Interaction of Plasma Lipoproteins With Human Platelets

By Linda K. Curtiss and Edward F. Plow

Human plasma low density lipoproteins (LDL) and high density lipoproteins (HDL) were radioiodinated and their interaction with washed human platelets was assessed. Both ligands were bound by the platelet at 37 °C, and an apparent equilibrium was attained within two hours. Minimal binding was observed at 22 °C or 4 °C. The specificity of these interactions was indicated by the observations that: (a) labeled and nonlabeled lipoproteins interacted with the platelet with the same apparent affinity; (b) nonlabeled lipoproteins inhibited binding, whereas unrelated plasma proteins did not; and (c) the platelet-bound ligands exhibited the appropriate apoprotein chain compositions when analyzed by polyacrylamide gel electrophoresis. Binding of HDL and LDL was found to be independent of the state of platelet activation and did not require divalent ions. Binding of HDL to the platelet was saturable, and a class of sites that maximally bound 1,585 ± 390 HDL particles, with a dissociation constant of 3.1 x 10^{-8} mol/L, was identified. Binding of LDL to the platelet was more complex, but evidence for a class of sites that bound 7,075 ± 4,800 LDL particles, with a dissociation constant of 4 x 10^{-8} mol/L, was found. LDL was a poor inhibitor of ^125I-HDL binding to the platelet, whereas HDL was an effective inhibitor of ^125I-LDL binding. The capacity of HDL to bind or inhibit LDL binding was not dependent on its apoprotein E content. These results are most readily interpreted in terms of two types of lipoprotein interaction sites on platelets: (1) an HDL binding site that does not bind or interacts poorly with LDL, and (2) an LDL binding site that recognizes or is otherwise altered by HDL. The HDL site may be similar to the HDL receptor expressed by steroidogenic tissues in terms of apoprotein specificity. The LDL site is not the same as the LDL receptor of most extrahepatic cells.

Platelets and lipoproteins are intimately involved in the pathogenesis of a wide variety of diseases, including atherosclerosis, thrombosis, and coronary heart disease. Evidence accumulating over the past two decades suggests the possibility of a direct relationship between plasma lipoproteins and the hemostatic function of platelets. Particularly pertinent in this regard are the reports of abnormal platelet function in patients with familial type IIa hyperlipoproteinemia, a disease that is associated with a predisposition and early onset of atherosclerosis. Platelets from these patients exhibit enhanced aggregation and secretory responses to a variety of physiologic stimuli. Nicotinic acid and clofibrate, which reduce plasma lipoprotein levels, decrease the platelet hypersensitivity in these patients and reduce the responsiveness of platelets from normal subjects. In addition, the platelets from patients with type IIa hyperlipoproteinemia have increased cholesterol and phospholipid content. Because increases in cholesterol content are associated with platelet hypersensitivity to stimulation, these changes may provide a basis for the abnormalities in platelet function observed in the disease.

Recent studies have demonstrated that lipoproteins can alter the aggregation response of platelets. Aviram and Brooks found that low density lipoproteins (LDL) enhanced, whereas high density lipoproteins (HDL) reduced thrombin-induced platelet aggregation. Hassal et al showed that plasma LDL levels were correlated with enhanced platelet sensitivity to aggregation by epinephrine. These observations imply an interaction of these lipoproteins with the platelet surface as the initial mechanism for altering platelet function. Specific receptors for lipoproteins have been identified on a variety of circulating and tissue-fixed cells. LDL receptors, as expressed by most extrahepatic cells, possess a dual specificity that permits lipoproteins containing either apoproteins (apo) B or E to interact. This relaxed specificity allows either four apo B-containing LDL particles or one apo E-containing HDL particle to bind to a single LDL receptor. In hepatic as well as steroidogenic tissues, such as testes, ovaries, placenta, and adrenals, independent binding sites for HDL have been reported. Though not definitively established, a role for apo AI in determining the specificity of these HDL receptors has been suggested. Several laboratories have now demonstrated specific binding of certain lipoproteins to washed human platelets. Aviram and Brook have reported that gel-filtered platelets from normal subjects bind both LDL and HDL and have found minimal cross-reaction between the binding sites for these ligands. In contrast, Koller et al found that HDL inhibited LDL binding and that LDL inhibited HDL binding. In this study, we have
systematically examined the binding, specificity, and interrelationships between the interactions of HDL and LDL with human platelets.

