Lipoprotein and Hormone-Sensitive Lipases in Porcine Adipose Tissue

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ABSTRACT: Lipoprotein lipase (LPL) is an adipocyte enzyme that cleaves fatty acids from circulating lipoproteins. Fatty acids enter the cell to be oxidized or esterified. Hormone-sensitive lipase (HSL) is an adipocyte enzyme that cleaves fatty acids from intracellular triacylglycerol. The HSL is activated by phosphorylation. Assays for the two lipases are complex because the hydrophobic substrate, triacylglycerol, must be presented as a gum-based suspension or as a detergent-based emulsion to a relatively hydrophilic enzyme. A convenient, stable glycerol/phospholipid suspension of the substrate was used for measurement of porcine adipose tissue LPL and HSL in vitro. This substrate was excellent for LPL. It produced rates five times those using a more complex and less convenient gum-based substrate suspension. The LPL activity was released by heparin, had a pH optimum of approximately 8.5, was activated by serum, and was inhibited by NaCl and protamine. This LPL assay measures enzyme capacity. The same substrate was used to measure an adipose tissue lipase activity that had a pH optimum below 7, was not activated by serum, and was not inhibited by NaCl or protamine. These are all characteristics of HSL. Despite the convenience, this substrate was not appropriate for porcine adipose tissue HSL because the rates were only 30 to 50% of those produced with a more complex, less convenient gum-based substrate suspension. Furthermore, incubation of enzyme or tissue slices with insulin, or agents that elevate cAMP concentration, did not modulate this lipase activity, as expected. These incubations poorly modulated LPL activity.

Key Words: Pigs, Adipose Tissue, Lipoprotein Lipase, Triacylglycerol Lipase

Introduction

Lipoprotein lipase (LPL) activity is measured in adipose tissue as an indicator of the anabolic pathway providing fatty acids to the adipocyte directly from the plasma lipoproteins and indirectly from the diet. Fatty acids enter the adipocyte to be oxidized or esterified into phospholipids or triacylglycerol. Lipoprotein lipase activity is also used in cell culture systems as an early-appearing marker for adipocyte differentiation. Hormone-sensitive lipase (HSL) is the enzyme that initiates the catabolism of triacylglycerol in the adipocyte. Although HSL usually is present in an inactive form, activation is the terminal event in the cascade mechanism beginning with formation of a hormone-receptor complex (e.g., epinephrine and the β-adrenergic receptor) at the cell surface and ending with the phosphorylation of HSL.

Assays for LPL and other lipases, including HSL, are complex because they involve the presentation of a hydrophobic substrate (triacylglycerol) to an enzyme in a hydrophilic medium. Traditionally, the triacylglycerol is suspended with a gum or emulsified with a detergent. These substrate preparations are unstable and must be prepared immediately before use, typically employing ultrasonic dispersion. All factors combine to make these types of substrate preparation cumbersome and inconvenient. A simplified, relatively stable suspension of the triacylglycerol substrate has been used for LPL assay (Nilsson-Ehle and Schotz, 1976). I have optimized porcine adipose tissue LPL

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and HSL assays using this substrate and compared rates with those obtained using a gum arabic suspension of the substrate.

Methods

Standard LPL Enzyme Preparation. Crossbred pigs were obtained from several commercial sources. Female and castrated male pigs were used. They were fed a corn-soybean meal-based diet with approximately 16% calculated crude protein and <4% calculated fat. Pigs weighed 12 to 25 kg when killed by captive bolt gun coupled with exsanguination. The variable period of time pigs were fed the high-carbohydrate, low-fat diet was not expected to affect the results because 1) all pigs were fed the diet a minimum of 2 wk, 2) most studies did not compare lipase activity in different pigs but used a pig as a source of tissue for laboratory experiments to study variables affecting enzyme activity, and 3) when pigs were compared, they were fed the diet for a similar period of time. The animal protocol was approved by the Baylor College of Medicine Animal Care and Use Committee. Adipose tissue (usually subcutaneous from the dorsal neck region) was removed, placed in 35°C transport medium consisting of .9% NaCl, 5.6 mM glucose, 25 mM HEPES, with pH adjusted to 7.4 at room temperature. For the LPL fat depot study, adipose tissue represented the upper and middle layers of subcutaneous fat from the dorsal neck region, the perirenal depot, the fat in the mesentery of the greater omentum, and the subcutaneous fat from the lateral region of the thigh (i.e., ham).

