Lipoprotein Lipase in Cultured Pig Aortic Smooth Muscle Cells

Jean E. Vance, John C. Khoo, and Daniel Steinberg

Acetone powder extracts prepared from cultured pig aortic smooth muscle cells and the culture medium from these cells (particularly when incubated with heparin) were shown to contain a lipolytic enzyme which was identified as lipoprotein lipase by the following criteria: 1) stimulation by apolipoprotein C-II; 2) an optimal activity at approximately pH 8.0; 3) inhibition by NaCl, and 4) binding to a heparin-Sepharose affinity column. In addition, we found that cultured arterial smooth muscle cells from guinea pig and rabbit secreted a similar lipase into the culture medium. In contrast, studies using cultured bovine aortic endothelial cells yielded no evidence for either the synthesis or secretion of lipoprotein lipase by these cells. The production of lipoprotein lipase by the smooth muscle cells of the artery may play a role in the process of atherogenesis. (Arteriosclerosis 2:390-395, September/October 1982)

Lipoprotein lipase (LPL) occurs in many diverse tissues and animals including the arterial wall of pig,1 rabbit,2 cow,3,4 and man.5 Zilversmit6 has proposed that the presence of LPL on the luminal arterial endothelial surface may play an important role in the initiation of atherosclerosis by interaction of the LPL with the triacylglycerols of chylomicrons and/or the very low density lipoproteins present in the circulation. However, it is still uncertain which of the several cell types present in the arterial wall may be responsible for the production of the LPL. Recent studies in this laboratory have shown that cultured macrophages,7 rabbit alveolar macrophages, and human monocytes8 synthesize and secrete LPL into the culture medium. Thus, the secretion of LPL by these cells could be one important source. It has not been demonstrated whether smooth muscle cells, the major cell type in the arterial wall, can synthesize and secrete LPL.

In the present study, we showed that cultured pig aortic smooth muscle cells synthesize and secrete LPL. In contrast, we found no detectable LPL activity in cultured bovine aortic endothelial cells.

Methods

Culture and Harvest of Cells

Cultures of pig aortic smooth muscle cells (Passages 10–20) were maintained in 150 cm² polystyrene tissue culture flasks in 40 ml Dulbecco’s modified Eagle’s medium which contained 10% heat-inactivated fetal calf serum. The cells were prepared, maintained, and characterized as described by Weinstein et al.;9 they exhibited a typical “hill and valley” growth pattern, the presence of myofilaments, and multiple dense bodies. Before harvest, the cells were washed with phosphate-buffered saline. The cells were harvested and collected in culture medium, centrifuged at 1000 × g for 10 minutes, washed twice with cold phosphate-buffered saline, and washed once with buffer which contained 10 mM Tris-HCl, 1 mM EDTA, and 0.25 M sucrose, pH 7.4 (Medium 1). The packed cells were homogenized in 4 volumes of Medium 1. Arterial smooth muscle cells from guinea pig and rabbit were maintained and harvested in a similar manner.

The bovine aortic endothelial cells were a gift from Michael A. Gimbrone, Harvard Medical School. These cells were maintained in 150 cm² polystyrene culture flasks in 40 ml Dulbecco’s modified Eagle’s medium which contained 10% heat-inactivated calf serum.

For experiments in which LPL secretion into the culture medium was measured, the smooth muscle cells were grown for 5 to 7 days at 37°C, then
washed twice with Dulbecco's phosphate-buffered saline. Fresh medium (20 ml Dulbecco's modified Eagle's medium which contained 1% heat-inactivated fetal calf serum and 10 units/ml heparin) was added to the cells. The cells were maintained in this heparin-containing medium at 37°C for 2 hours unless otherwise stated. The culture medium was removed and centrifuged at 1000 \( \times \) g for 10 minutes before assay for LPL activity. The activity of LPL (usually in 0.2 ml culture medium) was measured using tri-1-\(^{14}\)C-oleoylglycerol as substrate. After removal of the medium, the cells were harvested, washed, and homogenized in 4 volumes of Medium 1. The protein content of the cell homogenates was determined by the method of Lowry et al.\(^{10}\)

### Assay of Lipoprotein Lipase

LPL was assayed as described by Khoo et al.\(^{11}\) The reaction mixture (final volume 0.4 ml) contained the enzyme (typically 0.05 ml of an extract of acetone powder or 0.2 ml of culture medium), 0.125 mM radioactive triolein containing 1-\(^{14}\)C-oleic acid distributed randomly among the acyl positions (3.2 \( \mu \)Ci/\( \mu \)mol), 1 to 5 \( \mu \)g/ml apolipoprotein C-II (apo C-II), 0.15 M NaCl, 5 mg/ml bovine serum albumin and 50 mM Tris-HCI, pH 8.2. The assay mixture was incubated at 30°C for 1 hour, during which time the enzyme reaction was linear with respect to time. The enzyme reaction was terminated by the addition of 3 ml of a solution which contained benzene:chloroform:methanol (20:10:24), and the radioactivity of the unesterified fatty acids in the aqueous phase was measured. One unit of LPL activity was defined as that which released one nmole oleic acid per hour at 30°C. Apo C-II was purified according to the method of Brown et al.\(^{12}\)

