Activation of Lipoprotein Lipase

EVALUATION OF CALCIUM, MAGNESIUM, AND AMMONIUM AS COFACTORS

By Thomas F. Whayne, Jr., and James M. Felts

ABSTRACT

Lipoprotein lipase (LPL) from rat heart acetone powders has been reported to depend on the presence of NH₄⁺, calcium, or other divalent cations for optimal activity. In addition, the enzyme will not hydrolyze an artificial triglyceride emulsion unless it is converted to an active substrate by the addition of very low density lipoproteins, high density lipoproteins (HDL), or certain peptides contained in these complexes. We observed earlier that HDL that had been extensively dialyzed against 0.005M ethylenediaminetetraacetic acid (EDTA), which probably removed most of the divalent cations, produced little lipolytic activity in LPL from rat heart acetone powders. This observation led us to evaluate the cation requirements for LPL. LPL was prepared in soluble form from rat heart acetone powders. HDL was isolated from rat serum by ultracentrifugation. The assay system contained a phospholipid-stabilized triglyceride emulsion as substrate. The addition of the whole rat serum to the LPL assay system produced high LPL activity. The addition of dialyzed HDL isolated from the same volume of serum produced low LPL activity. The addition of calcium in physiological concentration to the assay containing HDL increased LPL activity markedly. The high enzyme activity produced by both serum and HDL plus calcium was inhibited by ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), a specific calcium chelating agent. The addition of NH₄⁺ or magnesium at several concentrations did not replace calcium in stimulating LPL activity. We conclude that calcium has a cofactor function in the LPL lipolytic reaction and that the optimal enzyme reaction rate is attained in the presence of calcium concentrations found in serum.

KEY WORDS postheparin lipolytic activity enzyme activation heparin cofactor triglycerides lipoproteins heart rat lipolysis albumin

Lipoprotein lipase (LPL) will hydrolyze an artificial triglyceride emulsion only if the emulsion is converted to an active lipoprotein substrate by the addition of serum, lipoprotein fractions, or specific serum peptides from lipoproteins (1-5). In addition, heparin has a marked influence on the rate of hydrolysis of the activated substrate. We have recently reported a study of the activation factors present in rat serum and the specific role of heparin in modulating lipolysis (5). Cofactors other than serum lipoproteins and heparin may also influence the reaction. In 1955, Korn and Quigley suggested that optimum enzyme activity was also dependent on the presence of NH₄⁺, calcium, or other divalent cations. Using LPL from rat heart acetone powders and an activated coconut oil emulsion as substrate, they observed no enzyme activity when NH₄⁺ or cations were present in low concentrations.
concentrations. LPL activity was optimal when calcium was present at a concentration of 10 μmoles/ml or when NH₄⁺ was present at a concentration of 50 μmoles/ml. Their results also suggested an interaction between calcium and heparin in stimulating LPL activity (6).

We recently observed that a triglyceride emulsion which had been activated with purified high density lipoproteins (HDL) from rat serum was slowly hydrolyzed by purified high density lipoproteins (HDL) which probably removed most of the divalent cations. Thus it appeared that the heart acetone powder system was deficient in an activation factor other than serum lipoproteins. These observations led us to reevaluate the cation dependence of LPL activity from rat heart acetone powders.

**Methods**

Acetone-ether powders were prepared from rat hearts. Long Evans rats weighing 325 to 375 g were fasted overnight and anesthetized with ether. Two rat hearts were removed and placed in an ice-cold 250-ml homogenizer. Ice-cold acetone (150 ml) was added and the hearts were homogenized for 30 to 60 seconds in a Waring Blender. The finely homogenized suspension was filtered on Whatman no. 1 filter paper in a Buchner funnel. The precipitate was washed twice with 20 ml of acetone at 0°C and twice with ether at 20°C. It was air-dried for 5 minutes under suction and placed in a desiccator under vacuum for 2 hours. The acetone powders were then homogenized in a glass homogenizer in 0.025M NH₄Cl buffer, pH 8.6 at 0°C, and allowed to stand for 1 hour with frequent stirring. The acetone powders were suspended in 7.0 ml of the NH₄Cl buffer. The particulate matter was removed by centrifugation at 1,000 X g for 10 minutes. Rat postheparin serum was obtained from fasted male rats anesthetized with ether. Midline laparotomies were performed and 10 ml of blood was collected from the abdominal aorta and allowed to clot. Serum was obtained by centrifugation at 1,000 X g for 10 minutes.