A pool of tissue slices (.4 mm thick) was prepared and collected in .9% NaCl at 37°C. Slices were homogenized in ice-cold 25 M sucrose, 50 mM Tris buffer, and 80 U heparin/mL (Sigma Chemical, St. Louis, MO; H431) with pH adjusted to 8.5 at 21°C. Tissue slices (1 g) were homogenized with 4 mL of homogenization medium using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) with three 20-s bursts at a rheostat setting of 6. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The fat cake was removed and the infranate was decanted and filtered through a single layer of cheesecloth. One milliliter of the infranate was diluted with 4 mL of homogenization medium and kept on ice until assay; this is referred to as working enzyme. The working enzyme preparation from 1 g of tissue was diluted to 25 mL. This enzyme preparation was essentially the same as that of Steffen et al. (1978).

Adipocyte size and number were determined on osmium-fixed tissue slices using an electronic particle size/number analyzer according to Mersmann and MacNeil (1986).

Steffen LPL Assay. The assay (Steffen et al., 1978) was a modification of that described by Bensadoun et al. (1974) and contained 100 mM NaCl, 10 mM CaCl₂, 20 mg fatty acid poor bovine serum albumin (Intergen, Purchase, NY; CRG-7 Bovuminar: catalog #3160-00), .1 mL pig serum (a pooled sample from three pigs that was previously heated to 62°C for 10 min), 2.5 mg gum arabic, 13.56 mM triolein (Sigma Chemical; T-7140), .3 μCi tritiated triolein with [9,10-³H(N)]-oleic acid moieties (NEN Life Sciences Products, Boston, MA; NET-431), and 200 mM Tris with pH at 8.5 in a total volume of 1.0 mL. The gum arabic-triolein suspension was prepared daily by sonication. The reaction contained .1 to .2 mL enzyme preparation, and the incubation was for 30 min at 30°C. Extraction of the released fatty acid products was the same as indicated below for the standard LPL assay; the original Steffen assay used an ion-exchange procedure to separate substrate from products.

Standard LPL Assay. The concentrated substrate was prepared by mixing 600 mg triolein with 36 mg phosphatidylycerine from egg yolk (Sigma Chemical; P-2772) and 200 μCi of tritiated triolein in chloroform. The solvent was evaporated in a stream of nitrogen. The dried lipids were emulsified in 10 mL of glycerol (12.5 g) by homogenization using a Polytron homogenizer with a PT-10 generator, at a rheostat setting of 6. The tube was covered with Parafilm to prevent aerosol dispersion during homogenization. The tube was immersed in an ice bath during the homogenization to prevent excessive heating. This optically clear, concentrated substrate could be stored at room temperature for at least 6 wk. After extended storage, the components settled but could be resuspended by rehomogenization. The substrate was that of Nilsson-Ehle and Schotz (1976).

The working substrate for assay was prepared each day by diluting one volume of concentrated substrate (using a positive displacement pipette to dispense this viscous solution) with four volumes of .2 M Tris-HCl buffer (with pH adjusted to 9.5 at room temperature) containing 3% wt/vol fatty acid poor bovine serum albumin and one volume of porcine serum. The substrate was mixed for 5 s with a vortex mixer. This working substrate was stable for at least 6 h.

The enzyme was assayed by incubating .1 mL of working assay substrate with .1 mL of working enzyme at 37°C for 30 min. After combining the working enzyme with the working substrate, the pH of the standard LPL assay was 8.5 at 37°C. The reaction was stopped by addition of 3.25 mL of methanol: chloroform:heptane (1:41:1.25:1, vol/vol/vol). After mixing, 1.03 mL of .1 M potassium carbonate/borate buffer at pH 10.5 was added and mixed. The extraction was at room temperature. The extract was vigorously mixed for 15 s, then centrifuged for 15 min at 3,000 × g. The extraction procedure was that of Belfrage and Vaughan (1969). A 1-mL portion of the methanol/water upper phase, containing the fatty acids released by LPL, was counted in a liquid scintillation counter using 10 mL of scintillation.
solution (Research Products International Corporation, Mount Prospect, NY; Budget-Solve).

The recovery of the fatty acid in the extraction medium was determined using \[9,10^{-2}H(N)\]oleic acid (NEN Life Sciences Products; NET-289) in a reaction mixture containing all components except tritiated triolein. The tubes received the extraction solvent before the enzyme and represent the equivalent of zero-time incubations. When carried through the extraction procedure, 31.5% of the fatty acid was recovered (mean for 14 tubes). For each assay, the recovered fatty acid released by LPL was corrected for percentage recovery.

The LPL activity (1 unit = nmol·30 min\(^{-1}\)-mg protein\(^{-1}\)) represents fatty acid released from the triolein substrate.

The glycerol-phospholipid-triolein substrate was used to evaluate porcine adipose tissue LPL in various centrifugal fractions, its stability to cold and freezing, and the effect of various assay conditions including pH, serum albumin, porcine serum, substrate concentration, NaCl and protamine inhibition, and heparin release of the enzyme.

