Neutral Triglyceride Lipase in Macrophages

John C. Khoo, Jean E. Vance, Eileen M. Mahoney, Dennis Jensen, Edward Wancewicz, and Daniel Steinberg

High levels of neutral triglyceride lipase activity have been demonstrated in several types of macrophages (J774 cells, human monocyte/macrophages, rabbit alveolar macrophages, and resident mouse peritoneal macrophages). The pH optima ranged from 6.5 to 7.4 depending upon the buffer and the conditions of incubation. The addition of bovine serum albumin stimulated activity at low concentrations, as expected for a fatty acid-releasing reaction, but strongly inhibited at higher concentrations; maximal activity was observed in the presence of 0.625 mg/ml of bovine serum albumin. The enzyme was remarkably thermostable, showing no apparent loss of activity at 50°C for as long as 6 hours. The lipase was inhibited 80% by 0.1 M NaCl. Assayed under optimal conditions, the specific activity of the neutral triglyceride lipase from J774 cells was more than 100-fold greater than the activity of lipoprotein lipase or neutral cholesterol esterase from those cells; this activity was 10-fold greater than the levels of hormone-sensitive lipase from 3T3-L1 adipocytes. This neutral triglyceride lipase may play an important role in the degradation and mobilization of cytosolic triglyceride in macrophage-derived foam cells.

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The lipid that accumulates in eruptive xanthomatosis, mostly triglycerides, is found in histiocytes or foam cells, cells that are probably derived from circulating monocytes. In patients with hyperchylomiconemia, these triglyceride-rich cells are found in the spleen and liver as well as in the skin; similar triglyceride-rich cells are seen in severe forms of diabetic hyperlipemia. When the hyperlipemia is brought under control, the eruptive xanthomata may resolve very rapidly, sometimes within a day or two. Serial biopsy studies by Parker et al. showed that the triglyceride content of the lesions fell much more rapidly than the cholesterol ester content. Electron microscopic studies show that most of the stored lipid in foam cells is contained in cytoplasmic vesicles lacking a limiting membrane. A number of lines of evidence suggest that these cytoplasmic lipid droplets result from lysosomal degradation of lipids taken up from chylomicrons (and/or very low density lipoproteins) and then are resynthesized in the cytoplasmic compartment from products escaping the lysosome. Mobilization of these lipid stores presumably involves either movement back into the lysosomal compartment for hydrolysis or the action of cytoplasmic hydrolases.

Previous studies in this laboratory have demonstrated the presence of a neutral cytoplasmic cholesterol esterase in homogenates of several types of macrophages. These cells were also shown to contain and to secrete a lipoprotein lipase similar in most respects to postheparin plasma lipoprotein lipase. Being a triglyceride lipase, the latter enzyme might theoretically play a role in the mobilization of stored triglycerides, but lipoprotein lipase in adipose tissue and other tissues functions exclusively in the uptake of plasma lipoprotein triglycerides, not in the mobilization of stored triglycerides. Our initial attempts to demonstrate additional neutral triglyceride lipase activities in macrophages were unsuccessful. Because of a chance observation while studying lipolytic activity in the J774 cell line of mouse macrophages, we subsequently found conditions that unmasked a high level of neutral triglyceride lipase activity in addition to that of lipoprotein lipase. We have also demon-
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strated the presence of a neutral triglyceride lipase in resident mouse peritoneal macrophages, rabbit alveolar macrophages, and human monocyte/macrophages.

Methods

Cell Preparations

Suspensions of J774 cells (clone G8), an established line derived from mouse ascites tumor, were maintained in alpha-minimal essential medium containing 5% heat-inactivated newborn calf serum. After 2 to 3 days the cells reached a density of about 5 x 10^6 cells per ml and were harvested by centrifugation (250 x g for 5 minutes); the packed cells were then stored at -70°C. The frozen cells were thawed and then homogenized in 4 volumes of a buffer containing 0.25 M sucrose, 1 mM EDTA and 25 mM Tris-HCl (pH 7.4). The homogenate was centrifuged successively at 1,000 x g for 10 minutes, 40,000 x g for 1 hour, and 120,000 x g for 1 hour at 4°C; the supernatant fraction and pellet were assayed for lipase activity at each step. The 40,000 x g supernatant fraction was designated S_{40} and the 120,000 x g supernatant fraction, S_{120}.