Rat postheparin serum was obtained from fasted rats weighing 325 to 375 g. Heparin, 20 U/kg, was injected into a mesenteric vein of rats and heparin in stimulating LPL activity from postheparin serum was assayed. Very low density lipoproteins and low density lipoproteins were removed by flotation at a density of 1.063 for 24 hours at 0°C (9). We altered this solution to contain EDTA, crystalline bovine albumin (Sigma), pH 8.6; and one or more of the following: ethylenediaminetetraacetic acid (EDTA), heparin, CaCl₂ or NH₄Cl as stated in the tables. The final incubation volume was 7.0 ml. Released free fatty acids were extracted by the method of Dole, as modified by Trout et al. (7). The free fatty acids were then titrated by a modification of the method of Sahaman and Robinson (8). One unit of LPL activity is equivalent to 1.0 μmole of free fatty acids released/ml of serum/hour. Titrations were made on aliquots removed at 0 and 60 minutes from the assay system in which the reaction was previously shown to be linear over the 60-minute period.

Rat serum was obtained from fasted male rats anesthetized with ether. Midline laparotomies were performed and 10 ml of blood was collected from the abdominal aorta and allowed to clot. Serum was obtained by centrifugation at 1,000 X g for 10 minutes.

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ACTIVATION OF LIPOPROTEIN LIPASE

Addition to assay system | Units of LPL activity | Expt. 1 | Expt. 2
--- | --- | --- | ---
NaCl, 0.15M (0.4 ml) | 0.49 | 0.00
Serum (0.4 ml) | 9.7 | 6.8
Serum (0.4 ml) + Heparin (7 U) | 15 | 
CaCl$_2^*$ | 0.49 | 0.46
HDL | 2.1 | 1.9
HDL + CaCl$_2^*$ | 7.1 | 2.9
HDL + heparin (7 U) | 4.9 | 4.2
HDL + heparin (7 U) + CaCl$_2^*$ | 13 | 13

Assay system: 0.3 ml 5% Intralipid, 0.75 ml 1.35M Tris/1.35 ml 25% albumin, 1.0 ml of enzyme extract in 0.025M NH$_4$Cl buffer plus 0.15M NaCl or additions shown above in a final volume of 7.0 ml, pH 8.4. Each value for LPL activity represents an average of two independent assays.

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Results

**LPL Activation by Serum, Calcium, Heparin, and HDL (Table 1).**—Extracts of rat heart acetone powders were assayed for LPL activity in the assay system in which the triglyceride emulsion had not been activated by preincubation with serum or HDL and which contained no added calcium or heparin. The system contained NH$_4$Cl at a final concentration of 0.004M. With no addition to the system, i.e., the NaCl control, LPL activity was very low. The addition of whole rat serum, 0.4 ml to the assay system, caused a marked increase in LPL activity which was further increased by heparin addition. Addition of calcium alone caused little or no stimulation of LPL activity; addition of HDL which had been dialyzed against EDTA caused relatively little stimulation. The combination, however, stimulated LPL activity markedly. Heparin further increased LPL activity in experiments with HDL alone and with HDL plus calcium.

**Elevation of the Calcium Requirement by Rat Heart LPL (Table 2).**—In this study, whole rat serum was used to activate the triglyceride emulsion. It was calculated that the serum addition contained approximately 1.0 /imole of total calcium. When 2.0 /imoles of EGTA were added to the assay system, activity was reduced 59%. In the heparin-stimulated system, EGTA reduced activity 66%. When calcium was added to the assay system in an amount equivalent to the added EGTA, activity was completely restored, indicating that EGTA was inhibiting by calcium sequestration and not by direct enzyme inhibition.

