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Interaction of Very Low Density Lipoprotein With Chicken Oocyte Membranes

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The interaction of hen ^{125}I-VLDL (very low density lipoprotein) with chicken oocyte membranes was characterized using a rapid sedimentation assay. Equilibrium and kinetic studies showed an apparent dissociation constant (K_d) 8.7–9.1 $\times$ 10^{-8} M or 43.5–45.5 μg VLDL protein/ml. Binding capacity was 2.0 μg VLDL protein/mg membrane homogenate protein. The apparent rate constants were $k_1 = 2.4 \times 10^5$ $M^{-1}min^{-1}$ and $k_2 = 2.1 \times 10^{-2}$ min^{-1}. Specific binding required the presence of divalent cations. Whereas binding was completely restored after treatment with EDTA by the addition of Mn^{++}, only 60% of binding was restored using Ca^{++}.

Key words: very low density lipoprotein, hen, oocyte membranes

Yolk formation in oocytes of hens requires large quantities of proteins to be sequestered during oogenesis [1]. Among the major proteins deposited in the yolk is very low density lipoprotein (VLDL, d < 1.006 g/ml) [2,3]. Plasma VLDL is selectively transferred to oocytes of laying hens by receptor-mediated endocytosis [4,5]. In an earlier paper we reported that chicken oocyte membranes possess specific receptors for VLDL [6].

In view of the fact that plasma VLDL increases tremendously at the onset of egg production [7], VLDL appears to be the lipoprotein class mainly responsible for the transport of lipids into oocytes [8]. Owing to the apparent importance of VLDL in oogenesis, we have examined the interaction of VLDL in greater detail and under different conditions than described earlier [6]. In the present report a kinetic analysis of VLDL interaction is performed and the requirement of this interaction for divalent ions is examined. For this purpose, we assayed the binding activity of ^{125}I-VLDL in homogenates of membrane fragments using a rapid sedimentation assay of receptor-bound ^{125}I-VLDL.

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MATERIALS AND METHODS

Lipoprotein

VLDL was prepared from laying hens' plasma containing Na_2EDTA (1 mg/ml) by sequential ultracentrifugation [9] as described for chicken blood by Chapman et al [10]. Testing of purity of chicken lipoproteins was evaluated by electrophoresis in agarose gel [11] and in 3% polyacrylamide gel [12] as shown elsewhere [6]. The various lipoprotein fractions, prestained with Sudan black, separated into distinct bands. In polyacrylamide gels, the VLDL band was located near the point of application on the anodic side, while low density lipoprotein (LDL, d 1.006–1.063 g/ml) moved further away toward the anode. VLDL was radiolabeled with ^{125}I according to McFarlane [13]. Unbound iodine was separated by extensive dialysis [6]. The concentration of the lipoprotein is expressed according to protein content determined by the Bio-Rad Protein Assay [14].

Oocyte Membranes

Oocyte membrane fragments were prepared [6] from oocytes, which were obtained from the ovaries of freshly killed White leghorn hens obtained at a local slaughterhouse or from an in-house colony of laying hens. The fragments, consisting of the oocyte plasma membrane, a perivitelline layer, a monolayer of follicle cells, and the basement membrane, were immediately placed in ice-cold Dulbecco's phosphate-buffered saline (PBS). The fragments were then transferred to Buffer A (150 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-Cl, pH 7.3) at 4°C. Typically, dissected fragments from fifteen 2-cm-diameter oocytes were transferred to 1.5 ml of buffer. The fragments were briefly homogenized in a Pyrex glass tissue grinder using 4–5 strokes, followed by centrifugation at 2,500g min to obtain a suspension of greater homogeneity.