Resident mouse peritoneal macrophages were harvested from animals that had received intraperitoneal injections of mineral oil 3 days previously.

Alveolar macrophages were harvested from rabbits by lavage with phosphate-buffered saline, pH 7.4 (PBS). Human monocytes were prepared from blood of normal healthy donors as previously described with some minor modifications. Blood was collected in 50-ml syringes containing 0.2 volumes of an anticoagulant (0.8% citric acid/2.2% trisodium citrate/2.45% glucose) and then diluted 1:1 with PBS. The supernatant plasma (including many platelets) was removed after centrifugation at 300 x g for 15 minutes at 4°C. A mononuclear cell fraction was obtained by layering the buffy coat on Histopaque and centrifuging for 30 minutes at 800 x g at room temperature. The layer containing mononuclear cells was washed twice with PBS. This monocyte-enriched fraction was plated in 35-mm plastic culture dishes (about 2 x 10^6 cells per dish) containing 2 ml of RPMI 1640 synthetic medium (Gibco) supplemented with 2% autologous serum and 10 mM N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) (pH 7.4), penicillin (50 units/ml), streptomycin (50 µg/ml), fungizone (0.25 µg/ml), and 2 mM glutamine. After 30 minutes at 37°C, the culture medium and nonadherent cells were removed. Finally, the monocytes (> 90% pure) were cultured in 2 ml of RPMI medium containing 10% autologous serum and incubated at 37°C in a humidified CO₂ incubator (8%). The cells were maintained by changing the medium every 3 days. After 9 days, the monocyte-macrophages were harvested and homogenized in six volumes of a buffer solution containing 20% glycerol, 1 mM EDTA and 25 mM Tris-HCl (pH 7.4) for enzyme assay.

The 3T3-1 adipocytes were cultured as previously described by Kawamura et al.

Enzyme Assays

The triglyceride lipase of macrophages was assayed as described by Khoo et al. with modifications. For 10 assays, 0.1 ml of stock solution of tri[^14]C-oleoylglycerol in absolute ethanol was rapidly injected into 3.9 ml H₂O in a 50 ml plastic centrifuge tube, followed by vortexing and the addition of 2 ml 0.2 M Hepes buffer (pH 6.6). To initiate the reaction, 0.6 ml of this substrate mixture was added to a 13 x 100 mm disposable test tube containing 0.2 ml of enzyme protein source and bovine serum albumin (BSA). The final volume of 0.8 ml incubation mixture contained 0.125 mM tri[^14]C-oleoylglycerol (0.16 µCi), 50 mM Hepes (pH 6.6), 0.625 mg/ml BSA, and enzyme preparation (generally 20–40 µg). Assays were carried out at 30°C for 5 or 10 minutes. The activities of diglyceride lipase and monoglyceride lipase were assayed in the same manner using di[^14]C-oleoylglycerol and mono[^14]C-oleoylglycerol.

It was found that this macrophage triglyceride lipase behaves differently from other triglyceride lipases in that the BSA must be added to the substrate along with the enzyme as the last addition in order to obtain optimal activity; when substrate and BSA were allowed to interact prior to the addition of the enzyme, much lower activities were observed. The choice of buffer was also critical, especially in the case of J774 cells. Assays carried out in Hepes buffer were essentially linear for 15 minutes (Figure 1); assays carried out in piperazine-N,N'-bis(2-ethanesulfonic acid, Pipes) buffer were decidedly nonlinear, especially when higher concentrations of enzyme protein were added in the assay. A comparison of assays in two different buffers is shown in Figure 1.

Cholesterol esterase was assayed in an 0.8 ml incubation mixture containing 0.125 mM cholesteryl 1,14-C-oleate, 5 mg/ml BSA, 0.15 M NaCl, 10% glycerol, 2 mM EDTA, 50 mM Tris-HCl (pH 7.0) and homogenate prepared from J774 cells.

Lipoprotein lipase was assayed in an incubation mixture of 0.8 ml containing 0.125 mM tri[^14]C-oleoylglycerol, 0.15 M NaCl, 5 mg/ml BSA, 50 mM Tris-HCl (pH 8.2), 3 µg/ml of apoprotein (apo) C-II and homogenate prepared from J774 cells. The assays were carried out at 30°C for 1 hour. Lipoprotein lipase activity was expressed as the activity assayed in the presence of apo C-II minus the activity in its absence.