**Standard HSL Enzyme Preparation.** Tissue was removed from the animal to transport medium and slices were prepared as indicated for LPL. Slices were homogenized using 1 g of slices plus 4 mL of .25 M sucrose, 50 mM HEPES, and 10 mM NaHPO\(_4\), with pH adjusted to 6.8 at 21°C. Homogenization was on ice using a Polytron homogenizer, as indicated for LPL. The homogenate was centrifuged at 1,200 \(\times\) g for 30 min at 4°C. The infranate was filtered through cheesecloth and retained on ice until assay. The working enzyme from 1 g of tissue was diluted to 5 mL. This enzyme preparation is essentially the same as that of Steffen et al. (1978), except 1 mM EDTA was not present in the standard HSL enzyme preparation.

**Steffen HSL Assay.** This assay (Steffen et al., 1978) was a modification of Khoo and Steinberg (1974). It contained .2 M HEPES (pH 6.8), 10 mg fatty acid poor bovine serum albumin, 2.5 mg gum arabic, 22.6 mM triolein, and .5 \(\mu\)Ci tritiated triolein in a total volume of 1.0 mL. The gum arabic-triolein suspension was prepared daily by sonication. The reaction contained 1 to .2 mL enzyme. The incubation was for 60 min at 30°C. Extraction of released fatty acids was as indicated above for the standard LPL assay. The original Steffen method used an ion-exchange procedure to separate substrate from products.

**Standard HSL Assay.** The assay used the same concentrated substrate preparation as indicated for LPL. The working assay substrate was prepared by diluting one volume of concentrated substrate (triolein suspension) plus five volumes of .2 M HEPES, 3% (wt/vol) fatty acid poor bovine serum albumin with pH adjusted to 6.8 at 21°C. The assay used .1 mL of working substrate plus .1 mL of working enzyme. The standard assay duration was 30 min at 37°C. The released fatty acids were extracted from the triolein substrate as indicated above for the standard LPL assay. The HSL activity (1 unit = nmol·30 min\(^{-1}\)-mg protein\(^{-1}\)) represents fatty acid released from the triolein substrate.

**Slice Preincubation.** Adipose tissue slices were preincubated at 37°C in Krebs-Ringer bicarbonate buffer containing 5.6 mM glucose, .56 mM ascorbate, and 4% fatty acid poor bovine serum albumin in the presence of 5% CO\(_2\) in oxygen (Mersmann and Hu, 1987). Slices were preincubated with no additions, with \(10^{-5}\) M isoproterenol, or \(10^{-3}\) M theophylline, or isoproterenol plus theophylline, or 1,000 \(\mu\)units insulin/mL. After preincubation, slices were removed and rinsed in room-temperature .9% NaCl. An aliquot of the washed slices was then homogenized, centrifuged, and assayed for LPL or HSL activity using the standard procedures. The medium was also assayed for LPL activity.

**Results and Discussion**

**Lipoprotein Lipase**

Lipoprotein lipase cleaves fatty acids from the 1 and 3 position of the triacylglycerol molecule (Nilsson-Ehle et al., 1974). The enzyme is produced by adipose tissue as well as several other tissues, including heart and skeletal muscle. It is transferred to the surface of the capillary endothelium, where it cleaves the triacylglycerol of lipoproteins. The function of LPL in many tissues and its role in several disease states have been reviewed (Borensztajn, 1987). The LPL activity has been investigated a number of times in porcine adipose tissue (reviewed in Mersmann, 1986a) or in differentiating porcine adipocytes (e.g., Hausman et al., 1984).

In order to assay the cleavage of fatty acids from a relatively hydrophobic substrate (triacylglycerol) by an enzyme in a hydrophilic medium, the substrate must be emulsified, or at least suspended. Usually the substrate is emulsified or suspended with detergents and sometimes by the addition of various gums (e.g., gum arabic). The assay medium also must contain a sink to trap the fatty acids released by enzymatic cleavage; fatty acids are toxic because of their detergent activities. The usual sink is serum albumin, which specific binding sites for fatty acids. The complexity of the assay medium is such that the kinetics of the enzyme activity are totally different depending on the type of medium selected (Riley and Robinson, 1974; Nilsson-Ehle, 1987). The relatively stable suspension of triacylglycerol in phosphatidylcholine plus glycerol (Nilsson-Ehle and Schotz, 1976) provides a substrate for LPL that is convenient to make and use. It has not been examined as a substrate for porcine adipose tissue LPL.
**Enzyme.** Homogenates were prepared from a pool of tissue slices using 1 g of tissue plus 4 mL of homogenization medium. The homogenates were individually centrifuged to obtain a 600 × g, 10-min infranate, a 10,000 × g, 30-min infranate, or a 40,000 × g, 60-min infranate. The homogenate or 1 mL of each fraction was individually diluted with 4 mL of medium and then .1 mL was assayed using the standard LPL assay. The homogenate (100% activity) contained 27.9 nmol·30 min⁻¹·1 mL⁻¹·SD = 2.3 for two enzyme preparations, each from a different pig. The mean percentage (SD) for the 600 × g infranate = 95 (17), for the 10,000 × g infranate = 100 (10), and for the 40,000 × g infranate = 68 (only one animal). Although the volume of each infranate was not measured, precluding calculation of total recovery, each infranate represented the same amount of a tissue slice pool, homogenized in a constant volume. There was no major loss of enzyme activity except in the 40,000 × g infranate.