Binding Studies

Binding was performed in Siliclad-coated 1.5-ml polypropylene microfuge tubes in 0.12 ml of Buffer B (140 mM NaCl, 3 mM KCl, 8 mM KH_2PO_4, 1 mM Na_2HPO_4, 0.05 mM $CaCl_2$, 2% bovine serum albumin) at pH 5.5 with the indicated amounts of membrane homogenate protein, ^{125}I-VLDL, unlabeled lipoprotein, and EDTA with and without $CaCl_2$ or $MnCl_2$. The binding reaction was initiated by adding ^{125}I-VLDL and mixing. The tubes were incubated for 30 min in an ice bath. To determine the amount of ^{125}I-VLDL bound, a 0.10-ml aliquot of the reaction mixture was layered onto 0.15 ml of 4% sucrose in PBS in Siliclad-coated 0.40-ml Beckman microfuge tubes. The tubes were immediately centrifuged in a Beckman Microfuge at 60,000g min at 4°C, the tips of the tubes were cut off, and the radioactivity of the pellets contained in the tips were determined by gamma spectrometry.

The protein content of the suspension of membrane fragments was assayed after dissolution in 10 M NaOH [6] according to Lowry et al [15] with bovine serum albumin (fraction V; Sigma Chemical, St. Louis, MO) as a standard.

All experiments were performed at least three times either with freshly dissected membrane fragments or with fragments stored in liquid nitrogen.

Kinetic Analyses

The rate constants for association and dissociation of ^{125}I-VLDL were determined from the time course of ^{125}I-VLDL binding at different concentrations. Binding

of VLDL to its receptor was assumed to be represented by the simple bimolecular reaction [16]:

$$[L] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [L\text{-}R] \quad (1)$$

where ligand L interacts with the receptor R to form the ligand-receptor complex L-R. Estimates for rate constants using this model were obtained from:

$$y(t) = \text{bound}\ (t) = \text{bound}_{eq}\ (1 - e^{-kt}) \quad (2)$$

which is the pseudo-first order approximation of Equation 1. The equilibrium value of ^{125}I-VLDL (bound $_{eq}$) at each concentration was determined using Equation 2 in the MLAB modeling system [17]. The apparent rate constant k in Equation 2 is dependent on the concentrations of both ligand and its receptor as well as on k_1. The time ($t_{1/2}$) required for bound_{eq} to reach half of its equilibrium value was determined from a plot of the time course of binding. A plot of $(\ln 2)/t_{1/2}$ versus ligand (lipoprotein) concentration, analyzed by a least-squares linear regression, gave a straight line with slope k_1 and intercept k_2. An estimate of the apparent dissociation constant K_d can then be obtained from the ratio of the rate constants:

$$K_d = k_2/k_1 \quad (3)$$

RESULTS

Equilibrium Studies

Competitive inhibition studies of ^{125}I-VLDL binding by increasing concentrations of unlabeled VLDL were analyzed using the LIGAND program [18]. A representative study is shown in Figure 1. About 65% of ^{125}I-VLDL added were displaced by unlabeled VLDL. The remaining 35% may, therefore, be regarded as representing nonspecific binding. The linear graph indicates that the mathematical model of a single class of sites is sufficient. This program computes the apparent affinity constant K_a, which was 1.1 ($\pm$ 0.7) $\times$ 10^7 M^{-1}. The apparent dissociation constant K_d, the inverse of K_a, was 9.1 $\times$ 10^{-8} M or 45.5 μg/ml. The computed maximal binding capacity (R) was 1.6 ($\pm$ 0.9) $\times$ 10^{-8} M or 2.0 μg/mg protein when expressed in weight of VLDL protein relative to homogenate protein. It is assumed that the molecular weight of VLDL is 5 $\times$ 10^6, of which about 10% is protein [19].

The values for the binding parameters were reproduced within 50% of above values using competitive inhibition studies as well as saturation binding (not shown).