MATERIALS AND METHODS

Lipoprotein Isolation and Characterization

Blood was obtained from humans following approved institutional protocols in accordance with assurance GO 108 XB on file with Health and Human Services (HHS). Lipoproteins were isolated from pooled fresh fasting plasma that was obtained from normal healthy donors by plasmapheresis. During the course of these studies, the lipoproteins were isolated from eight different plasma pools by sequential ultracentrifugation using KBr for density adjustment, as previously described, and in the presence of 0.1% (wt/vol) EDTA, 1 mg/mL gentamicin sulfate, 0.2% sodium azide, 5 mmol/L benzamidine, 10 mmol/L diisopropylfluorophosphate, and 10 μg/mL soybean trypsin inhibitor. The lipoprotein fractions used were LDL (density = 1.019 to 1.063 g/mL) and HDL (density = 1.063 to 1.25 g/mL). Lipoproteins were dialyzed thoroughly against 0.15 mol/L NaCl, 0.3 mmol/L EDTA, 5 mmol/L benzamidine, and 0.005% alpha-toocopherol, pH 7.4, filter sterilized and stored at 4°C for no more than 14 days. The total protein content of the lipoproteins was analyzed by a modification2 of the method of Lowry, using a bovine serum albumin standard, and all lipoprotein concentrations are expressed on the basis of protein. The apoprotein composition of each of the lipoprotein classes was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as outlined below.

For selected experiments, apoprotein E-containing lipoproteins were removed from HDL by heparin-agarose affinity adsorption. Heparin-agarose (lot No. 20526, Bethesda Research Laboratories, Gaithersburg, Md), containing 0.55 mg heparin/mL swollen gel, was equilibrated in 5 mmol/L Tris, pH 7.5, containing 50 mmol/L NaCl and 25 mmol/L MnCl₂. Adsorptions were performed by incubating 0.5 mL of lipoprotein, containing between 25 and 200 μg of apo E, with 0.2 mL of packed heparin-agarose or unsubstituted agarose for 30 minutes at 24°C, followed by removal of the agarose by low-speed centrifugation. Removal of apo E was monitored immunochemically as described below.

Radioiodination

LDL and HDL were enzymatically radioiodinated with Na⁻¹²⁵I (500 mCi/mL, Amersham, Arlington Heights, Ill) using immobilized lactoperoxidase and glucose oxidase (Enzymobeads; BioRad, Richmond, Calif) as described. The specific activities for ¹²⁵I-LDL and ¹²⁵I-HDL ranged from 1.4 to 3.0 μCi/μg and 2.0 to 3.1 μCi/μg, respectively. In all cases, greater than 90% of the ¹²⁵I in the lipoproteins was precipitated by incubation with 5% (wt/vol) trichloroacetic acid and 10% (wt/vol) phosphotungstic acid for one hour at 4°C. Half of the nonprecipitable radioactivity was free iodide, as measured in a Sienco dual channel aggregometer) to 5 mU/mL of thrombin and 4 μg/mL of collagen. Adenosine diphosphate (ADP) (5 μmol/L) induced aggregation-required addition of exogenous fibrogen. Thrombin-induced secretion of 3H-serotonin was complete at 5 μL/mL of thrombin. Platelet counts were determined electronically. For selected experiments, platelet lysis induced by LDL or HDL was assessed using ⁵¹Cr-labeled platelets.

Binding of the Lipoproteins to Platelets

All labeled and nonlabeled lipoprotein preparations were dialyzed into Tyrode's buffer immediately before use, and the labeled ligands were precentrifuged for five minutes at 11,000 rpm in a Beckman microfuge. In a typical experiment, platelets at a final concentration of 10⁶/mL and ¹²⁵I-HDL or ¹²⁵I-LDL at 5 to 20 μg/mL were mixed and incubated at the desired temperature. At selected time points, tricarboxylic acid aliquots were removed and layered onto 0.3 mL of 20% sucrose in Tyrode's-albumin buffer. The tubes were centrifuged at 22°C for 2.5 minutes at 11,000 rpm in a Beckman microfuge, and the tips of the tubes containing the platelets were amputated and counted for radioactivity. Platelets spun at 37°C or 22°C gave comparable binding. Calculation of the bound lipoproteins was based on the specific activities of the radiolabeled ligands, and results are expressed as nanograms of lipoprotein protein bound per 10⁶ platelets. Binding isotherms were subjected to nonlinear curve-fitting analysis, utilizing the “Ligand Program” of Munson and Rodbard on the Hewlett-Packard HP 9836 computer.