The enzyme specific activity was enriched in the 10,000 × g, 30-min infranate (155%) and in the 600 × g, 10-min infranate (142%) compared to the total tissue homogenate (100% = 419 units; SD = 127 for two enzyme preparations, each from a different pig). The specific activity was not enriched in the 40,000 × g, 60-min infranate (103%). The 10,000 × g, 30-min infranate contained the highest specific activity and essentially all the activity; it was found appropriate for use in the standard LPL assay.

The assay of LPL was linear with time and enzyme concentration (Figure 1, LPL). The 30-min duration of the standard LPL assay was found appropriate for continued use, with .1 mL of the working enzyme preparation (the enzyme concentration indicated as 1× in Figure 1, LPL).

**Cold and Freezing.** Many enzyme assays place tissue on ice immediately after removal from the animal to slow degradative processes. However, adipose tissue often is kept at room temperature or at elevated temperatures to keep fatty acids from solidifying at lower temperatures.

To test the effect of cold and freezing on the enzyme activity, tissue was removed from the pig and placed in either ice-cold or 35°C transport medium. Tissue slices, homogenates, and the working enzyme were prepared using standard procedures. A portion of the LPL working enzyme prepared from warm or cold tissue was frozen in liquid nitrogen and stored at −70°C until assay. After 1 to 3 wk of frozen storage, the enzyme was rapidly thawed at 37°C and placed on ice for assay. A portion of the fresh tissue also was frozen in liquid nitrogen immediately after removal from the pig and was stored at −70°C. It was removed from the freezer, placed in liquid nitrogen, pulverized by impact, and homogenized from the frozen state. The working enzyme was prepared as for the fresh tissue and kept cold until assay. There was no difference among the five types of preparations when analyzed using a repeated measures ANOVA (there were four sets of tissue, each from a different animal; P = .16). The units of LPL were as follows: warm tissue-immediate assay = 588; cold tissue-immediate assay = 652; warm tissue-frozen enzyme = 505; cold tissue-frozen enzyme = 684; and immediately frozen...
tissue = 506. The pooled SD = 117. The data suggest that the tissue may be handled in any of these ways.

In addition, the LPL activities in five sets of warm and cold tissue (each set from a different animal) were compared. There was no difference (paired t-test) in the mean (SD) units of LPL: warm tissue = 172 (120) and cold tissue = 180 (121). Frozen tissue retained only 48% (SD = 13%) of the specific activity of the fresh tissue (two preparations, each from a different pig). Warm tissue-immediate assay was compared with warm tissue-frozen enzyme (seven enzyme preparations, each from a different animal). The mean LPL units (SD) were 589 (390) for warm tissue-immediate assay. The warm tissue-frozen enzyme retained 88% (SD = 28%; P = .41 by paired t-test) of the activity of the fresh preparations. Although the enzyme activity seems relatively stable, three of the seven frozen preparations had <65% of the activity of the fresh preparation.

For the standard LPL assay, I prefer the warm tissue-immediate assay because this has been a routine approach for other porcine adipose tissue enzymes in this laboratory and none of the alternative methods was clearly better. Rule et al. (1996) demonstrated a major reduction in adipose tissue LPL activity in frozen compared to fresh tissue, as well as loss of the effects of animal diet composition on enzyme activity.

Protease Inhibitors. The addition of protease inhibitors may preserve the integrity of various proteins after disruption of cells and tissues. Some protease inhibitors in the homogenization medium had no significant effects on LPL activity (repeated measures ANOVA). Leupeptin at 1 mM yielded 115% of control activity, pepstatin at 1 mM yielded 101% of control activity, and the combination of leupeptin and pepstatin yielded 118% of control activity. The addition of 7.5 mM EGTA, .2 mM PMSF, or both to the homogenization medium reduced the specific activity to <40% of the enzyme activity compared to no additions to the homogenizing medium. I recommend using no protease inhibitors in the homogenization medium for the standard porcine adipose tissue LPL enzyme preparation.