Kinetic Studies

Kinetic analyses of VLDL-receptor interaction were performed to evaluate the validity of (a) the model of a single class of sites and (b) to obtain an independent estimate for the equilibrium constants. Binding was measured at several concentrations of ^{125}I-VLDL in the presence and absence of 2 mM of EDTA, which was used

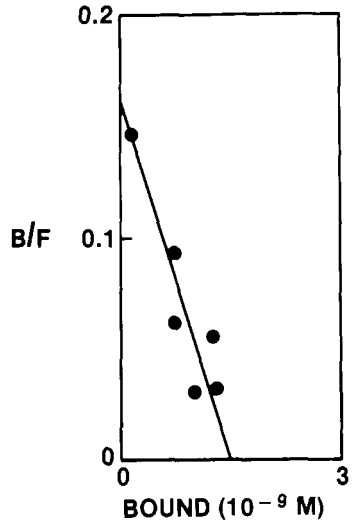

Fig. 1. Competitive inhibition study of VLDL binding to membrane homogenate. Homogenate protein (39 μg) was incubated with 20 μg/ml or 4×10^{-8} M of ^{125}I-VLDL protein (42 cpm/ng) for 30 min at 4°C with increasing concentrations of unlabeled VLDL protein (●) as indicated. Binding parameters were computed using LIGAND program [18].

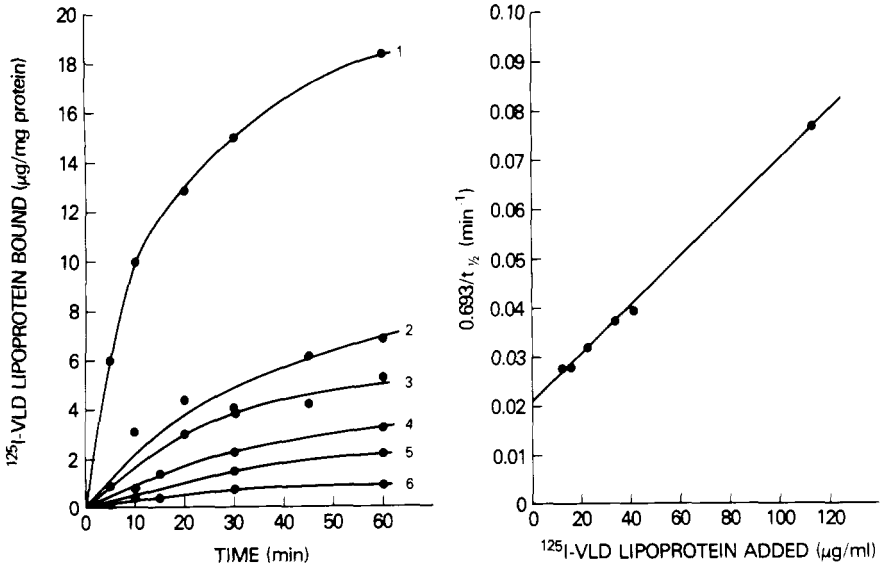

Fig. 2. Time course of specific VLDL binding. Left) Membrane homogenate protein (52 μg) was incubated for the indicated times at 4°C with ^{125}I-VLDL (42 cpm/ng) at varying concentrations with and without a 50-fold excess of unlabeled VLDL (●) in each case. The following six concentrations, expressed in μg ^{125}I-VLDL protein/ml, were used: 115 (1), 42 (2), 34 (3), 22 (4), 15 (5), and 12 (6). Right) Plots of (ln 2)/$t_{½}$ were made for each concentration. The slope of the line drawn through these points indicates the rate of association (k_1) and the intercept indicates the rate of dissociation (k_2).