**Activation of Rat Heart Acetone Powder LPL in the Presence of High NH$_4$Cl.**—Experiments were carried out to test the effects of activators of LPL (HDL, heparin, and calcium) in the presence of NH$_4$Cl. A

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**Effect of EGTA on Rat Heart Acetone Powder LPL Activated with Serum and Serum plus Heparin (Table 2).**

<table>
<thead>
<tr>
<th>Addition to assay system</th>
<th>Units of LPL activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl, 0.15M (0.4 ml)</td>
<td>0.35</td>
</tr>
<tr>
<td>Serum</td>
<td>6.9</td>
</tr>
<tr>
<td>Serum + EGTA (2 /imoles)</td>
<td>2.8</td>
</tr>
<tr>
<td>Serum + EGTA (2 /imoles) + CaCl$_2$ (2 /imoles)</td>
<td>7.3</td>
</tr>
<tr>
<td>Serum + heparin (7 U)</td>
<td>19</td>
</tr>
<tr>
<td>Serum + heparin (7 U) + EGTA (2 /imoles)</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Assay system: Same as described in Table 1. Each value for LPL activity represents an average of two independent assays. All serum was 0.4 ml of rat serum, which contains approximately 1 /imole total calcium.
This concentration of NH₄⁺ was selected since Korn and Quigley (6) reported this to be optimum for enzyme activity in their rat heart acetone powder preparation. The results of our study showed that the pattern of stimulation of LPL induced by calcium in the presence of HDL or in the presence of HDL plus heparin was not altered by high NH₄⁺. Calcium (2.5 μmoles/ml in the assay) added to the assay containing HDL (0.2 mg HDL protein/incubation) increased activity from 0.87 to 2.11 units. Calcium addition to the assay containing HDL plus heparin (7 U/incubation) increased activity from 1.91 to 11 units.

Evaluation of Effects of NH₄⁺ and Calcium on Serum-Activated Rat Heart Acetone Powder LPL.—Experiments were carried out to evaluate the interaction of NH₄⁺ and calcium in altering LPL activity. The assay system contained LPL from rat heart acetone powders and the triglyceride substrate was activated with whole rat serum (0.4 ml). When NH₄⁺ (50 μmoles/ml assay) was added to the assay system, LPL activity was decreased from 8.5 to 2.7 units. When NH₄⁺ (50 μmoles/ml assay) was added to the assay mixture which contained EGTA (4 μmoles), LPL activity was decreased from 0.91 to 0.46 units. It is clear that the NH₄⁺ concentration, which Korn and Quigley found to be optimal in their assay system (6), was inhibitory under all experimental conditions used here. Also, NH₄⁺ could not stimulate LPL activity when calcium was sequestered by EGTA.