Polyacrylamide Gel Electrophoresis

The apoproteins were analyzed by polyacrylamide slab gel electrophoresis of the lipoproteins in the presence of 1% SDS. The gels were prepared according to Laemmli, using a 25 mmol/L Tris-glycine buffer at pH 8.6, as described previously. The stacking gel contained 1% SDS and 3% acrylamide, and the lower running gel was either a 3% to 6% or a 7.5% to 20% acrylamide gradient that also contained 1% SDS. Lipoproteins were delipidated by boiling for three minutes in electrophoresis buffer containing 1% SDS and electrolysed for 18 hours at 35 mA for the 7.5% to 20% gel or 8 mA for the 3% to 6% gel. The gels were stained for protein with 0.1% Coomassie Brilliant Blue R250 in 50% trichloroacetic acid, destained, photographed, and dried. Dried gels containing radioiodinated lipoproteins were analyzed by autoradiography (X-Omat; Eastman Kodak, Rochester, NY) and the autoradiographs quantitated by soft laser densitometric scanning.
Radioimmunoassays

The apoprotein Al, B, CI, and E content of each of the plasma lipoprotein classes was quantitated immunochemically in a solid-phase competitive radioimmunoassay. Assays were performed, as described,22 in flexible round-bottom polyvinyl chloride microtiter plates (Dynatech, Alexandria, Va). The wells were coated, as described,22 so as to achieve a final bound lipoprotein concentration of 50 ng/well. To accomplish this, very low density lipoprotein (VLDL) was used at 50 µg/mL, LDL at 4.4 µg/mL, and HDL at 24 µg/mL. After postcoating, 0.025 mL of lipoprotein (0.1 to 1,000 g/mL) was added and allowed to compete with the bound antigen for binding to limiting amounts of ascites fluid containing human apoprotein-specific monoclonal antibodies. The antibodies specific for apo Al and apo CI were IgGκ antibodies secreted by cloned and stable hybridomas generated from the fusion of P3 X 63.653 mouse myeloma cells with spleen cells of mice immunized with the isolated apoproteins. They were used at dilutions of 1:90,000 and 1:300,000, respectively. The apo B-specific antibody (B24) has been extensively characterized22.25 and was used at a dilution of 1:1,000,000. The apo E-specific antibody (Emab-3) is a mouse IgGκ monoclonal antibody that binds all apoprotein E isomers and was a generous gift of Dr. L. C. Smith, Baylor.24 It was used at a dilution of 1:5,000. After incubation for 18 hours at 4°C, the wells were washed free of unbound material. The amount of mouse monoclonal antibody bound was quantitated by a second four-hour incubation at 4°C with 10 ng/well of an immunochemically purified 125I-goat anti-mouse Ig, which has been previously described.22 Nonspecific binding was determined by replacing the specific ascites fluids with similar dilutions of their respective control mouse myeloma ascites fluids. Apo Al, apo CI, and apo E were quantitated by reference to standard curves generated with the purified apoproteins. The apoproteins were used at 0.1 to 20 µg/mL, were a gift of Drs. L. C. Smith and J.T. Sparrow, Baylor, and were isolated as described.34 Apo B was quantitated by reference to a standard curve generated with LDL of density 1.032 to 1.035 g/mL as competitor and was used at 0.1 to 10 µg/mL. This LDL standard did not contain immunochemically detectable apo Al, CI, E, or human serum albumin.

RESULTS

Human plasma HDL (density, 1.063 to 1.25 g/mL) and LDL (density, 1.019 to 1.063 g/mL) were isolated by density gradient ultracentrifugation in KBr and radioiodinated to specific activities of 1 to 3 µCi/µg. Interaction of these radiolabeled ligands with washed human platelets was assessed by centrifuging the platelets through 20% sucrose to separate bound from free ligand. As previously described, this centrifugation system provides greater than 90% platelet recovery without cell lysis or induction of platelet secretion.29,36 In the absence of platelets, the application of 5.5 to 6.0 × 104 cpm of 125I-HDL or 125I-LDL onto the sucrose solution resulted in the recovery of less than 100 cpm in the tip of the tube after centrifugation.

In initial experiments, the interaction of the two lipoproteins with washed human platelets was assessed as a function of time and temperature. As shown in Fig 1, each lipoprotein bound to the washed platelets at 37°C, and the time course of the binding reaction was similar for 125I-HDL and 125I-LDL. In each case, binding increased linearly for approximately 90 minutes and reached an apparent equilibrium by 120 minutes. At equilibrium, approximately sixfold more 125I-LDL was bound to the cells than 125I-HDL on a protein basis. With both ligands, the interactions were markedly temperature dependent. At 22°C and 4°C, binding of each lipoprotein at 180 minutes was less than 5% of that observed at 37°C. In all subsequent studies, lipoprotein binding was measured at 37°C.

