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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stratified 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 × 10^6 cells per ml and were harvested by centrifugation (250 × 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 × g for 10 minutes, 40,000 × g for 1 hour, and 120,000 × g for 1 hour at 4°C; the supernatant fraction and pellet were assayed for lipase activity at each step. The 40,000 × g supernatant fraction was designated S_{40} and the 120,000 × g supernatant fraction, S_{100}.

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 × g for 5 minutes; the packed cells were then stored at -70°C. The frozen cells were thawed and homogenized in 4 volumes of a buffer solution containing 20% glyceral, 1 mM EDTA and 25 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 100,000 × g for 1 hour, and 120,000 × g for 1 hour at 4°C; the supernatant fraction and pellet were assayed for lipase activity at each step. The 120,000 × g supernatant fraction was designated S_{120}.

The 3T3-L1 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 trioleoylglycerol in absolute ethanol was rapidly injected into 3.9 ml H_2O 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 × 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 trioleoylglycerol (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[^14C]-oleoylglycerol and mono[^14C]-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 1,4-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[^14C]-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[^14C]-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[^14C]-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 × g supernatant</td>
<td>1643</td>
<td>82</td>
</tr>
<tr>
<td>1000 × g pellet</td>
<td>550</td>
<td>28</td>
</tr>
<tr>
<td>40,000 × g supernatant</td>
<td>1769</td>
<td>89</td>
</tr>
<tr>
<td>40,000 × g pellet</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>120,000 × g supernatant</td>
<td>1437</td>
<td>72</td>
</tr>
<tr>
<td>120,000 × 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.

BSA and homogenate prepared from J774 cells. The assays were carried out at 30°C for 30 min.

All assays were terminated by the addition of 3 ml of chloroform/methanol/benzene (1:2.4:2, v/v). 14C-oleic acid was extracted by the addition of 0.1 ml of 1M NaOH and quantified as described.

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 S120 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

Figure 1. The effects of Hepes and Pipes buffers on the rate of reaction of triglyceride lipase from J774 macrophages. The enzyme in the S120 fraction was assayed with 50 mM Hepes (○), or Pipes (△) at pH 6.6 in the presence of 0.625 mg/ml BSA. Enzyme assays were carried out as described in Methods.

Figure 2. The effects of increasing concentrations of BSA on neutral triglyceride lipase (○), neutral cholesterol esterase (●), and lipoprotein lipase (●) activities in homogenates prepared from J774 cells, and the hormone-sensitive triglyceride lipase in 3T3-L1 adipocyte-like cells (△). Enzyme assays were carried out as described in Methods. The activities of all four enzymes were normalized and expressed relative to the maximal activities at their respective optimal BSA concentrations. The maximal activities for neutral triglyceride lipase, neutral cholesterol esterase, lipoprotein lipase and hormone-sensitive lipase were 2850, 15.8, 17.4, and 213 nmol/mg protein/hour, respectively.
cells was obtained at 1.25 mg/ml BSA, and the activity decreased by about 30% at higher BSA concentrations (Figure 2). As shown in Figure 3A, the reaction rate was nonlinear in the absence of BSA but became linear for at least 10 minutes in the presence of 0.625 mg/ml BSA. Then at 5 mg/ml BSA there was a marked initial inhibition and some recovery at later time-points. Under optimal conditions for the assay, the activity observed was linear in relation to the enzyme concentration as shown in Figure 3B. At BSA concentrations just above or below the optimum, the slope of the enzyme-activity curve was lower but close to linearity up to 25 μg enzyme protein per assay.

The triglyceride lipase activity in the homogenates prepared from J774 cells, rabbit alveolar macrophages, human monocyte-macrophages, and resident mouse peritoneal macrophages was examined as a function of pH. Single peaks were observed with an optimum at pH 6.6 for rabbit alveolar macrophages, J774 cells, and mouse peritoneal macrophages and an optimum at pH 6.9 for human monocyte-macrophages (Figure 4). When bis-(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (bis-Tris) was used as buffer for assay instead of HEPES, optimal activities were observed between pH 7.2 to pH 7.4 (data not shown).

This lipase was remarkably stable to heat treatment. As shown in Figure 5, there was virtually no loss in activity (there was actually a slight increase in activity) of neutral triglyceride lipase in the homogenates prepared from J774 cells and human monocyte-macrophages after incubation at 50°C for as long as 6 hours. In contrast, lipoprotein lipase lost about 90% of its activity after 3 hours at 50°C, neutral cholesterol esterase lost 70% of its activity after 6 hours of incubation at 50°C, and acid triglyceride lipase lost 60% of its activity after 4 hours of incubation at 50°C.

As emphasized by the comparisons shown in Table 2, the specific activity of neutral triglyceride lipase from the macrophage was extraordinarily high when assayed under optimal conditions. The specific activity in J774 cells was more than 100 times
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-

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

### 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>

The S₁₂₀ 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.

### Discussion

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

Khoo et al.

Active neutral triglyceride lipase, a distinctive intracellular enzyme, has been described in several types of macrophage in vitro. Its activity is essential for the normal release of free fatty acids from triglyceride-loaded macrophages, leaving 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.

Index Terms: cytosolic triglyceride lipase • macrophages, J774 cells • hypertriglyceridemia, foam cells • xanthomata
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