/mg protein/min) by heparin treatment whereas the activity at pH 6.0 (initial activity 75.14 ± 7.6 pmol oleic acid/mg protein/min), however, was only partially (47.0 ± 19.7%) released (Table 1). Since lipoprotein lipase is readily released from membranes by heparin [21,22], this indicates that placental MVM contain a unique non-heparin-releasable triacylglycerol hydrolase with pH optima at 6.0, in addition to heparin-releasable triacylglycerol hydrolase with pH optima 8.0.

Furthermore, triacylglycerol hydrolase activity of MVM was assayed at pH 6.0 and 8.0, either in the presence or absence of 15% (v/v) fetal calf serum (FCS). FCS stimulated the triacylglycerol hydrolase activity at pH 8.0 by as much as 250 times (0.08 ± 0.3 pmol oleic acid/mg protein/min vs. 19.8 ± 0.2 pmol oleic acid/mg protein/min, P < 0.005, n = 5). In contrast, however, the triacylglycerol hydrolase activity of MVM at pH 6.0 (75.14 ± 0.06 pmol oleic acid/mg protein/min) was abolished by the presence of serum (0.01 ± 0.002 pmol oleic acid/mg protein/min, P < 0.005, n = 7). This indicates that triacylglycerol hydrolase activity at pH 8.0 of MVM was a lipoprotein lipase (consistent with criteria such as serum stimulation), whereas at pH 6.0, the activity was unique in that it was almost abolished by serum.

As a final step, the tracylglycerol hydrolase activity at pH 8.0 of MVM was determined in the presence of serum to activate the enzyme to its fullest extent.

Table 1

<table>
<thead>
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<th>pH</th>
<th>Triacylglycerol hydrolase activity (pmol oleic acid released/mg protein/min)</th>
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<td></td>
<td>Initial activity present in microvillus membranes</td>
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<tr>
<td></td>
<td>Membrane</td>
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<tr>
<td>8.0</td>
<td>27.19 ± 4.27</td>
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<tr>
<td>6.0</td>
<td>75.14 ± 7.6</td>
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Triacylglycerol hydrolase activities of microvillus membranes (with and without treating with heparin) and their supernatant were determined at pH 6.0 and pH 8.0, as described in Section 2. Results are expressed as mean ± S.E.M. (n = 3).

<sup>a</sup>P < 0.005 relative to initial triacylglycerol hydrolase (pH 8.0 optima) activity of microvillus membranes.

<sup>b</sup>P < 0.01 relative to initial triacylglycerol hydrolase (pH 6.0 optima) activity of microvillus membranes (t-test).

Fig. 3. Western blot analysis of placental crude homogenates, placental cytosol and microvillus membranes. (A) Lane 1, molecular weight protein standards, bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3 dehydrogenase (36 kDa), carbonic anhydrase (20 kDa) and α-lactalbumin (14.2 kDa); lane 2, crude placental homogenates (15 µg); lane 3, placental cytosol (15 µg); lane 4, placental microvillus membranes (15 µg). (B) Representative Western blot of the proteins showing immunoreactivity with human placental anti-hormone sensitive lipase antisera. The Western blots were carried out as described in Section 2. The lanes were as for A.
and then the effect of 1.0 M NaCl on hydrolase activity was investigated. Under these incubation conditions, the triacylglycerol hydrolase activity (20.36 ± 1.03 pmol oleic acid/mg protein/min) at pH 8.0 was inhibited by up to 80% (4.17 ± 0.02, \( P < 0.05, n = 5 \)) whereas NaCl was without effect on the triacylglycerol hydrolase activity of MVM at pH 6.0 (70.14 ± 0.10 vs. 69.26 ± 0.6 pmol oleic acid/mg protein/min, \( P > 0.5, n = 7 \)).

Trypsin digestion of MVM abolished triacylglycerol hydrolase activity at pH 6.0 by 80% (\( P < 0.05 \)), whereas the activity at pH 8.0 was abolished only by 20% (\( P = 0.05 \)).

All the above studies demonstrated clearly that placental MVM contain a new triacylglycerol hydrolase activity with pH 6.0 optimum, in addition to previously described lipoprotein lipase with pH 8.0 optima [14].