Hormone-sensitive lipase was assayed in an incubation mixture of 0.8 ml containing 0.125 mM tri[^14]C-oleoylglycerol, 50 mM sodium phosphate (pH 7.0), 5 mg/ml BSA and homogenate prepared from 3T3-L1 adipocytes. The assay was carried out at 30°C for 30 min.

Acid triglyceride lipase was assayed in an incubation mixture containing 0.125 mM tri[^14]C-oleoylglycerol, 50 mM sodium acetate (pH 4.6), 0.625 mg/ml
Table 1. Subcellular Distribution of Triglyceride Lipase in the J774 Macrophage-Like Cell

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Total activity (µmol/hr)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1996</td>
<td>100</td>
</tr>
<tr>
<td>1000 x g supernatant</td>
<td>1643</td>
<td>82</td>
</tr>
<tr>
<td>1000 x g pellet</td>
<td>550</td>
<td>28</td>
</tr>
<tr>
<td>40,000 x g supernatant</td>
<td>1769</td>
<td>89</td>
</tr>
<tr>
<td>40,000 x g pellet</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>120,000 x g supernatant</td>
<td>1437</td>
<td>72</td>
</tr>
<tr>
<td>120,000 x g pellet</td>
<td>79</td>
<td>4</td>
</tr>
</tbody>
</table>

The assays were carried out at 30°C for 10 minutes, as described under Experimental Procedures, using 50 mM Hepes (pH 6.6).

*Total activity in 30 ml of homogenate prepared from 7.5 ml of frozen packed cells in a buffer containing 0.25 M sucrose, 1 mM EDTA, and 25 mM Tris-HCl, pH 7.4.

Results

The J774 macrophage-like cell line was found to contain a very active neutral triglyceride lipase, most of it recovered in the high speed supernatant fractions. As shown in Table 1, about 89% of the total homogenate activity was recovered in the S120 fraction, and about 72% in the S40 fraction. These results suggest a cytoplasmic location for the lipase activity, but since frozen cells were used in all these studies, that conclusion remains tentative.

Neutral lipase activity was strongly dependent on BSA concentration. Maximal activity was obtained using BSA in the range of 0.313 to 0.625 mg/ml (Fig. 2). These concentrations of BSA gave a three-fold increase in activity over that observed in the absence of BSA; however, higher concentrations of BSA were markedly inhibitory. At 2.75 mg/ml, the activity obtained was even lower than that assayed in the absence of BSA. This inhibitory effect was observed with several preparations of BSA, including highly purified fractions and fractions treated to remove free fatty acids. Furthermore, several other nonspecific proteins (thyroglobulin, lysozyme and histone) inhibited at high concentrations. In contrast, in the case of hormone-sensitive triglyceride lipase of 3T3-L1 adipocyte-like cells and neutral cholesterol esterase of J774 cells, maximal activities were obtained at BSA concentrations ranging from 2.75 to 6.25 mg/ml. Maximum activity for the lipoprotein lipase of J774 was observed with several preparations of BSA, including highly purified fractions and fractions treated to remove free fatty acids. Furthermore, several other nonspecific proteins (thyroglobulin, lysozyme and histone) inhibited at high concentrations. In contrast, in the case of hormone-sensitive triglyceride lipase of 3T3-L1 adipocyte-like cells and neutral cholesterol esterase of J774 cells, maximal activities were obtained at BSA concentrations ranging from 2.75 to 6.25 mg/ml. Maximum activity for the lipoprotein lipase of J774 was observed with several preparations of BSA, including highly purified fractions and fractions treated to remove free fatty acids. Furthermore, several other nonspecific proteins (thyroglobulin, lysozyme and histone) inhibited at high concentrations. In contrast, in the case of hormone-sensitive triglyceride lipase of 3T3-L1 adipocyte-like cells and neutral cholesterol esterase of J774 cells, maximal activities were obtained at BSA concentrations ranging from 2.75 to 6.25 mg/ml. Maximum activity for the lipoprotein lipase of J774 was observed with several preparations of BSA, including highly purified fractions and fractions treated to remove free fatty acids. Furthermore, several other nonspecific proteins (thyroglobulin, lysozyme and histone) inhibited at high concentrations. In contrast, in the case of hormone-sensitive triglyceride lipase of 3T3-L1 adipocyte-like cells and neutral cholesterol esterase of J774 cells, maximal activities were obtained at BSA concentrations ranging from 2.75 to 6.25 mg/ml.
Figure 3. The effects of incubation time and enzyme concentration on the rate of reaction of neutral triglyceride lipase from J774 cells. A. The enzyme in the S_{120} fraction was incubated at 30°C for 20 minutes in the absence of BSA (•), in the presence of 0.625 mg/ml BSA (○), and 5 mg/ml BSA (■). B. The assays were carried out at 30°C for 10 minutes with increasing concentrations of \( S_{120} \) fraction. The buffer used for the assays was 50 mM Hepes (pH 6.6).