Assay Conditions. To ensure the assay of LPL and not other lipases or esterases, the pH optimum must be alkaline. The LPL activity was measured at a pH of 7.5, 8.0, 8.5, and 9.0 (final pH of the complete reaction medium, including the enzyme). The mean (SD) specific activities for two enzyme preparations, each from a different animal and expressed as a percentage, were 71 (10)% at pH 7.5, 66 (9)% at pH 8.0, 95 (8)% at pH 8.5, and 95 (7)% at pH 9.0. The highest LPL activity for a given enzyme preparation was indicated as 100%. The mean LPL units = 1,411. The activity of LPL was greatest at pH 8.5 and 9.0, as expected for porcine LPL (Bensadoun et al., 1974). The data suggest the standard LPL assay should continue to use a pH of 8.5.

Almost all assays for LPL contain bovine serum albumin to bind the fatty acids released by the enzyme (Nilsson-Ehle, 1987). The 3% concentration of albumin in the standard LPL assay produced the same activity as 1 or 5% albumin (data not indicated). I recommend the standard LPL assay use 3% albumin.

Serum is added to LPL assays as a source of apolipoprotein C-II, a required cofactor that is a constituent of the natural substrates, chylomicrons and very-low-density lipoproteins (reviewed in Quinn et al., 1982). The serum is heated to inactivate lipase and esterase enzymes that would be detectable with the triolein substrate and interfere with the LPL assay. With no serum present in the assay, the porcine adipose tissue LPL activity was only 10% of that with serum. When the amount of serum was lowered to 25 or 50% of the amount in the standard assay, the LPL activity was reduced to < 50%. There was approximately 20% variation in the activity measured with sera from different pigs (data not indicated); consequently, I recommend a single pooled sample of serum be used for all experiments.

The substrate concentration for the standard LPL assay was 5.65 mM triolein. This concentration saturated the enzyme with a specific activity of 666 units. This triolein concentration also saturated the rat adipose tissue LPL using the same substrate presentation (Nilsson-Ehle and Schotz, 1976). There was 94% of the activity at 2.83 mM triolein, 86% of the activity at 1.43 mM triolein, and 25% of the activity at .57 mM triolein.

The addition of NaCl to the assay inhibited the LPL activity, as expected. With .5 M NaCl, there was 54% of the activity without NaCl, whereas with 1 or 2 M NaCl, there was approximately 25% of the activity without NaCl. The control with no NaCl had a mean (SD) specific activity = 452 (303) units for two enzyme preparations, each from a different animal. The mean (SD) LPL activity in the presence of 1 mg protamine/mL was 71 (23)% of the control. The control with no protamine had a mean (SD) specific activity of 543 (637) units. The inhibition of enzyme activity by NaCl and by protamine has been demonstrated for porcine LPL (Enser, 1973; Bensadoun et al., 1974; Steffen et al., 1978) and suggests that the activity assayed under the standard LPL assay conditions is primarily LPL and not other lipases or esterases.
The current assay evaluated LPL activity in porcine adipose tissue. Mammalian heparin is more convenient and recommended for the standard LPL assay.

LPL in Different Adipose Tissue Depots (Table 2). As adipocytes grow, the amount of LPL activity per cell is expected to increase (Hietanen and Greenwood, 1977). In pigs, the LPL activity increases with age or weight (Lee and Kauffman, 1974; Steffen et al., 1978). Because different adipose tissue depots develop at different rates in pigs (Anderson and Kauffman, 1973), at a given age in young pigs, adipocytes in individual depots are expected to be at different stages of development both with regard to cell size and to LPL activity. Four pigs with mean weight of 33.6 kg (SD = 4.0) were used to measure adipose tissue depot variation in LPL activity. The upper and middle subcutaneous depot from the neck region, the perirenal depot, the mesenteric depot, and the subcutaneous depot from the ham were sampled. Standard LPL working enzyme was prepared from each depot and assayed using the standard LPL assay. The greatest LPL activities in these young pigs were in the subcutaneous depots. The cell size between depots was greatest in the upper and middle subcutaneous depots. The cell size between depots was greatest in the upper and middle subcutaneous depots.

Table 1. Effect of preincubation of tissue with heparin on lipoprotein lipase (LPL) activity released to the medium

<table>
<thead>
<tr>
<th>Heparin, µg/mL</th>
<th>Activitya</th>
<th>%</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>13</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>68</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>88</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>101</td>
<td>—</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>100</td>
<td>—</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

n = 3. The 450 µg heparin/mL homogenization medium to release LPL from the tissue (Steffen et al., 1978) or 80 U heparin/mL in the preincubation medium results in 40 U heparin/mL in the standard LPL assay. The LPL activity with 0, 40, 60, or 80 U heparin/mL homogenization medium was 35, 89, 101, and 100%, respectively. The 100% activity = 786 units. Thus, the 80 U heparin/mL in the homogenization medium (452 µg/mL for the lot of heparin used) is recommended for the standard LPL assay.