### Preparation of Acetone Powders

We added 1 ml cell homogenate (protein concentration 3–8 mg/ml) dropwise, with stirring, to 30 ml cold (-20°C) acetone. After 30 minutes at -20°C, the white precipitate was removed by centrifugation at 2000 \( \times \) g for 10 minutes at 4°C. The precipitate was washed once with cold (-20°C) acetone, once with cold (-20°C) diethyl ether, and was finally lyophilized. The resultant powder was extracted with 1.0 ml of Medium 1 at 4°C. In experiments with the fat pads of guinea pigs and mice, the tissue was homogenized in 2 volumes Medium 1. The hearts of guinea pigs and mice were homogenized in 4 volumes of Medium 1. The acetone powder of these homogenates were prepared as described above.

## Results

Extracts of acetone powders of cultured pig aortic smooth muscle cells contained lipolytic activity, 10.8 units/mg cell protein under the standard conditions of assay. The culture medium from these cells that was incubated in the presence of heparin for 2 to 4 hours also contained lipolytic activity – 416 units/mg cell protein (table 1). The lipolytic activities both in the cells and in the culture medium shared the characteristic properties of LPL, as detailed below.

As shown in figure 1, both activities were stimulated by apo C-II, a known activator of LPL.\(^{13}\) Maximal stimulation by apo C-II was observed when 1–5 \( \mu \)g/ml of apo C-II were added to the assay mixtures. In the extract of the acetone powder, maximal lipase activity was approximately threefold higher in the presence of apo C-II. In the culture medium there was an approximately 30-fold stimulation by apo C-II. The pH optimum of the lipase in the acetone powder extract was 7.6 to 8.0 (figure 2); at pH 5.0, there was less than 20% of the maximal lipase activity. In the culture medium, the pH optimum of the lipolytic activity was 8.0 to 8.2. An alkaline pH optimum is characteristic of purified LPL. Sodium chloride (0.5-1.0 M) inhibits LPL activity, and a typical inhibition of the lipase from the pig aortic smooth muscle cells was observed in the presence of NaCl at concentrations within this range (figure 3). The required con-

### Table 1. Triacylglycerol Lipase Activity In the Culture Medium from Heparin-treated Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Total lipase activity in presence of apo C-II (1 ( \mu )g/ml, units/mg cell protein)</th>
<th>Total lipase activity in absence of apo C-II (units/mg cell protein)</th>
<th>Average percentage stimulation by apo C-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig aortic smooth muscle cells*</td>
<td>416 ± 100 (3)</td>
<td>26 ± 10 (3)</td>
<td>1494%</td>
</tr>
<tr>
<td>Guinea pig aortic smooth muscle cells†</td>
<td>362 ± 48 (3)</td>
<td>8 ± 3 (3)</td>
<td>4541%</td>
</tr>
<tr>
<td>Rabbit carotid artery smooth muscle cells*</td>
<td>102 (2)</td>
<td>0 (2)</td>
<td>0%</td>
</tr>
<tr>
<td>Bovine aortic endothelial cells*</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>0%</td>
</tr>
</tbody>
</table>

Values = mean values ± so. The number of samples is indicated in parentheses. *Medium assayed after incubation with heparin for 2 hours. †Medium assayed after incubation with heparin for 19 hours.
Figure 1. Lipase activity assayed in duplicate, as a function of apo C-ll concentration, in an acetone powder extract from cultured pig aortic smooth muscle cells (●) and in the culture medium from heparin-treated cultured pig aortic smooth muscle cells (○). The cells were incubated with 10 units/ml heparin for 2 hours before LPL assay.

Figure 2. Lipase activity in duplicate assays, in an acetone powder extract of cultured pig aortic smooth muscle cells, as a function of pH. In the pH range 5.0–6.0, 50 mM acetate buffer was used, whereas 50 mM Tris-HCl was used for incubations at pH 7.6–8.6.

Figure 3. Effect of NaCl on the lipase activity assayed in duplicate in an extract of the acetone powder (●) and the culture medium (○) of cultured pig aortic smooth muscle cells treated with 10 units/ml of heparin for 4 hours. NaCl was added during the assay at the concentrations indicated.

centration of NaCl for optimal lipase activity in the acetone powder extract was 0.2 M; at a NaCl concentration of 0.6 M, the enzyme activity was only 20% of maximum, and only 30% of maximum at a NaCl concentration of 0.05 M. Similarly, NaCl inhibited the lipase activity in the culture medium at concentrations above 0.1 M (figure 3).