Four approaches were utilized to establish that the binding of the lipoproteins to the platelet was specific. First, it was determined that the observed interaction was not due to radioiodination of the lipoproteins. Each radiolabeled lipoprotein and homologous nonlabeled lipoprotein was added to the platelets in varying proportions, while a constant total lipoprotein concentration was maintained. At 120 minutes, plots of the cpm bound vs percent of the radiolabeled lipoprotein present were found to be linear for both HDL and LDL (Fig 2). The linear correlation coefficients of r ≥ .991.
Fig 2. Each radiolabeled lipoprotein interacts with the platelet with the same apparent affinity as its nonlabeled counterpart. Varying proportions of labeled and nonlabeled lipoproteins were incubated with 10^10 platelets/mL at 37 °C for 120 minutes. Constant concentrations of 7.5 μg/mL HDL (^125I-HDL plus HDL) and 15 μg/mL LDL (^125I-LDL plus LDL) were maintained. Results were plotted as mean cpm recovered with the platelets after centrifugation vs the percent of ^125I-lipoprotein added. Linear correlation coefficients (r) were ≥ 0.991 for both HDL and LDL.

indicated that each labeled and nonlabeled pair of lipoproteins interacted with the platelet with the same apparent affinity. In separate experiments, ^125I-HDL labeled with the Bolton-Hunter reagent or by the lactoperoxidase procedure were found to be equivalent in their reactivity with the platelet.

Because less than 2% of the added ^125I-HDL and ^125I-LDL was bound by the platelets, we attempted to verify that the observed binding was not limited to a small population of each of the added ^125I-ligands. A constant amount of ^125I-HDL (7.5 μg/mL) or ^125I-LDL (15 μg/mL) was added to platelets in the absence or presence of a 20-fold excess of its homologous nonlabeled ligand. After one hour at 37 °C, the unbound fraction of each ^125I-ligand was transferred to a fresh platelet suspension for a second one-hour incubation at 37 °C. This same second unbound fraction was then transferred successively to two additional platelet suspensions for identical incubations. Binding of the radioiodinated lipoprotein in the presence of excess nonlabeled lipoprotein in each instance was less than 10% of the total binding observed in the absence of nonlabeled lipoprotein. No decrease in the percent ligand bound to platelets was observed from the first through the fourth incubation. Therefore, the observed specific binding of ^125I-HDL and ^125I-LDL to platelets was not limited to a small subpopulation of the HDL and LDL ligands.

As a third criterion of specificity, the radiolabeled material binding to the platelets was characterized. ^125I-HDL and ^125I-LDL were incubated with platelets for 120 minutes. After phase separation, the unbound ligands and the ligands extracted from the platelet pellets with SDS were analyzed by polyacrylamide gel electrophoresis. The autoradiograms shown in Fig 3 indicate a qualitative identity in apoprotein composition of the starting, bound, and unbound ligands for either HDL or LDL. From densitometric scans, > 95% of the radioactivity of the ^125I-LDL preparation was associated with large molecular weight apo B, and this percentage remained the same in the bound and unbound ligand. No apo B was detected in the bound, unbound, or starting preparation of ^125I-HDL. In the starting ^125I-HDL, apo AI and apo AI all constituted 54% and 33% of the radioactivity, respectively, and this proportion was maintained in the unbound ^125I-HDL (57% and 34%, respectively). In the platelet-bound ^125I-HDL, apo AI and apo AI all also accounted for > 90% of the radioactivity, but their relative proportion was altered to 75% apo AI and 15% apo AI all. In addition, the low molecular weight peptides present in the starting and unbound ^125I-HDL

![Polyacrylamide gel electrophoresis of platelet-bound ^125I-LDL and ^125I-HDL.](image)

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(likely apo AII monomers or apo C) were not observed in the platelet-bound 125I-HDL. The increase in the apo Al content of the platelet-bound 125I-HDL ligand was observed in two separate analyses with different preparations of HDL. Thus, the platelet-bound lipoproteins were authentic HDL and LDL, and a selection of HDL containing only apo Al may occur in binding to the platelet. Because all HDL particles contain apo Al and all LDL particles contain apo B, these observations are consistent with the studies above indicating that platelet binding was a function of a major rather than a minor component of the labeled ligands.

In separate experiments, the acid precipitability of the extracted radioactivity in 10% phosphotungstic acid was assessed. For 125I-HDL, the platelet-bound radioactivity was 85.3% precipitable compared to 93.0% precipitation of 125I-HDL incubated at 37 °C for two hours in the absence of platelets. For 125I-LDL, 88.2% of the ligand and 90.3% of the platelet-bound radioactivity were precipitable. Thus, protein-associated radioactivity accounted for at least 85% of the bound radioactivity, and no apparent degradation of the apoprotein chains of the platelet-associated lipoproteins was detected during the 120-minute incubation at 37 °C.