As adipocytes grow, the amount of LPL activity per cell is expected to increase (Hietanen and Greenwood, 1977). In pigs, the LPL activity increases with age or weight (Lee and Kauffman, 1974; Steffen et al., 1978). Because different adipose tissue depots develop at different rates in pigs (Anderson and Kauffman, 1973), at a given age in young pigs, adipocytes in individual depots are expected to be at different stages of development both with regard to cell size and to LPL activity. Four pigs with mean weight of 33.6 kg (SD = 4.0) were used to measure adipose tissue depot variation in LPL activity. The upper and middle subcutaneous depot from the neck region, the perirenal depot, the mesenteric depot, and the subcutaneous depot from the ham were sampled. Standard LPL working enzyme was prepared from each depot and assayed using the standard LPL assay. The greatest LPL activities in these young pigs were in the subcutaneous depots. The cell size between depots was greatest in the upper and middle subcutaneous depots.

Table 2. Adipose tissue depot lipoprotein lipase (LPL) activity

<table>
<thead>
<tr>
<th>Depot</th>
<th>LPLprotein</th>
<th>Cell diameter</th>
<th>Cell volume</th>
<th>LPLcell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous-upper</td>
<td>905e</td>
<td>57.3</td>
<td>99,764</td>
<td>699y</td>
</tr>
<tr>
<td>Subcutaneous-middle</td>
<td>784y</td>
<td>59.2</td>
<td>111,319</td>
<td>520y</td>
</tr>
<tr>
<td>Subcutaneous-ham</td>
<td>974e</td>
<td>56.2</td>
<td>93,353</td>
<td>937x</td>
</tr>
<tr>
<td>Perirenal</td>
<td>439Y</td>
<td>57.0</td>
<td>98,028</td>
<td>422Y</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>521y</td>
<td>52.8</td>
<td>78,623</td>
<td>466Y</td>
</tr>
<tr>
<td>SD</td>
<td>327</td>
<td>4.7</td>
<td>18,655</td>
<td>220</td>
</tr>
<tr>
<td>P</td>
<td>&lt;1</td>
<td>.19</td>
<td>.24</td>
<td>.04</td>
</tr>
</tbody>
</table>

*LP_{protein} = nmol fatty acid released·30 min^{-1}·mg protein^{-1}.

*Cell diameter is the average diameter (µm) of particles beginning at 20 µm.

*Cell volume was calculated from average diameter as \( \frac{1}{6} \pi d^3 \) and expressed as µm^3.

*LPL_{cell} = LPL per gram of tissue = cell number per gram of tissue. The LPL per gram of tissue was extrapolated from the homogenization of 1 g of tissue slices + 4 mL of medium, centrifugation, dilution of 1 mL of the infranate + 4 mL medium, and assay of 0.1 mL of enzyme in a total volume of 0.2 mL. The average cell number per gram of tissue = 10^{12} µm^3 ÷ average cell volume, where 10^{12} = volume of a cube, 1 cm on a side or approximately 1 g of tissue. Units for LPL are nanomoles of fatty acid released·30 min^{-1}·10^6 cells^{-1}.

*Values in a column with different superscripts are significantly different using Tukey's procedure for mean separation (P < .05) after a significant F-value (P < .05) for the repeated measures ANOVA.
not significantly different. Extrapolation of the data to a per-cell basis did not change the rank order of the LPL activities.

Effect of Insulin and Isoproterenol on Enzyme Activity. It is generally recognized that insulin and the \( \beta \)-adrenergic receptor agonists are primary regulators of adipose tissue LPL (Cryer, 1981, 1987; Eckel, 1987). Increased insulin concentrations tend to cause an increase in adipose tissue LPL activity. Increased \( \beta \)-adrenergic agonist concentrations or other agents that cause an increase in tissue cAMP concentration (e.g., theophylline) tend to cause a decrease in adipose tissue LPL activity. The demonstration of hormonal regulation of LPL in vitro may be difficult because of the complexities of the system, requiring precise timing and exact conditions.

Addition of \( 10^{-5} \) M isoproterenol, \( 10^{-3} \) M theophylline, isoproterenol plus theophylline, or \( 1,000 \mu U \) insulin/mL to the standard LPL assay had no effect (repeated measures ANOVA, \( P = .54 \)) on the enzyme activity. These attempts to regulate LPL activity in the standard enzyme preparation may not have been successful because many of the components of the insulin and \( \beta \)-adrenergic receptor cascade systems would be expected to be in centrifugal fractions other than the one used for LPL activity measurement. Furthermore, the physical disruption of the receptor cascade systems by homogenization, centrifugation, and dilution probably impedes their function.