4. Discussion

The studies presented in the paper attempt to further characterise triacylglycerol hydrolase activities in human placenta. We have detected the presence of cholesterol acid hydrolase, hormone sensitive lipase (on the basis of immunological data only) and lipoprotein lipase which are, however, reported to occur in human placenta. In addition to lipoprotein lipase (pH 8.0), MVM contain, hitherto unknown, a major triacylglycerol hydrolase activity with a pH 6.0 optima. In contrast to lipoprotein lipase, this enzyme activity was only partially released from the membranes by heparin treatment, was inactivated by serum and was unaffected by the presence of high concentrations of NaCl. The triacylglycerol hydrolysing activity at pH 6.0 represented up to 80% of the total triacylglycerol hydrolysing capability of the MVM. All these data suggest that presence of a unique, but major, lipase activity in MVM hitherto unknown at pH 6.0 in human placenta. By important diagnostic criteria (pH optima, heparin releasability, serum and salt effect), this activity was clearly different from authentic lipoprotein lipase. Previous studies indicated that placental lipoprotein lipase may not be authentic lipoprotein lipase based on one important criteria, i.e. lack of serum stimulation of placental lipase [13,21]. However, these studies were performed using crude placental homogenates and therefore they could not identify the important and major triacylglycerol hydrolase activity at pH 6.0 on MVM. Our study demonstrates that authentic lipoprotein lipase activity is rather a minor activity in purified MVM, which is consistent with the findings of Bonet et al. [15].

Serum stimulation of lipoprotein lipase is a physiologically relevant phenomenon, residing in the apolipoprotein (apo) C-II – a component of chylomicron, VLDL and HDL. It enables the lipoprotein lipase, acting at a site on the luminal surface of the capillary, to be maximally active in the presence of its substrates, triacylglycerol-rich lipoproteins [22]. Therefore, it is possible that the inhibitory effect of serum on triacylglycerol hydrolase activity at pH 6.0 of MVM may not allow it to act on maternal lipoproteins, but only on intracellular triacylglycerol stores in placenta. The mode of fatty acid transport of placenta to the fetus is not well known, but some studies suggest that FFA are released from the placenta [23,24]. If it is true, then this membrane-bound triacylglycerol hydrolase (optima pH 6.0) may play an important role in releasing FFA by hydrolysing the placental stored triacylglycerol. This unique triacylglycerol hydrolase activity at pH 6.0 therefore may be involved in the packaging and/or releasing.

Fig. 4. pH dependence of triacylglycerol hydrolase activity of human placental microvillous membranes. Placental microvillous membranes (20 µg of protein) were incubated with 250 µM glycerol tri[9,10(n)-3H]oleate (5 µCi/µmol) for 30 min at 37°C. For experimental details please see Section 2. Each point represents the mean of triplicate determinations of three placentas ± S.E.M.
FFA from the placenta, whereas lipoprotein lipase, which is mostly present in placental macrophages, may be responsible for hydrolysis of maternal plasma lipoproteins as suggested by Bonet et al. [15].

Apart from lipoprotein lipase, other membrane bound lipases, such as microsomal triacylglycerol hydrolase(s), have been reported to occur in many tissues [19,25,26]. Although the function of the microsomal triacylglycerol hydrolase activity is still not clearly understood, they may be involved in mobilisation of intracellular triacylglycerol for VLDL assembly and secretion [19,25]. Microsomal triacylglycerol hydrolase(s) appear to be different from this human placental MVM triacylglycerol hydrolase activity (pH 6.0) both in their pH optima (varies from pH 4.5 to pH 8.0) and membrane location [25,26]. However, further work, such as molecular characterisation of the newly identified activity at pH 6 in MVM, is required for definitive conclusions as to its relationship with lipases and its role in placental lipid transport and metabolism.

Previous studies have shown the lipoprotein lipase (pH 8.0) was not affected in clinical conditions, such as diabetes, whereas acid cholesterol hydrolase activity (pH 4.0) was increased [27]. However, no information was available on pH 6.0 activity; therefore, further work is now in progress to examine the activity of pH 6.0 activity under these clinical conditions.

In conclusion, we report for the first time the presence of a unique, but major, triacylglycerol hydrolase activity in purified microvillous membranes of human placenta. This newly identified activity may play a major role in transporting placental derived maternal FFA to the fetus.

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