As a fourth approach to validate specificity, the capacity for the nonlabeled lipoproteins and unrelated proteins to inhibit the platelet interaction of 125I-HDL and 125I-LDL was assessed. As shown in Table 1, nonlabeled HDL and LDL inhibited the binding of the homologous radiolabeled ligands by greater than 90%, which confirmed previous estimates of a nonspecific binding of ≤10%. In six experiments using at least two different preparations of each labeled ligand, the nonspecific binding (residual binding in the presence of excess nonlabeled lipoproteins) was 6.4% ± 1.5% and 5.7% ± 3.4% for 125I-HDL and 125I-LDL, respectively. Nonlabeled LDL and HDL also inhibited the binding of 125I-HDL and 125I-LDL, respectively, and this cross-competition will be considered in detail later. However, a tenfold molar excess of unrelated proteins produced no inhibition of the binding of either ligand (Table 1).

It was noted that 125I-LDL binding was inhibited 33% by a 50-fold molar excess of ovalbumin, but not the other proteins. In summary, the preceding experiments demonstrated that: (a) radiolabeled and nonlabeled lipoproteins interacted with the platelet with the same apparent affinity; (b) the observed binding was not mediated by a minor component of the added lipoproteins; (c) authentic HDL and LDL were bound to the platelet; and (d) the interaction was specific for the lipoproteins.

To establish optimal conditions for interaction of the lipoproteins with the platelets, the effects of divalent ions and platelet stimulation on ligand binding were evaluated. Previous interactions had been measured in the absence of added divalent cations. The observation that 3 mmol/L EDTA did not affect the binding of either 125I-HDL or 125I-LDL verified divalent ion independence of the interactions (Table 2). Moreover, calcium and magnesium inhibited the binding of both HDL and LDL in a dose-dependent fashion. At a 1 mmol/L concentration of calcium, specific 125I-LDL binding was inhibited 100%. The inhibition of specific 125I-HDL binding was less extensive and did not exceed 59%, even at concentrations of 2 mmol/L calcium. The residual binding of 125I-HDL in the presence of calcium or magnesium was specific, as inclusion of a 100-fold excess of nonlabeled HDL in the reaction mixtures inhibited the total ligand binding by ≥95% in the presence or absence of the divalent ions. Similar

| Table 1. Specificity of HDL and LDL Binding to Human Platelets |
|---------------|------------------|------------------|
| Competitor    | Total ng Bound/10⁹ Platelets | ¹²⁵I-HDL | ¹²⁵I-LDL |
| None          | 471 ± 65                 | 3,587 ± 140   |
| LDL           | 101 ± 25                 | 175 ± 26     |
| HDL           | 48 ± 6                  | 191 ± 13     |
| Fibrinogen    | 543 ± 85                | 3,616 ± 250  |
| Transferrin   | 487 ± 15                | 3,596 ± 114  |
| Ovalbumin     | 432 ± 50                | 3,630 ± 341  |
| Myoglobin     | ND                      | 3,575 ± 226  |
| Soybean trypsin inhibitor | ND              | 3,608 ± 157  |
| RNAase        | ND                      | 3,625 ± 121  |

| Table 2. Effect of Divalent Ions on LDL and HDL Binding to Human Platelets |
|---------------|------------------|------------------|
| Addition      | Total ng Bound/10⁹ Platelets | ¹²⁵I-HDL | ¹²⁵I-LDL |
| None          | 590 ± 12                 | 2,400 ± 40     |
| EDTA (3 mmol/L)| 550 ± 30                | 2,670 ± 280   |
| Calcium (2 mmol/L)| 259 ± 26            | ND            |
| Calcium (1 mmol/L)| 290 ± 90             | 130 ± 3       |
| Calcium (0.01 mmol/L)| 500 ± 10           | 1,320 ± 78    |
| Magnesium (1 mmol/L)| 280 ± 15             | 380 ± 10      |
| Magnesium (0.01 mmol/L)| 415 ± 25           | 2,170 ± 85    |