Preincubation of slices for 10 or 60 min in the presence of \( 10^{-5} \) M isoproterenol caused slice LPL to be increased 13% (\( P < .05 \)), the opposite of the expected result. Preincubation with \( 1,000 \mu U \) insulin/mL with isoproterenol \( + 10^{-3} \) M theophylline for 10 min had no effect. Preincubation for 60 min with isoproterenol or insulin caused slice LPL to be increased 13%, whereas preincubation with isoproterenol + theophylline had no effect. The medium LPL activity was increased with isoproterenol (56%), isoproterenol + theophylline (41%), or insulin (45%), after 60 min of preincubation The absolute activity in the medium was only about 8% of that in the slices (medium basal activity = .93 and slices basal activity = 11.45 nmol·30 min\(^{-1} \)·.004 g tissue\(^{-1} \)). Incubation of adipose tissue (Cryer, 1987; Eckel, 1987), including that from pigs (Steffen et al., 1978) with various hormones, produced only modest changes in LPL activity.

Hormone-Sensitive Lipase

The activation of HSL is controlled by a cAMP cascade system that begins with the binding of a hormone to its specific cell surface receptor. In the case of the \( \beta \)-adrenergic receptor, binding of a \( \beta \)-adrenergic receptor agonist activates the receptor. The activated hormone-receptor complex interacts with a Gs protein to release the \( \alpha \)-subunit that activates adenylyl cyclase. Activated cyclase catalyzes the synthesis of cAMP from ATP. The regulatory subunit of protein kinase A binds cAMP with subsequent release and activation of the catalytic subunit. One of the intracellular proteins phosphorylated by the activated kinase is HSL. Phosphorylated adipocyte HSL is the active form of this enzyme that begins the sequential removal of fatty acids from triacylglycerol molecules (i.e., lipolysis). The 1,3 ester bonds of triacylglycerol are the targeted cleavage sites, although the specificity is not as great as that of LPL (Fredrikson and Belfrage, 1983).

Discussion of porcine HSL activity is limited because the Steffen et al. (1978) assay was superior to the standard HSL assay. Furthermore, there was no stimulation of HSL activity by the \( \beta \)-adrenergic agonist isoproterenol. Thus, the lipase assayed may not be HSL.

Enzyme. The addition of 1 mM EDTA as a heavy metal chelator to the standard HSL homogenization medium did not alter the HSL activity. The enzyme activity was not enriched in the 600 × g, 10-min infranate, the 1,200 × g, 30-min infranate, the 9,000 × g, 15-min infranate, or the 40,000 × g, 60-min infranate compared to the homogenate (data not indicated). The standard HSL assay, using the 1,200 × g, 30-min infranate, was linear with time and enzyme concentration (1× Enz in Figure 1, HSL).

Cold and Freezing. Experiments similar to those reported for LPL using warm and cold tissue, fresh and frozen enzyme, and frozen tissue indicated no significant effect (\( P = .16 \)) on HSL activity. As for LPL, I recommend warm tissue-immediate assay of HSL.

Assay Conditions. Hormone-sensitive lipase should have a pH optimum between 6 and 7 (Khooh and Steinberg, 1974). The porcine adipose tissue HSL assayed at pH 6.0, 6.5, and 7.0 using ACES buffer in place of HEPES was not different (\( P = .98 \)). Enzyme from the same animals was assayed at pH 6.5, 7.0, 7.5, 8.0, and 8.5 using HEPES buffer. The enzyme activities were similar at each pH (\( P = .16 \)). The nonsignificant increases at pH 8.0 (16%) and 8.5 (26%) suggest a contribution from LPL with its pH optimum at approximately pH 8.5. I recommend the standard HSL assay be at a pH of 6.8.

The standard HSL assay uses 3% bovine serum albumin to sequester fatty acids released from triacylglycerol. Enzyme activity at 0% albumin was 38% of that at 3% albumin; there was no difference in enzyme activities from 1 to 5% albumin.

Serum is a mandatory addition to the LPL assay to provide cofactor. Addition of serum to the standard HSL assay did not increase the activity. The specific activity was 51.9 units without serum and 36.7 units with serum (\( P = .37 \)). The numerical decrease in lipase activity in the presence of serum suggests that some of the activity assayed may be that of a unique porcine adipose tissue lipase with properties somewhat distinct from either LPL or HSL (Matsumura et al., 1976).
The standard HSL assay used 5.65 mM triolein as substrate. This concentration saturated the enzyme.

One of the major distinctions between LPL and HSL enzyme activity assays is that LPL activity is inhibited by NaCl and by protamine, whereas HSL is not inhibited by either compound (Matsumura et al., 1976). Sodium chloride added to the porcine adipose tissue HSL assay at .5, 1.0, and 2.0 M did not inhibit the activity (P = .17).