Figure 4. The influence of pH on the activities of triglyceride lipase in homogenates prepared from J774 cells (○), rabbit alveolar macrophages (■), human monocyte-macrophages (△), and resident mouse peritoneal macrophages (▴). The buffer used for the assays was 50 mM Hepes.
greater than the activities of lipoprotein lipase and neutral cholesterol esterase in this same cell line (all enzymes assayed under conditions optimal for their activity in whole homogenates). The specific activity was even greater than that of hormone-sensitive li-

Table 2. Comparison of Lipolytic Activities in Whole Homogenates Prepared from Various Types of Cells

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Activities</th>
<th>nmol/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774</td>
<td>Neutral triglyceride lipase</td>
<td>2,850</td>
</tr>
<tr>
<td>J774</td>
<td>Lipoprotein lipase</td>
<td>15.8</td>
</tr>
<tr>
<td>J774</td>
<td>Neutral cholesterol esterase</td>
<td>17.4</td>
</tr>
<tr>
<td>3T3-L1 adipocyte</td>
<td>Hormone-sensitive lipase</td>
<td>213</td>
</tr>
</tbody>
</table>

Discussion

The triglyceride-loaded foam cells that characterize eruptive xanthomas almost certainly arise from macrophages. In familial hyperchylomicronemia and other severe forms of hypertriglyceride-

Figure 6. A. Substrate specificity of the thermostable lipase activity from J774 macrophages. The S_{120} fraction was heated at 50°C for 2 hours. Aliquots containing 19 μg protein were assayed for activity toward 0.125 mM tri {¹⁴C}-oleoylglycerol (♀) in 0.125 mM di {¹⁴C}-oleoylglycerol. Activity toward 0.125 mM mono {¹⁴C}-oleoylglycerol (★) was assayed using 98 μg protein (expanded in B). All three activities were assayed in the presence of 0.625 mg/ml BSA and 50 mM Hepes buffer at pH 6.6. Specific activities toward trioleoylglycerol, dioleoylglycerol, and monooleoylglycerol were 4285, 5154, and 134 nmol/mg protein/hr.

Figure 5. The effects of incubation at 50°C on the activities of neutral triglyceride lipase (♀), acid triglyceride lipase (○), neutral cholesterol esterase (▲), and lipoprotein lipase (●) in homogenates prepared from J774 cells; and the neutral triglyceride lipase in homogenate prepared from human monocyte-macrophages (▲). The original activities before heating were 3,368, 22.2, 17.4, and 15.8 nmol/mg protein/hour, respectively, for neutral triglyceride lipase, acid triglyceride lipase, neutral cholesterol esterase, and lipoprotein lipase from J774 cells. The neutral triglyceride lipase activity in human monocyte-macrophages before heating was 3,500 nmol/mg protein/hour.

The S_{120} fraction previously heated at 50°C for 2 hours was assayed for activities toward triolein, diolein, and monoolein. As shown in Figure 6, the levels of activities toward triolein and diolein were almost the same, whereas on the same scale there was virtually no activity toward monoolein. The activity toward monoolein, plotted on a different scale, is shown in the inset to Figure 6. It was only about 3% of the activity toward triolein. These results predict that when triolein is used as substrate, the end products are probably a mixture of free oleic acid and monoolein. In fact, analysis of the hydrolytic products by thin layer chromatography revealed that about 50% of the end products were monoolein.
miae, these lesions can appear rapidly — when plasma lipids rise — and disappear almost as rapidly when lipid levels are normalized. The uptake and storage of lipids from triglyceride-rich lipoproteins have been studied in several types of macrophage in vitro.\textsuperscript{15-19} This process occurs in part via saturable, receptor-mediated pathways, the best characterized being the pathway for the uptake of beta-VLDL or "remnants".\textsuperscript{15} and in part through phagocytosis.\textsuperscript{16-18} The recent discovery by Khoo et al.,\textsuperscript{7} that macrophages synthesize and secrete lipoprotein lipase, elucidates additional mechanisms that may be relevant. As shown by Ostadlund-Lindqvist et al.,\textsuperscript{16} and by Lindqvist et al.,\textsuperscript{17} the free fatty acids released from triglyceride-rich lipoproteins by lipoprotein lipase can be subsequently taken up into the macrophage. These authors also showed that the action of lipoprotein lipase increased the uptake of chylomicron and VLDL apoproteins, presumably reflecting the more rapid uptake of remnants.