¹²⁵I-HDL or ¹²⁵I-LDL, at final concentrations of 7.5 or 15.0 µg/mL, respectively, were added to a platelet suspension (10⁶ cells/mL) containing the indicated competing proteins. Each competitor was added at ≥10 molar excess, relative to the final concentration of the radiolabeled ligand. Binding was measured after a two-hour incubation at 37 °C. Results are the mean of triplicate determinations. ND, not determined.
inhibitory patterns for both 125I-LDL and 125I-HDL binding also were obtained when sulfates rather than the chloride salts of the divalent ions were utilized. Evidence presented in Table 3 indicated that interaction of the lipoproteins with the platelet was also independent of platelet stimulation. ADP and thrombin were utilized as representative platelet stimuli, and, to insure the absence of stimulation during the binding reaction, a combination of prostaglandin E1 (PGE1) and theophylline was utilized. These stimuli and inhibitors had little effect on the quantitative binding of either 125I-HDL or 125I-LDL. The greatest change was noted with LDL binding in the presence of thrombin, and this represented only a 14% increase in the total nanograms bound compared to the nonstimulated cells. In separate experiments, we verified that the kinetics of ligand binding were not altered by these stimuli and inhibitors.

Utilizing nonstimulated platelets in the absence of divalent ions and a two-hour incubation at 37 °C, the capacity to saturate the platelet binding sites for the lipoproteins was assessed. In these experiments, increasing concentrations of radiolabeled 125I-HDL or 125I-LDL were added to the platelets in the absence or presence of a 50-fold excess of the homologous nonlabeled lipoprotein. Residual binding of the radiolabeled ligands in the presence of excess nonlabeled lipoprotein was defined as being “nonspecific” and was subtracted from the binding in the absence of nonlabeled ligand to yield the “specific” binding. The specific binding isotherm for 125I-HDL is shown in Fig 4A. When the concentrations of 125I-HDL added exceeded 15 μg/mL, saturation was attained. Scatchard analysis of the data (Fig 4B) could be interpreted in terms of a linear Scatchard plot (Fig 5A), with a linear correlation coefficient of r = .93.

Assuming a mean particle protein molecular weight of 2.5 × 10^2 for LDL, duplicate experiments with two preparations of 125I-HDL yielded a Kd of 3.1 ± 2.8 × 10^-8 mol/L with 1.585 ± 390 HDL particles bound per platelet. In addition, the data from a single experi-

Table 3. Effect of Platelet Stimulation on HDL and LDL Binding to Human Platelets

<table>
<thead>
<tr>
<th>Addition</th>
<th>Total ng Bound/10^9 Platelets</th>
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<tbody>
<tr>
<td></td>
<td>125I-HDL</td>
</tr>
<tr>
<td>None</td>
<td>504 ± 15</td>
</tr>
<tr>
<td>ADP (10 μmol/L)</td>
<td>510 ± 5</td>
</tr>
<tr>
<td>Thrombin (2 U/mL)</td>
<td>499 ± 22</td>
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<tr>
<td>PGE1 (1 μg/mL)</td>
<td>518 ± 14</td>
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<tr>
<td>+ theophylline (1 μmol/L)</td>
<td>58 ± 7</td>
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Stimuli and inhibitors were added to platelets (10^9/mL) in divalent ion-free Tyrode’s albumin buffer, pH 7.2, followed immediately by addition of either 125I-HDL (7.5 μg/mL) or 125I-LDL (15.0 μg/mL). Binding was measured in triplicate after two hours at 37 °C. Nonspecific binding in the presence of a 20-fold molar excess of cold ligand was 38 ± 12 and 162 ± 14 ng/10^9 platelets for HDL and LDL, respectively.

![Fig 4. Concentration-dependent binding of 125I-HDL to platelets. (A) Platelets at a final concentration of 5 × 10^7/mL were incubated at 37 °C for 120 minutes with increasing concentrations of 125I-HDL. Data have been corrected for nonspecific binding. (B) Scatchard plot of the data shown in A. “Bound/Free” is the 125I-HDL bound (ng/10^9 platelets)/ng 125I-HDL added.](https://www.bloodjournal.org/doi/abs/10.1182/blood-2017-02-744854)
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Fig 5. Concentration-dependent binding of $^{125}$I-LDL to platelets. (A) Platelets at a final concentration of $5 \times 10^8$/mL were incubated at 37 °C for 120 minutes with increasing concentrations of $^{125}$I-LDL. Data have been corrected for nonspecific binding. (B) Scatchard plot of the data shown in A. "Bound/Free" is the $^{125}$I-LDL bound (ng/10^9 platelets)/ng $^{125}$I-LDL added.