Previously (Steffen et al., 1978), this laboratory assayed HSL using a homogenization procedure and centrifugal fraction similar to the standard HSL enzyme preparation, but with a gum arabic-triolein substrate. This assay was directly compared to the current standard HSL assay by using a common standard HSL enzyme. For three enzyme preparations, each from a different animal, the current standard HSL assay yielded 513.3 units and the previous Steffen HSL assay yielded 157.9 units. The standard deviations were significantly different in the two groups, so the data were log-transformed. A paired t-test analysis of the log-transformed data indicated the Steffen assay tended (P = .08) to yield greater activity than the current standard HSL assay. For measurement of optimal activity, the Steffen assay is recommended. Unpublished observations by Steffen indicated HSL has distinct kinetic characteristics when the triolein concentration is varied in constant proportion to the gum arabic and serum albumin concentrations compared to variation of triolein with constant gum arabic and albumin concentrations. The LPL activity also has distinct kinetic characteristics with each type of substrate presentation; however, they were opposite those for HSL. Thus, it is not surprising that the use of the triolein-lecithin-glycerol suspension used in the current standard LPL and HSL assays was not an optimal substrate for both LPL and HSL.

Heparin. Another distinction between LPL and HSL is the release of LPL activity by heparin. Even when assayed at low pH, 80 U heparin/mL of homogenization medium caused a numerical increase in lipase activity (control = 58 units and heparin = 84 units).

Preincubation of porcine adipose tissue slices with 1 to 450 μg heparin/mL for 45 min at 37°C had no effect (P = .67) on tissue HSL specific activity assayed in a standard enzyme preparation (data not presented). The medium lipase specific activity (assayed with the standard HSL conditions) numerically doubled after preincubation with heparin. Although this increase was not significant (P = .32) by repeated measures ANOVA, it suggested that heparin caused release of LPL that was partially measured in the HSL assay. The numerical increases in activity measured with the standard HSL assay when heparin was added to the homogenization medium or was present during preincubation of tissue slices preclude the presence of heparin at any stage of accurate measurement of HSL activity.

Effects of Insulin and Isoproterenol. Exposure of mammals to an elevated insulin concentration lowers the lipolytic rate, whereas exposure to an elevated β-adrenergic agonist concentration increases the lipolytic rate. These effects are usually demonstrable when tissues or isolated adipocytes are incubated in vitro with insulin or β-adrenergic agonists. The inhibition of lipolysis by insulin and the stimulation of lipolysis by β-adrenergic agonists have been demonstrated in pigs in vivo and in porcine adipose tissue in vitro (Mersmann, 1986b, 1987).

It is expected that the modulation of lipolysis by insulin and β-adrenergic agonists would be reflected in changes in HSL activity. When isolated HSL is incubated with protein kinase A in vitro, HSL is phosphorylated and activated (Corbin et al., 1970; Huttunen and Steinberg, 1971). There was no effect of isoproterenol, theophylline, isoproterenol plus theophylline, or insulin when present in the standard HSL incubation (data not presented), as observed by Steffen et al. (1978). There was no change in HSL activity measured in homogenates of tissue slices preincubated with the same hormones (data not presented; P = .32). Incubation of rat adipose tissue with insulin in vitro caused a decrease in HSL activity (Khoo et al., 1973). However, porcine adipocytes are relatively insensitive to exogenous insulin in vitro (Mersmann, 1989). Incubation of rat adipose tissue with epinephrine or norepinephrine in vitro (Vaughan et al., 1964) or rat adipocytes with epinephrine in vitro (Crum et al., 1971) caused an increase in HSL activity. The lack of modulation of porcine HSL by these hormones was unexpected. Preincubation conditions were optimal to demonstrate the effects of insulin and β-adrenergic agonists on porcine lipolysis in vitro (Mersmann, 1987; Mersmann and Hu, 1987).

In conclusion, the simplified, stable medium with triolein suspended in glycerol/phospholipid was an excellent substrate for measurement of porcine adipose tissue LPL. This substrate was not optimal for porcine adipose tissue HSL. Porcine adipose tissue standard enzyme preparations for LPL and HSL assay were not modulated by addition of insulin or agents to increase cAMP concentration. Preincubation of tissue slices with insulin and agents to increase cAMP yielded only slight modulation of LPL activity and did not alter HSL activity.

Implications

Adipocyte lipoprotein lipase (LPL) releases fatty acids from plasma lipids. The fatty acids are imported into the adipocyte for oxidation or storage. Hormone-sensitive lipase (HSL) cleaves fatty acids from intracellular triacylglycerol for oxidation and export. Lipase measurement is complex because the substrate is hydrophobic and the enzyme plus medium are
hydrophilic. A convenient, simplified substrate was used to measure porcine adipose tissue LPL and HSL. It was excellent for LPL but inappropriate for HSL. Although both enzymes are modulated by insulin and agents that increase cyclic adenosine monophosphate in vivo, these effects could not be demonstrated in vitro for porcine adipose tissue HSL and were marginal for LPL.

**Literature Cited**