Evaluation of Optimal Calcium Requirement for Rat Heart Acetone Powder LPL (Fig. 1).—Increasing concentrations of calcium were added to an assay system which contained rat heart acetone powder LPL and in which the triglyceride substrate was activated with dialyzed HDL from rat serum. From this study, it appears that the optimal calcium concentration is between 1.25 and 2.5 μmoles/ml, which is similar to the calcium concentration of rat serum (experimentally determined to be 2.3 μmoles/ml). The enzyme preparation used in this study was the supernatant obtained by centrifugation at 1000 x g which contained the usual fine suspension of particulate material. Two questions arose with respect to this preparation. (1) How much bound calcium was present in the enzyme preparation? (2) Was the calcium having its effect by solubilizing more of the enzyme from the particulate matter? The calcium content of this enzyme was determined by atomic absorption spectrometry, and the calcium content was found to be only 0.27 μmoles/ml. The supernatant obtained by centrifugation at 200 x g was then centrifuged at 105,000 x g for 10 minutes. The clear supernatant contained all of the enzyme activity and also contained 0.27 μmoles calcium/ml, indicating that LPL enzyme activity and calcium were not bound to the particulate matter. Also, LPL activity of the clear supernatant was stimulated to the same extent by increasing the concentration of calcium to 2.5 μmoles/ml, i.e., activity increased from 1.9 to 4.6 units.
Evaluation of Optimal Magnesium Requirement for Rat Heart Acetone Powder LPL—Since Korn and Quigley (6) reported that the cation requirement could be satisfied by divalent cations other than calcium, we have also evaluated the effect of increasing concentrations of magnesium added as MgCl₂ (pH 8.6). The addition of magnesium over a wide range of concentrations (0.375 to 12.0 μmoles/ml assay) had little effect on LPL activity as compared to physiological levels of calcium. Whereas 2.5 μmoles of calcium increased LPL activity from a base-line activity of 1.5 to 7.2 units, magnesium had a variable effect and increased activity from a base-line activity of 1.5 to approximately 3.1 units in the physiological range (experimentally determined to be 1.1 μmoles/ml serum in the rat). No optimum was observed.

Effect of EGTA on Rat Heart Acetone Powder LPL—Increasing amounts of EGTA were added to the assay system which contained rat heart acetone powder LPL and in which the triglyceride substrate had been activated with rat serum (0.4 ml). The added rat serum contained approximately 1.0 μmole total calcium. Increasing the EGTA addition to 2.0 μmoles reduced activity by approximately 65%, from 4.7 to 1.7 LPL units. This was decreased to only 1.4 units by increasing the EGTA eightfold.

Effect of EGTA on Rat Postheparin Serum LPL Activity.—To establish that calcium is required for the postheparin serum enzyme as well as the heart enzyme, we added increasing amounts of EGTA to the assay system for postheparin serum. The volume of serum added to the assay system contained approximately 5.5 μmoles of calcium. The addition of an equal number of μmoles of EGTA reduced activity by 54%, from 21 to 9.7 LPL units. Increasing the EGTA eightfold (44 μmoles) decreased activity by only 58%, from 21 to 8.8 LPL units.

Discussion
Korn and Quigley (8) studied the cation requirement for LPL from rat heart acetone powders suspended in dilute NH₄⁺ buffer. They found no activity in the absence of added cations, even though their substrate had been activated with whole normal human serum. The cation additions which they studied in greatest detail were calcium and NH₄⁺ and they concluded that either calcium or NH₄⁺ could function as cofactors for the enzyme. However, it is possible that two different processes were involved in these effects. NH₄⁺ may have increased LPL activity by aiding in the solubilization of the enzyme from the whole acetone powder used in the incubation. Calcium may have had a more specific cofactor role which was also fulfilled by the other divalent cations studied. However, the most abundant divalent cations in situ which might function as cofactors for LPL are calcium and magnesium.

The results of the experiments described in our study differ from those of Korn and Quigley. We carried out these studies with an enzyme preparation which was completely solubilized. Under these conditions, NH₄⁺ at 0.05M, the concentration reported by Korn and Quigley to be optimal, inhibited LPL activity about 56%. We may tentatively conclude, therefore, that other than its ability to solubilize the enzyme from the particulate matter of the rat heart acetone powder, NH₄⁺ does not play a direct role in the lipolytic reaction.

Korn and Quigley found that the optimal calcium concentration was 10 μmoles/ml in their assay system for rat heart acetone powders. Doizaki and Zieve found an optimum at 50 μmoles/ml in an assay for postheparin plasma lipolytic activity (11). We found that the optimal calcium concentration is similar to the calcium concentration present in serum, i.e., 1.25 to 2.5 μmoles/ml. The marked differences between our results and the results of earlier investigations could possibly be due to the albumin preparation used in the assay system. Citrate is a common contaminant of albumin preparations (12) and would undoubtedly sequester added calcium. In our assay system, we used crystalline serum albumin which contained very small amounts of citrate (less than 50
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References


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