methods

A quantitative assay for the non-covalent association between apolipoprotein[a] and apolipoprotein B: an alternative measure of Lp[a] assembly

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Abstract **Increasing evidence suggests that the assembly of lipoprotein[a] (Lp[a]) proceeds in two steps. In the first step, non-covalent interactions between apolipoprotein[a] (apo[a]) and apolipoprotein B (apoB) of low density lipoprotein (LDL) form a dissociable apo[a]:LDL complex. In the second step, a covalent disulfide linkage forms the stable Lp[a] particle. Several methods are currently used to study the assembly of Lp[a], however, these methods are laborious, time-consuming, and not suitable for a high throughput screening. We report here the development of a rapid and simple assay based on the binding of labeled LDL to a Lp[a]/apo[a] substrate which is immobilized on the surface of a microtiter plate. Quantification of bound LDL provides a measure of the extent of complex formation. Labeled LDL bound to both Lp[a] and apo[a] substrates with similar affinity. Plasma lipoproteins containing apoB as well as free apo[a] were capable of competing with LDL binding. The binding of LDL to Lp[a]/apo[a] was inhibited by L-proline and lysine analogs, which are known to inhibit the non-covalent association between apo[a] and apoB. Using this method we have found that nicotinic acid and captopril are able to inhibit the association of apo[a] with apoB. This method is compatible with automation and can be applied to a high throughput screening of inhibitors of Lp[a] formation.**—Dardik, B. N., C. D. Schwartzkopf, D. E. Stevens, and R. E. Chatelain. **A quantitative assay for the non-covalent association between apolipoprotein[a] and apolipoprotein B: an alternative measure of Lp[a] assembly.** ***J. Lipid Res.*** **2000.** 41: **1013–1019.**

Supplementary key words Lp[a] formation • nicotinic acid

Elevated levels of lipoprotein[a] (Lp[a]) are associated with an increased risk for premature coronary heart disease and stroke (1–3). Lp[a] is a heterogeneous lipoprotein formed by the covalent linkage of apolipoprotein[a] (apo[a]) to the apolipoprotein B-100 (apoB) of a low density lipoprotein (LDL) particle (4, 5). Plasma levels of Lp[a] are genetically determined and appear to be controlled by rates of synthesis rather than catabolism (6–9). Concentrations of Lp[a] are relatively insensitive to dietary changes and to most lipid-lowering drugs. Niacin, neomycin, and sex steroid hormones are the only agents known to decrease Lp[a] in humans (10–12) and the development of new therapies has been hampered by the limited understanding of the regulation of apo[a] synthesis and secretion.

Although apo[a] and apoB are synthesized in the liver, the main site of Lp[a] assembly in vivo is still unclear. Studies using primary baboon hepatocytes and transfected cell lines have convincingly demonstrated the extracellular assembly of Lp[a] (13–16). Other studies, however, suggest that apo[a] and apoB may be covalently linked before secretion (17, 18). Increasing evidence suggests that Lp[a] assembly proceeds in two steps. In the first step, non-covalent interactions between apo[a] and apoB form a dissociable apo[a]:LDL complex. In the second step, a disulfide bond forms the stable Lp[a] particle (19–23). Consequently, inhibition of Lp[a] assembly could provide a new approach to reduce plasma levels of Lp[a].

Several analytical methods have been used to examine the molecular interactions of apo[a] and Lp[a] with LDL, including sedimentation analysis (19), affinity chromatography (24), electrophoresis followed by fluorography or immunoblotting (23–25), ligand blotting (26), and immunochemical assays (21). These methods, however, are laborious, time consuming, and not suitable for high throughput screening.

The objective of the work reported here was to develop a simple, rapid, and high throughput assay mimicking the apo[a]:apoB interaction(s) occurring during assembly of the Lp[a] particle. We developed a method compatible with a microtiter plate format and consisting of immobilized Lp[a] and labeled LDL as the source of the apo[a] substrate and the ligand, respectively. This method can be used to screen for new inhibitors of Lp[a] formation as

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; apoB, apolipoproteinB; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ε-ACA, 6-amino-n-hexanoic acid.