An additional characteristic of LPL is that it binds tightly to a heparin-Sepharose affinity column14 and is usually eluted from such a column with a solution of NaCl having a concentration of 1.0 M or higher. An extract of the acetone powder from the cultured pig smooth muscle cells was applied to a heparin-Sepharose 4B column (figure 4) and the column was eluted with 0.3 to 2.0 M NaCl. No lipase activity was eluted by 0.3 M NaCl; 1.0 M NaCl eluted 90% of the applied lipase activity. The lipase recovered from the column was stimulated sixfold by 1 μg/ml of apo C-ll and the pH optimum was between 7.5 and 8.0.

A comparison of the LPL activities in acetone powders prepared from various tissues and cell types is presented in table 2. The lipase activities in acetone powder extracts from the cultured pig smooth muscle cells and J774 macrophages were approximately equal in the presence of apo C-ll (approximately 10 units of lipase activity/mg cell protein). Acetone powders prepared from heart and epididymal fat pads of fed mice yielded higher activities (two- and 50-fold higher, respectively). Similar activities were observed in guinea pig heart and lumbar fat pads (20.9 and 465 units/mg protein, respectively). In contrast,
Heparin-Sepharose 4B affinity chromatography of the lipase activity in an extract of an acetone powder from cultured pig aortic smooth muscle cells. A 1.5 ml extract of the acetone powder was mixed with 1.5 ml of Buffer A (25 mM Tris-HCl, 20% glycerol, 1 mM EDTA, pH 7.4). NaCl was added to give a final concentration of 0.3 M. This sample was mixed gently with 1 ml of heparin-Sepharose 4B (prepared as described by Iverius14) and allowed to stand overnight at 4°C. The suspension was poured into a column (4 mm × 6 mm) and eluted with 3 ml of each of the solutions of NaCl in Buffer A in this sequence of concentrations: 0.3 M, 0.5 M, 1.0 M and 2.0 M NaCl. The lipase was eluted with Buffer A containing 1.0 M NaCl. Aliquots of 0.1 ml of column effluent were assayed in duplicate for LPL activity in the presence (•) and absence (○) of 1 μg/ml apo C-Ⅱ. Of the lipase activity applied to the column, 90% was recovered. The NaCl concentration of the effluent is represented by the dotted line.

Figure 4: Heparin-Sepharose 4B affinity chromatography of the lipase activity in an extract of an acetone powder from cultured pig aortic smooth muscle cells.

Table 2. Triacylglycerol Lipase Activities (at pH 8.2) of the Acetone Powder Extracts from Various Tissues and Cultured Cells

| Tissue/cell type                        | Total lipase activity in presence of apo C-Ⅱ (1 μg/ml) (units/mg protein) | Total lipase activity in absence of apo C-Ⅱ (units/mg protein) | Average percentage stimulation by apo C-Ⅱ
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Cultured pig aortic smooth muscle cells</td>
<td>10.8 ± 2.8 (4)</td>
<td>2.9 ± 0.6 (4)</td>
<td>272%</td>
</tr>
<tr>
<td>Cultured J774 macrophages</td>
<td>9.7 ± 3.1 (3)</td>
<td>3.6 ± 1.2 (2)</td>
<td>269%</td>
</tr>
<tr>
<td>Cultured guinea pig aortic smooth muscle cells</td>
<td>2.3 ± 0.5 (4)</td>
<td>2.8 ± 0.5 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Cultured bovine aortic endothelial cells</td>
<td>24.1 (2)</td>
<td>27.8 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Ovarian fat pads from fed mouse</td>
<td>497 (1)</td>
<td>54.6 (1)</td>
<td>810%</td>
</tr>
<tr>
<td>Heart from fed mouse</td>
<td>21.3 (1)</td>
<td>6.7 (1)</td>
<td>218%</td>
</tr>
<tr>
<td>Lumbar fat pads from fed guinea pig</td>
<td>465 (2)</td>
<td>111 (2)</td>
<td>320%</td>
</tr>
<tr>
<td>Heart from fed guinea pig</td>
<td>20.9 (2)</td>
<td>3.3 (2)</td>
<td>533%</td>
</tr>
</tbody>
</table>

Values = mean values ± sd. The number of samples is indicated in parentheses.
Figure 5. Release of LPL into the medium of cultured pig aortic smooth muscle cells as affected by heparin and by cycloheximide. At Time 0, the medium was removed from the cells and replaced with Dulbecco's modified Eagle's medium which contained 1% heat-inactivated fetal calf serum with (●) or without (○) heparin (10 units/ml). The medium was completely removed and LPL assayed at the times indicated. At each time point, fresh medium was added to the cells. Data represent cumulative secretion of enzyme. When medium containing both heparin (10 units/ml) and cycloheximide (0.5 μg/ml) was used, LPL secretion was almost completely arrested after 1 hour (▲).