Radiolabeled lipoprotein was inhibited by both nonlabeled lipoproteins. This phenomenon was further investigated by examining the effects of increasing concentrations of the nonlabeled lipoproteins on the binding of each ligand. In preliminary experiments, it was established that concentrations of nonlabeled LDL and HDL as high as 1 mg/mL did not cause platelet lysis during a two-hour incubation at 37 °C, as assessed by the recovery of $^{51}$Cr-labeled platelets. The effects of varying concentrations of HDL and LDL on $^{125}$I-HDL binding are shown in Fig 6A. As anticipated, nonlabeled HDL effectively inhibited the binding of radiolabeled HDL. The concentration of nonlabeled HDL required for 50% competition was 5.7 µg/mL ($2.3 \times 10^{-8}$ mol/L) and was in reasonable agreement with the $K_d$ for the binding of $^{125}$I-HDL. Nonlabeled LDL was only inhibitory at very high concentrations. An LDL concentration of 200 µg/mL ($4 \times 10^{-7}$ mol/L) was required for 50% inhibition of $^{125}$I-HDL binding; no competition was observed with LDL at 50 µg/mL, a concentration that approached apparent saturation of the measurable LDL binding sites on the platelet (see Fig 5A). The effect of the higher LDL doses on $^{125}$I-HDL binding could reflect occupancy of a second low-affinity LDL binding system or nonspecific effects. With $^{125}$I-LDL as the ligand (Fig 6B), nonlabeled LDL produced 50% inhibition at 16 µg/mL ($3 \times 10^{-8}$ mol/L), and this also was consistent with the $K_d$ of the interaction derived from Scatchard analyses. However, HDL not only produced complete inhibition, but also was more effective than LDL in inhibiting the binding of $^{125}$I-LDL. A concentration of 2 µg/mL ($0.8 \times 10^{-8}$ mol/L) HDL was required for 50% inhibition, and this was fourfold less than required with nonlabeled LDL.

On cells such as fibroblasts, the binding of HDL to the LDL receptor is dependent on its apo E content. To determine if the capacity of HDL to bind to platelets or to compete with $^{125}$I-LDL binding was dependent on its apo E component, apo E-free HDL was prepared by heparin-agarose absorption. A control sample was generated in parallel by treating the same HDL preparation with unsubstituted agarose beads. The apoprotein A1, B, C1, and E content of sample was quantitated by solid-phase radioimmunoassays with monoclonal antibodies to each apoprotein (Table 4). No apo E was detected in the heparin-agarose-adsorbed HDL, representing a reduction of at least 97% in apo E. The low content of apo B present in the HDL preparation was also reduced by the heparin-agarose absorption, whereas the apo A1 and C1 content were unaltered. Each of the HDL preparations was then tested for its capacity to inhibit the binding of $^{125}$I-HDL or $^{125}$I-LDL to the platelet. The concentrations required for 50% inhibition are summarized in Table 5. With $^{125}$I-HDL (untreated) as the ligand, similar concentrations of HDL absorbed with heparin-agarose or agarose were required for 50% inhibition. These preparations also inhibited $^{125}$I-LDL binding at...
similar concentrations. Thus, HDL binding or its capacity to inhibit LDL binding was independent of its apo E content.

DISCUSSION

The abnormalities in platelet composition and function in patients with hyperlipoproteinemia suggest that the circulating levels of lipoproteins influence the properties of the platelet. On this basis, we have sought to evaluate the direct interaction of plasma lipoproteins with the platelet utilizing LDL and HDL as prototype lipoprotein particles. When radiiodinated, each of the lipoprotein ligands bound to the platelets in a time-dependent and temperature-dependent interaction. The specificity of the interaction for lipoproteins was established by the observations that: (a) labeled and nonlabeled lipoproteins interacted with the platelet with the same apparent affinities; (b) a constant proportion of the labeled ligands was bound in sequential binding assays; (c) the platelet-bound HDL and LDL resembled the added lipoprotein in apoprotein composition; and (d) the interaction was inhibited by nonlabeled lipoproteins, but not by unrelated proteins or glycoproteins. Characteristics of the interaction included: (a) a marked temperature dependence; (b) a lack of divalent ion dependence; (c) independence of the state of platelet stimulation; and (d) absence of extensive apoprotein degradation during a two-hour incubation. At 37°C in the absence of divalent ions and platelet stimulation, a saturable component of both HDL and LDL could be demonstrated, indicating the presence of a discrete number of binding sites for these lipoproteins on the platelet. Scatchard analyses of the specific binding isotherms for either HDL or LDL could be interpreted in terms of a single class of binding site with respect to affinity. The dissociation constants for the binding of HDL and LDL to the platelets were similar (3 to 4 x 10⁻⁸ mol/L), but the capacity for LDL was 4.5-fold higher. The existence of an additional high-capacity, low-affinity LDL binding site could not be experimentally excluded.