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demonstrated by its ability to detect the inhibitory activity of nicotinic acid and captopril.

MATERIALS AND METHODS

Bovine serum albumin (fatty acid-free), fluorescein isothiocyanate (FITC), 6-amino-n-hexanoic acid, l-proline, tranexamic acid, captopril, nicotinic acid, and Tween 20 were purchased from Sigma Chemical Company (St. Louis, MO). ^{125}I-labeled LDL was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Microtiter plates (MicroFLUOR) were obtained from Dynex Technologies (Chantilly, VA). All chemicals and reagents were of analytical grade.

Isolation of lipoproteins

Lipoproteins were prepared from freshly drawn plasma obtained from healthy subjects with low (<1 mg/dl) and high (25–40 mg/dl) Lp[a] levels as determined by ELISA (ApoTek, Intracel Corp.). Blood was collected in EDTA (2 mm) and protease inhibitors were added to a final concentration of 2 μg/ml aprotinin, 10 μm leupeptin, and 1 μm pepstatin.

LDL (d 1.019–1.050 g/ml) and Lp[a] (d 1.075–1.12 g/ml) were isolated by sequential ultracentrifugation by adjusting densities with potassium bromide (27). All the solutions used to adjust the flotation density of the lipoproteins contained 1 mm EDTA and protease inhibitors. After isolation, lipoproteins were dialyzed against 0.15 m sodium phosphate buffer (PBS) pH 7.4 containing 1 μm EDTA, sterilized by passage through a 0.22 μm filter (Millipore Corp., Bedford, MA) and stored under nitrogen at 4°C. To remove contaminant HDL, the Lp[a] fraction was purified by affinity chromatography over heparin Sepharose 4B (Amersham Pharmacia Biotech) (28). A parallel Lp[a] fraction was purified by lysine-Sepharose (Amersham Pharmacia Biotech) (29).

All fractions were assayed for protein content with bovine serum albumin as a standard (Pierce BCA protein assay, Pierce), total cholesterol (Sigma Diagnostics), and Lp[a] protein (ApoTek, Intracel Corp.). The purity of the isolated lipoproteins was assessed by sodium dodecyl sulfate (SDS) electrophoresis in 2% agarose gels. Electrophoresis (11 V/cm) was carried out on a vertical apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 18°C. LDL preparations isolated from the plasma of low Lp[a] donors were free of Lp[a] as determined by gel electrophoresis and ELISA. Likewise the purified Lp[a] was devoid of contaminant LDL and HDL.

Apo[a] was obtained from purified Lp[a] by a procedure described by Edelstein et al. (30) involving a mild reductive reaction to cleave apo[a] from Lp[a].

All concentrations of lipoproteins and apo[a] were expressed in terms of their protein content.

Lipoprotein labeling

Low density lipoprotein (1 mg/ml) in 0.1 m $NaHCO_3$ (pH 9.0) was admixed with 10 μl/ml of FITC (2 mg/ml in dimethyl sulfoxide) (31). The mixture was gently mixed by inverting the tube followed by slow rocking at room temperature under argon for 1 h. The unreacted dye was removed by gel filtration on a Sephadex G-25 column equilibrated with PBS-EDTA buffer. The fluorophore/protein ratio was calculated from the emission intensity of FITC-LDL at 530 nm (excitation 485 nm) and the absorbance at 492 and at 280 nm. The integrity of the labeled LDL was assessed by agarose gel electrophoresis.

Immobilization of Lp[a] on microtiter plates

MicroFLUOR plates were incubated with 100 μl/well of Lp[a] at 10 μg/ml in PBS containing 10 μm EDTA, pH 7.4, for 18 h at 4°C. To prevent non-specific binding, wells were blocked with 200 μl of PBS containing 3% BSA for a 2-h period at room temperature. The wells were rinsed three times with PBS/BSAT (PBS, 0.5% BSA, and 0.01% Tween 20) and 100 μl of PBS/EDTA containing sodium azide was added to each well. The plates can be stored with this solution for no more than 3 days at 4°C, or dry for up to 3 months at −70°C. Plates were coated with free apo[a] at 1 μg/ml as described for Lp[a].