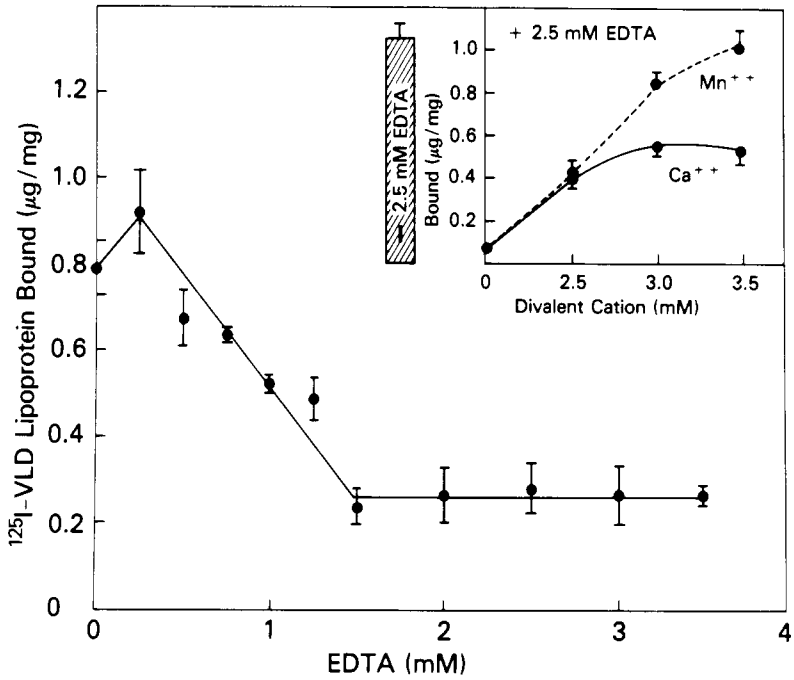

Fig. 3. The dependence of VLDL binding on divalent cations. Divalent cation requirements were determined by incubating 100 μg of membrane homogenate protein for 30 min at 4°C with 12 μg/ml of ^{125}I-VLDL protein (175 cpm/ng) in the presence and absence of 600 μg/ml of unlabeled VLDL protein and the indicated concentrations of EDTA (●). Inset: In a separate experiment the recovery of specific binding was determined. Homogenate protein (58 μg) was incubated with 12 μg/ml of ^{125}I-VLDL protein (67 cpm/ng) with and without 600 μg/ml of unlabeled VLDL protein, 2.5 mM of EDTA, and the indicated concentrations of either Ca^{++} (—●—) or Mn^{++} (---●---). The hatched column at left shows specific binding in the absence of EDTA. Each value represents the mean ± SD of duplicate measurements.

to define nonspecific binding (see next section). The rate constants for ^{125}I-VLDL association and dissociation were computed as described in Methods: $k_1 = 2.4 \times 10^5$ M^{-1} min^{-1} and $k_2 = 2.1 \times 10^{-2}$ min^{-1} (Fig. 2). The K_d value estimated from these values was 8.7×10^{-8} M or 43.5 μg/ml.

Divalent Cation Requirements

The dependence of ^{125}I-VLDL on divalent cations is shown in Figure 3. Binding was reduced 66% by 1.5 mM EDTA. This EDTA inhibited binding is assumed to represent specific ^{125}I-VLDL binding. The addition of either Ca^{++} or Mn^{++} restored binding (Fig. 3, inset). However, whereas Mn^{++} restored binding to 100% of control values, Ca^{++} restored only 60% of binding. The control values were determined in the absence of EDTA.

DISCUSSION

We have used a rapid centrifugal assay to obtain additional information of VLDL binding to chicken oocytes. Based on an assay in which repeated washings were used after the initial interaction of the lipoprotein with diced membranes, we reported earlier that oocyte membranes possess receptors for VLDL [6]. However,

as VLDL interaction was reversible, the repeated washings would cause equilibrium of the VLDL-receptor complex to be shifted in the direction of free VLDL (Methods, Eq. 1). Since the VLDL remaining bound after the last wash would only be a fraction of the amount of VLDL initially bound to the receptors, we have reexamined the VLDL-receptor interaction using a different assay. The major advantages in using the present centrifugal assay are (a) the omission of repeated washings and (b) the use of homogenized membrane fragments, which allows for more uniform sampling than diced fragments [20,21].

