Activation of Lipoprotein Lipase

EVALUATION OF CALCIUM, MAGNESIUM, AND AMMONIUM AS COFACTORS

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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 ethyleneglycol bis(β-aminoethylether)-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 lipase cofactor triglycerides lipoproteins heart rat albumin
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.

We recently observed that a triglyceride emulsion which had been activated with purified high density lipoproteins (HDL) from rat serum was slowly hydrolyzed by LPL. However, this substrate was rapidly hydrolyzed by LPL from rat heart acetone powders. Acetone 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 allowed to stand for 1 hour with frequent stirring. Each acetone powder was suspended in 7.0 ml of 0.025M NH₄Cl buffer, pH 8.6 at 0°C, and then removed by centrifugation at 1,000 X g for 10 minutes.

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 allowed to stand for 1 hour with frequent stirring. Each acetone powder was suspended in 7.0 ml of the NH₄Cl buffer. The particulate matter was then removed by centrifugation at 1,000 X g for 10 minutes at 4°C, and the cloudy supernatants were removed and combined. Sufficient enzyme was obtained from two hearts for four LPL incubations using 1.0 ml of the extract per incubation. LPL activity was assayed in duplicate. The triglyceride substrate for the assay system consisted of three parts Intralipid (A.B. Vitrum, Stockholmen) and four parts serum, HDL solution, or 0.15M NaCl, preincubated at 37°C for 5 minutes. The final assay system consisted of 1.0 ml enzyme extract; 0.7 ml of the above triglyceride substrate; 0.75 ml of 1.35M Tris, pH 8.6; 1.35 ml of a 2% (w/v) solution of 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 1000 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 anesthetized with ether. Over the 2 to 3 minutes following injection, 10 ml of blood was rapidly withdrawn from the abdominal aorta into a glass test tube. The blood was allowed to clot, and following centrifugation at 1000 X g, the postheparin serum was removed and kept on ice. When LPL activity from postheparin serum was assayed, 1.0 ml of postheparin serum was substituted for the acetone powder extract in the assay system described above.

Electrophoretically pure HDL were prepared as follows. One part of a solution of 0.05M EDTA and 0.5M phosphate buffer, pH 7.5, was added to 49 parts of rat serum to give a final concentration of 0.005M EDTA and 0.01M phosphate buffer. To inhibit bacterial growth, polymyxin B sulfate (Aerosporin, Burroughs Wellcome) was added to give a serum concentration of 0.5 U/ml. Rat serum HDL were prepared in a 40.3 rotor on a model L2-65 B ultracentrifuge (Beckman). Densities were adjusted with a NaCl-KBr solution (9). We altered this solution to contain EDTA, 0.005M, and phosphate buffer, 0.01M, pH 7.5. Very low density lipoproteins and low density lipoproteins were removed by flotation at a solvent density (d) of 1.003 for 24 hours at 40,000 rpm (1.51 X 10⁹ mean g·min). The HDL were then isolated by flotation at d = 1.210 for 24 hours.
ACTIVATION OF LIPOPROTEIN LIPASE

Activation of Lipoprotein Lipase from Rat Heart Acetone Powders by Serum, HDL, Calcium, and Heparin

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 |
CaCl₂* | 0.49 | 0.46 |
HDL | 2.1 | 1.9 |
HDL + CaCl₂* | 7.1 | 5.9 |
HDL + heparin (7 U) | 4.9 | 4.2 |
HDL + heparin (7 U) + CaCl₂* | 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 NH₄Cl 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.

*Final concentration = 2.5 μmoles/ml in assay.

The system contained NH₄⁺ at a final concentration of 0.004M NaCl, containing 0.005M EDTA, 0.01M phosphate buffer, pH 7.5, and polymyxin B sulfate, 25 U/ml. Following dialysis, the HDL were stored at 4°C in glass test tubes for up to 2 weeks prior to use. HDL stored under these conditions showed no alteration in electrophoretic characteristics. HDL protein concentrations were determined by the method of Lowry et al. (10). The isolated rat HDL was electrophoretically pure as assessed by agarose gel electrophoresis using a modification of Noble's method (5).

Calcium concentrations in heart acetone powder preparations and rat serum and magnesium concentrations in rat serum were determined by atomic absorption spectrometry on perchloric acid filtrates.

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 NH₄⁺ 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 μmole of total calcium. When 2.0 μmoles 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 NH₄⁺.—Experiments were carried out to test the effects of activators of LPL (HDL, heparin, and calcium) in the presence of NH₄⁺ (50 μmoles/ml). 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.

Effect of EGTA on Rat Heart Acetone Powder LPL Activated with Serum and Serum plus Heparin

Addition to assay system | Units of LPL activity | Expt. 1 | Expt. 2
---|---|---|---
NaCl, 0.15M (0.4 ml) | 0.35 |
Serum | 6.9 |
Serum + EGTA (2 μmoles) | 2.8 |
Serum + EGTA (2 μmoles) + CaCl₂ (2 μmoles) | 7.3 |
Serum + heparin (7 U) | 19 |
Serum + heparin (7 U) + EGTA (2 μmoles) | 6.5 |

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 μmole total calcium.
μmoles/ml, pH 8.6). 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 × 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 × g was then centrifuged at 105,000 × 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.

![Graph](http://circres.ahajournals.org/lookup/figure/1/)

**FIGURE 1**

Effect of calcium concentration on activation of rat heart acetone powder LPL. Assay system same as described in Table 1 except these incubations contained 0.2 mg HDL protein, which is approximately equivalent to the amount contained in 0.4 ml rat serum. HDL was dialyzed against a very low concentration of EDTA (0.0005M) to reduce calcium-binding potential of this addition. Each value for LPL activity represents an average of two independent assays.
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 (6) 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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