Binding and quantification of bound FITC-LDL

Binding of FITC-LDL to immobilized Lp[a] was performed with labeled LDL (1–2 μg/ml, 100 μl/well) for 1 h at room temperature. The incubation buffer was discarded and, after three rinses with PBS/BSAT, the bound FITC-LDL was solubilized by adding 100 μl of 1% SDS (pH 9.0). Fluorescence was measured in a microtiter plate fluorometer (Fluorolite 1000, Dynex) with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. A standard curve with known FITC-LDL concentrations was obtained for each assay to convert relative fluorescence units to ng.

Quantification of bound ^{125}I-labeled LDL

MicroFLUOR removawells strips were used in experiments using ^{125}I-labeled LDL, but otherwise binding conditions were the same as described above. After three washes the individual wells were removed from the strips and the bound radioactivity was determined by gamma counting (LKB 1282 COMPUGAMMA, Wallac Inc., Gaithersburg, MD).

Analysis of binding data was performed by using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Assay development

In initial experiments several buffer systems were evaluated for coating the plates with Lp[a]. Adsorption of Lp[a] to the surface of the wells was slightly better in tris- or phosphate buffer, pH 7.4, than in carbonate buffer, pH 9.6 (data not shown).

The optimal coating concentration of Lp[a] to obtain maximum binding of LDL was determined in wells coated at different Lp[a] concentrations. A constant amount of 2 μg/ml of FITC-LDL was used for binding and concentrations of Lp[a] in the range of 0.5–20 μg/ml were used for coating. There was a gradual increase in LDL binding up to a Lp[a] coating concentration of 10 μg/ml, followed by a plateau at higher concentrations (**Fig. 1**). Similar results were obtained with three different Lp[a] preparations. Therefore, a concentration of 10 μg/ml was used for coating the wells in all the subsequent experiments. Plates coated under these conditions were stored up to 3 months at −70°C with no change in binding capacity. The optimal coating concentration of free apo[a] determined in a similar manner was 1–2 μg/ml.

The binding of labeled LDL to immobilized Lp[a] was studied as a function of time using a concentration of labeled LDL of 2 μg/ml. Binding was rapid and already significant at 10 min, reached 70% of the maximal binding at 1 h, and slowly continued to increase up to 5 h. Based on these results, a 1-h binding time period was chosen for the assay (data not shown).

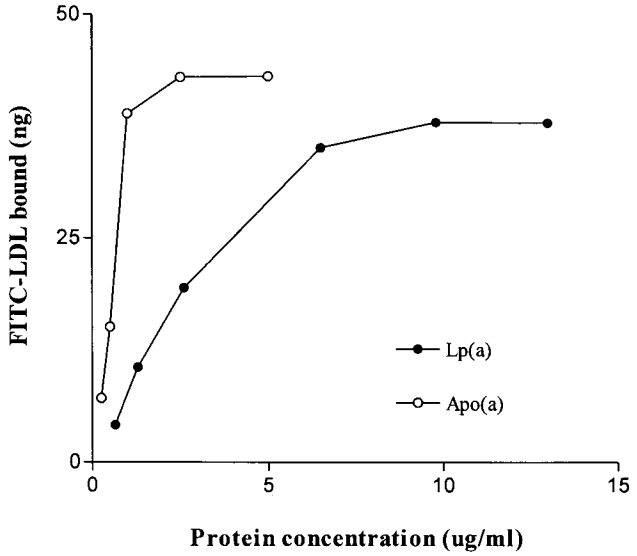

Fig. 1. Effect of Lp[a] and apo[a] coating concentration on LDL binding. Wells were coated with 100 μl of increasing concentrations of Lp[a] and apo[a] in phosphate buffer, pH 7.4, for 18 h at 4°C. FITC-LDL at 2 μg/ml was added for 1 h at 25°C. Coating concentrations represent total protein. Bound LDL was determined as described in Materials and Methods. Values are mean ± SEM (n = 3).