We reported earlier that the optimum pH values of chicken lipoprotein binding to chicken oocytes was pH 5.3 for VLDL and an indistinct pH near 7.3 for LDL. The pH optimum of hen LDL binding to diced fragments from chicken oocytes is similar to the pH value used in measurements of human LDL binding to human cultured fibroblasts [22]. Because our previously reported binding data of VLDL were to be directly compared to LDL binding, we used a pH value of 7.3. However, in the current study where VLDL is studied, we have performed all binding studies near the pH optimum of 5.3–5.5. The pH of the binding reaction was rechecked at the end of the incubation period and was found unchanged.

Vitellogenin, the other principal serum protein specifically entering the oocytes, exhibits optimal binding at an acid pH value of 6.0. Woods and Roth [23] and Yusko et al [21] proposed that the acid pH value may be near the pH environment of the receptors. Since the oocyte surface is many cell layers from the circulation, a build-up of the lactate released by glycolysis in the oocytes may occur. This accumulation of lactate provides an acid microenvironment at the oocyte surface. Thus, it is likely that a receptor with a pH optimum near pH 5 or 6 evolved to facilitate the transport of VLDL and vitellogenin by increasing the specificity of the interactions. Using membrane preparations similar to those already described, Perry et al [5] have proposed, on the basis of electron microscopy studies, that receptors binding VLDL maximally at pH 5.3 are located in the basal lamina and not on the oocyte plasma membrane. On the other hand, receptors for LDL with maximal binding at pH 7.3 are located on the oocyte plasma membrane. At the same time, these investigators have observed that some plasma membrane samples bound more particles than others in acid conditions. This effect was ascribed to incomplete rinsing of native particles before binding. Alternatively, we propose that this effect was due to better preservation of the native environment, in which lipoprotein particles, VLDL and/or LDL, bound more abundantly than to extensively rinsed preparations. Using the described biochemical assay, it is not possible to determine the location of VLDL binding in this study. However, in view of the tremendous rise of VLDL in plasma of laying hens [7] and accumulation in the oocytes [8], we propose that VLDL receptors are located in the basal lamina as well as on the oocyte plasma membrane to account for VLDL transport during oogenesis.

Whereas the stepwise events involved in the selective uptake of LDL by cultured human skin fibroblasts have been described in detail [24], the mechanism responsible for the transfer of VLDL from plasma to chicken oocytes is not known. Hen VLDL contains two major apoproteins, apoprotein B and apoprotein VLDL-II [10,25]; human LDL contains mainly apoprotein B. Perry et al [5] have shown that avian lower density lipoproteins containing varying concentrations of apoprotein VLDL-II

bind to oocyte plasma membrane with similar dissociation constants. On the basis of these observations it was concluded that the chicken oocyte receptors recognize preferentially apoprotein B, a characteristic shared by the LDL receptor on cultured fibroblasts. Yet, the presence of VLDL-II in VLDL [26] may account for the differences between the binding of hen VLDL and LDL to chicken oocytes described earlier [6].

We have shown in this study that Mn^{++} is more effective than Ca^{++} in restoring the binding of VLDL after treatment with EDTA. In agreement with these observations are the findings by Perry et al [5] that the binding of hen VLDL occurred in the absence of Ca^{++} but not to full extent when EDTA was present. In contrast, the binding of human LDL to cultured fibroblasts depends on Ca^{++}.

Chicken oocytes differ from other cells in that they store nutrients for later use by the developing chick embryo. Among these nutrients are cholesterol, which is essential for growth. Whereas LDL is the main carrier of cholesterol to certain mammalian cells [24], VLDL, the predominant lipoprotein in the plasma of laying hens, may be the main carrier of cholesterol to the chicken oocytes [27]. The effectiveness of VLDL as a carrier of cholesterol was shown for human macrophages. In these cells the cholesterol content was doubled by incubation with VLDL [28]. On the other hand, in rats, whose predominant lipoprotein is high density lipoprotein, it appears that this predominant class carries cholesterol to the ovaries [29]. Thus, differences in lipid transport to various tissues in different animals appear to be based on need and availability.

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