was also released into the culture medium by these cells. This lipase had an almost absolute dependence on apo C-II for activity, showed an alkaline pH optimum, and was inhibited by NaCl concentrations greater than 0.1 M. It is interesting, however, that no apo C-II-dependent triacylglycerol lipase could be detected in the acetone powder prepared from the guinea pig smooth muscle cells (table 1). Acetone powders prepared from guinea pig hearts and fat pads yielded an apo C-II-dependent lipase (presumably LPL) which could be readily assayed. The absence of LPL from the acetone powder of the guinea pig cells might reflect the presence of inhibitors or a rapid degradation peculiar to these cultured cells. Alternatively, there might be a rapid secretion without significant accumulation within the cells.

The finding of LPL in cultured arterial smooth muscle cells and in the culture medium of these cells may have relevance to the role played by arterial smooth muscle cells in the atherogenic process. Experiments in which rabbits were fed a diet containing 0.25–0.5% cholesterol for 2 to 4 months demonstrated that the content of LPL in the aorta was directly proportional to the cholesterol content of the aorta. Ross and Glomset in their “response to injury” hypothesis of atherogenesis have proposed that, after the injury of the arterial endothelium, the proliferation of smooth muscle cells at that site is a key event in the formation of an atherosclerotic lesion. Lipoprotein lipase, produced by the smooth muscle cells at that site, would be available for hydrolysis of the triacylglycerols in the chylomicrons and very low density lipoproteins in the circulation. The action of LPL on these triacylglycerol-rich lipoproteins would produce cholesterol- and cholesterol ester-rich remnant particles, in addition to unesterified fatty acids. Subsequently, the arterial smooth muscle cells could internalize these lipoprotein remnants and degradation products and become “foam” cells. Floren et al. have demonstrated that uptake of remnant particles results in the intracellular deposition of neutral

Discussion

Cultured pig aortic smooth muscle cells synthesized LPL, which could be identified both in an acetone powder prepared from these cells and, particularly when the cells were incubated with heparin, in the culture medium. The triacylglycerol lipase activity produced by these cells has been identified as LPL by the following criteria: 1) stimulation by apo C-II; 2) an alkaline pH optimum; 3) inhibition by concentrations of NaCl above 0.2 M; 4) binding to heparin-Sepharose affinity column, requiring elution by 1.0 M NaCl; and 5) release into the culture medium in greater amounts in the presence of heparin. The enzyme in the medium was more stable than LPL from bovine milk, which can lose 90% of its activity in 5 minutes at 37°C. The pig smooth muscle enzyme was also more stable than a similar lipase released into the medium of heparin-treated J774 macrophages in culture. More than 50% of the activity of the lipase in the culture medium remained after storage for 48 hours at -70°C, and more than 70% of the activity remained when the culture medium was maintained at 37°C for 1 hour. The enzyme was stable under the conditions of the enzyme assay as indicated by linearity of the enzyme reaction with time.

Similar experiments have identified LPL in the culture medium of guinea pig arterial smooth muscle cells. However, there was no evidence for LPL in an acetone powder prepared from these cells. The lack of LPL in the acetone powder is probably not due entirely to the instability of the enzyme, since an acetone powder prepared from guinea pig hearts and fat pads yielded an apo C-II-dependent lipase (presumably LPL) which could be readily assayed. The absence of LPL from the acetone powder of the guinea pig cells might reflect the presence of inhibitors or a rapid degradation peculiar to these cultured cells. Alternatively, there might be a rapid secretion without significant accumulation within the cells.

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lipids. A similar process is thought to occur in macrophages.\textsuperscript{7}

In agreement with the findings of other workers,\textsuperscript{19} we found no evidence for the presence of LPL in cultured bovine aortic endothelial cells. It would appear that in the artery, LPL is most likely synthesized by the smooth muscle cells and transported by some undefined process to the surface of the endothelium. At this site, the lipase is readily available for degradation of the tricylglycerol moieties in chylomicrons and very low density lipoproteins in the circulation.

The relative contribution of LPL from arterial smooth muscle cells compared to that from other arterial cell types, especially at the site of atherosclerotic lesions, remains to be determined.

Any factors that influence the production of LPL by the arterial smooth muscle cells, or the proliferation of these cells may be important for regulation of the process of atherogenesis. Such factors may include hormones,\textsuperscript{20} growth factors derived from other associated cell types (for example, platelet-derived growth factor,\textsuperscript{21}) or variations in the supply of lipoproteins in culture media.\textsuperscript{22}

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References


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