Comparison of binding of LDL to immobilized Lp[a] and apo[a]

Comparison of binding of LDL to immobilized Lp[a] and immobilized free apo[a] was performed to validate the use of the Lp[a] particle instead of purified apo[a] as the substrate for this assay. As shown in **Fig. 2**, binding of FITC-LDL to Lp[a] and apo[a] increased in a dose-dependent manner and appeared to approach saturation at a concentration of 10 μg/ml. The binding affinity of LDL to both Lp[a] and apo[a] was very similar with an apparent K_d of 3.3 ± 0.3 nm for Lp[a] and a K_d of 1.5 ± 0.2 nm for apo[a]. Nonspecific binding was determined in wells incubated with unlabeled LDL at 500 μg/ml. At concentrations of labeled LDL of 1–2 μg/ml, nonspecific binding was generally less than 5–10% of the total binding whereas the nonspecific binding increased at concentrations above 10 μg/ml. A similar K_d (4.6 ± 0.6 nm) was obtained with immobilized Lp[a] that was affinity purified on lysine-Sepharose.

Binding of FITC-LDL and ^{125}I-labeled LDL to immobilized Lp[a]

To verify that FITC-labeling of LDL did not affect its functional integrity, the affinity of FITC-LDL to immobilized Lp[a] was compared to that of ^{125}I-labeled LDL. As shown in **Fig. 3**, typical concentration response isotherms were observed with both ligands. Non-linear regression analysis of the data revealed an apparent K_d of 3.2 ± 0.3 nm and 2.2 ± 0.2 nm for FITC-LDL and ^{125}I-labeled LDL, respectively. In addition, both FITC and ^{125}I-labeled LDL were able to compete with unlabeled LDL for binding to immobilized Lp[a] (Fig. 3) with the same inhibition constant (K_i) of approximately 3 nm.