To consider the relationship between HDL and LDL binding, the capacity of nonlabeled lipoproteins to inhibit binding of each radiolabeled ligand was assessed. With [¹²⁵I]-HDL, inhibition was observed with both HDL and LDL; however, LDL was a much poorer inhibitor (40-fold) and at least 100 μg/mL LDL was required for significant inhibition of [¹²⁵I]-HDL binding. From the specific binding isotherms of [¹²⁵I]-LDL, concentrations of 20 to 40 μg/mL LDL saturated the high-affinity platelet binding sites for this lipoprotein. This implied that the inhibition of [¹²⁵I]-HDL binding by LDL was not due to the interaction of LDL with this site on the platelet. Inhibition by LDL must, therefore, arise from nonspecific or indirect effects or from a lower affinity interaction with the platelet. On this basis, it is proposed that the platelet possesses a high-affinity binding site for HDL that does not bind LDL or binds LDL very poorly. HDL receptors have been identified on steroidogenic tissues, such as testes, ovaries, and adrenals. These receptors bind apo E-free HDL with high affinity in the absence of divalent ions, do not bind LDL, do not necessarily facilitate the degradation of the apoprotein chains of LDL, and have a suggested specificity for apo AI-containing HDL particles. These characteristics, including the capacity to bind apo E-free HDL and a selective specificity for apo AI-containing HDL, suggest that the platelet binding sites for HDL share many of the characteristics of the HDL receptors expressed by steroidogenic cells. However, only direct binding studies utilizing isolated apo AI will confirm this hypothesis.

Platelet binding of [¹²⁵I]-LDL was effectively inhibited by both HDL and LDL. Such cross-inhibition is characteristic of the LDL receptor of fibroblasts, which binds apo E-containing HDL and apo B-containing LDL. The platelet binding site for LDL did not, however, appear to recognize the apo E components of HDL. The temperature and divalent ion sensitivity of LDL binding further distinguished
the platelet binding site from the LDL receptor on extrahepatic cells. Based on these properties, LDL binding to platelets may be dependent on either the lipid or the apoprotein constituents of the lipoprotein. Inhibition of LDL binding by HDL also may reflect recognition of common nonapoprotein components of the particles, as lipoprotein receptors with specificities for both LDL and apo E free-HDL have been reported, and these receptors appear to interact with phospholipids as well as apoproteins. In addition, the data of Koller et al. suggest that such inhibition may be of a more complex variety than simple competition for the same binding site. 

In sum, our data suggest the existence of two binding sites for lipoproteins on platelets: an HDL binding site that interacts poorly with LDL, and an LDL binding site that either reacts well with HDL or is altered by HDL binding. The postulation of distinct HDL and LDL binding sites is consistent with the conclusions of Koller et al. In fact, the affinity and number of HDL and LDL particles bound per platelet measured by these investigators and in our study are quite similar (LDL, \( K_a = 6.2 \times 10^7 \text{L/mol} \) and 1,470 molecules/platelet compared to our data of \( K_a = 2.5 \times 10^7 \text{L/mol} \) and 7,075; HDL, \( K_a = 9 \times 10^7 \text{L/mol} \) and 3,200 molecules/platelet compared to our data of \( K_a = 3.2 \times 10^7 \text{L/mol} \) and 1,585 molecules/platelet). Nevertheless, certain characteristics of the interactions are quite distinct in the two studies. Specifically, we found that LDL was a poor inhibitor of HDL binding and that the binding of both ligands required approximately two hours to reach equilibrium, was temperature sensitive, and was inhibited by divalent ions. In contrast, Koller et al. observed that LDL was an effective inhibitor of HDL binding and that the binding of both ligands reached a rapid equilibrium (15 minutes), was temperature independent, and occurred in the presence of divalent ions. Our data, as well as that of Koller et al., contrast considerably with the observations of Aviram and Brooks, who found that 125I-LDL binding was not affected by HDL as well as in a number of other major parameters. Further studies are required to resolve the basis for these differences.

The role of these platelet binding sites in the maintenance or alteration of platelet function by lipoproteins is unknown. Presumably, such interactions initiate the exchange or transfer of lipids between the lipoproteins and the platelet, and this could result in either the loss of platelet lipid or the utilization and processing by the cells of lipid constituents of the lipoproteins, such as those recently discussed by Glass et al. In the system utilized in this study, physiologic concentrations of divalent ions inhibited high-affinity LDL binding to the platelet. However, this does not exclude an influence of LDL on platelet cholesterol content through a collisional exchange mechanism. In addition, a significant component (~50%) of HDL binding was not inhibited by calcium or magnesium. Therefore, from a physiologic perspective, there is good reason to examine in detail the high-affinity, EDTA-insensitive interaction of HDL with platelets and to assess the consequences of this interaction on platelet lipid composition and function.

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