The mechanisms involved in the mobilization of stored triglycerides from macrophages has received less attention. Since the stored lipids are in cytoplasmic droplets that do not appear to be membrane-limited, and since release of intact triglycerides is unlikely, either the triglycerides must be transferred to the lysosome for hydrolysis or there must be a cytoplasmic triglyceride lipase for that purpose. Our present studies describe such an enzyme that has remarkably high levels of activity. The specific activity of the neutral triglyceride lipase in the supernatant fraction of the J774 cell line was more than 100 times greater than that of the cytoplasmic cholesterol esterase, the intracellular lipoprotein lipase, or the lysosomal acid triglyceride lipase, all of which were measured under conditions optimal for their respective activities. The question of whether this enzyme might be identical to the cytoplasmic cholesterol esterase of macrophages previously described by Khoo et al.\textsuperscript{8} must be asked. In adipose tissue and in adrenal the two activities have never been fully resolved.\textsuperscript{20-22}

Also, the acid acylhydrolase activities in lysosomes of histiocytes responsible for the hydrolysis of cholesterol ester and triglyceride appear attributable to the same enzyme.\textsuperscript{23, 24} The question is answered clearly by the completely different heat sensitivities of the two activities in the macrophage: the supernatant neutral triglyceride lipase lost almost no activity at 50°C for 6 hours, whereas the cholesterol esterase activity fell by 80% under the same conditions. Thus it appears that the macrophage possesses a uniquely active neutral triglyceride lipase that may govern its ability to mobilize and rid itself of accumulated triglycerides, separate but functionally closely related to the previously described cholesterol esterase in these cells.\textsuperscript{8} The studies of Lindqvist et al.,\textsuperscript{17} showing that albumin in the medium facilitated the loss of stored triglycerides from macrophages, together with recent results from this laboratory (E. von Hodenberg, J.C. Khoo, J.L. Witztum and D. Steinberg, unpublished results) directly demonstrating net release of free fatty acids from triglyceride-loaded macrophages, leave little doubt that triglyceride hydrolysis precedes mobilization. Whether or not the enzyme activity described in this paper is the only determinant of such mobilization and whether it is subject to regulation remains to be determined.

The unusual properties of the neutral triglyceride lipase, particularly in the J774 cell line, deserve comment. Its low apparent activity in some buffers and its sensitivity to the concentration of albumin and other proteins may explain why it has not been previously reported and also may be significant with regard to its functional behavior. Other lipases have not shown this kind of selectivity and so the enzyme must at least differ in the way it interacts with substrates presented in various ways. The remarkable heat stability of the enzyme suggests an unusual enzyme protein.

When we consider the possible significance of this triglyceride lipase, we should note that triglyceride accumulation in macrophages is by no means limited to hyperchylomiconemia and other severe hypertriglyceridemic states. Tuberous xanthomata may contain a great deal of triglyceride even in patients whose plasma triglycerides are only modestly elevated,\textsuperscript{4} presumably due to the uptake of beta-VLDL, which contain less triglyceride than normal VLDL but considerably more than LDL. When both cholesterol and triglycerides accumulate, are they stored intermingled in the same cytoplasmic droplets? If so, must both the neutral cholesterol esterase and the triglyceride lipase, shown here to be apparently separate enzymes, be simultaneously active? In other words, could the presence of a second lipid in the droplet mask the other, making it less accessible to its hydrolase? An interesting possibility is that a deficiency in neutral triglyceride lipase could, on this basis, contribute to cholesterol ester accumulation and, conversely, that cholesterol esterase deficiency might contribute to triglyceride accumulation.

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