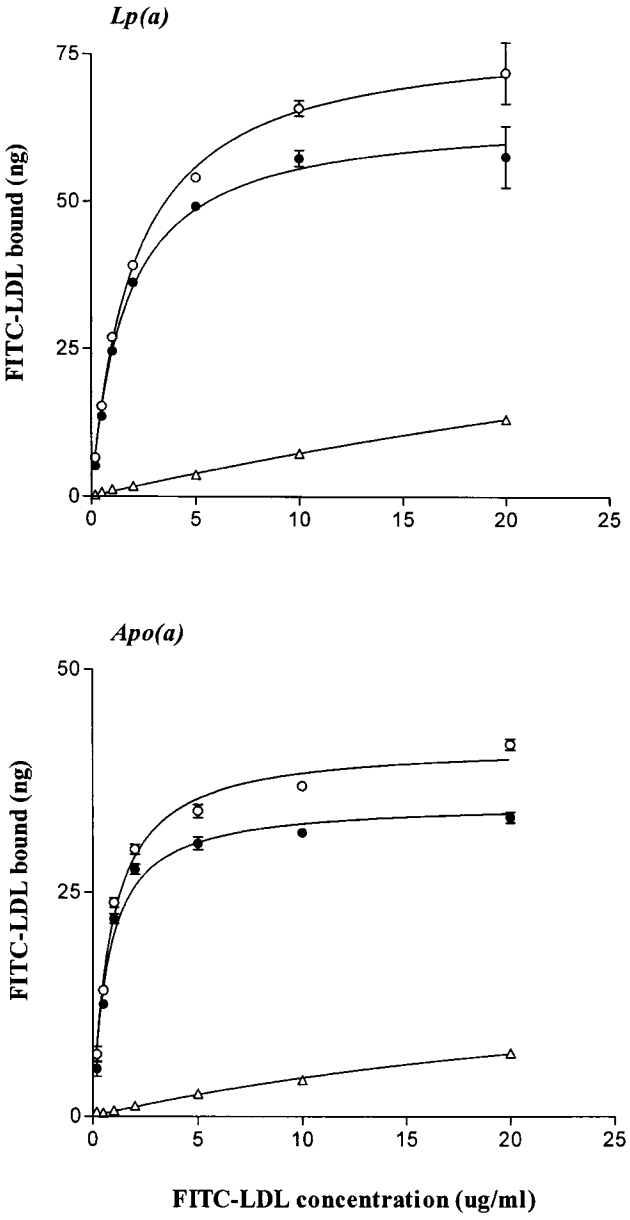

Fig. 2. Comparison of binding of LDL to Lp[a] and apo[a]. FITC-LDL at increasing concentrations (0–20 μg/ml) was incubated with immobilized Lp[a] or apo[a] for 1 h at 25°C. Coating concentrations were 10 μg/ml for Lp[a] and 1 μg/ml for apo[a]. Concentrations refer to total protein. Bound LDL was solubilized in SDS and the fluorescence was measured as described in Materials and Methods. Nonspecific binding was determined in the presence of 500 μg/ml of unlabeled LDL. Values are mean ± SEM (n = 3) for each experiment. Top panel: Lp[a]; bottom panel: apo[a]. Total binding (○), specific binding (●), and non-specific binding (△).

Inhibition of LDL binding to Lp[a] by apoB-containing lipoproteins and free apo[a]

To establish the specificity of the apoB:apo[a] interaction, competition binding studies were performed with FITC-LDL (2 μg/ml) in the presence of increasing concentrations of unlabeled LDL, VLDL (very low density lipoprotein), and HDL (high density lipoprotein). As shown

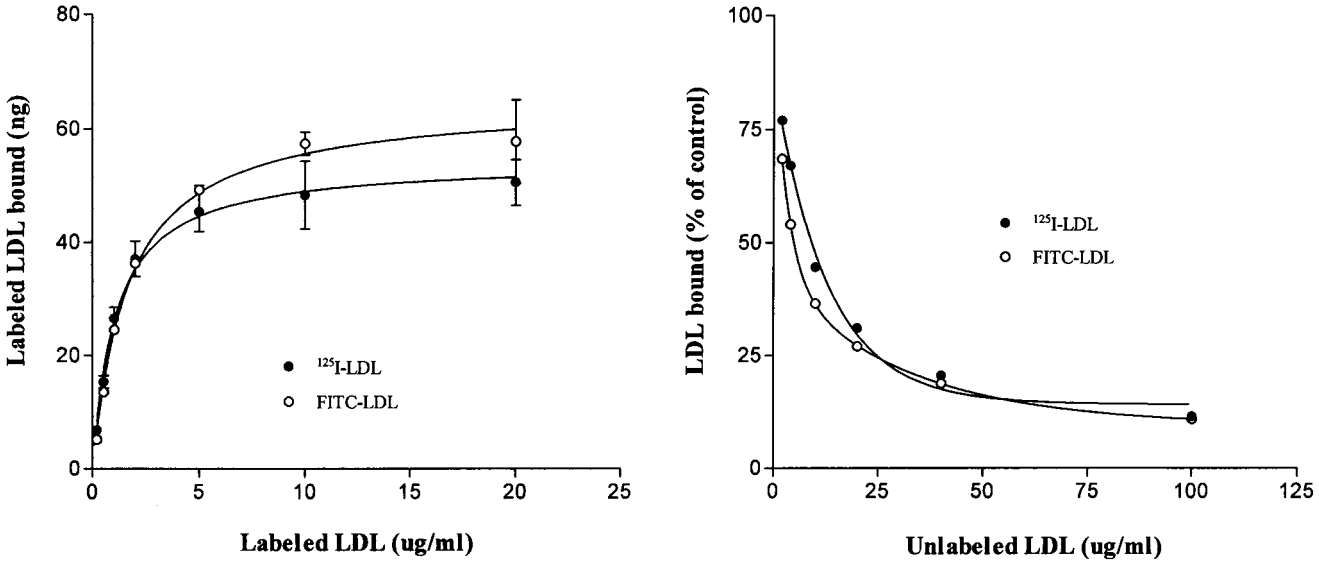

Fig. 3. Binding of FITC-LDL and ^{125}I-labeled LDL to Lp[a]. Left panel: Increasing concentrations (0–20 μg/ml)) of FITC-LDL and ^{125}I-labeled LDL were incubated with immobilized Lp[a] for 1 h at 25°C. Bound LDL was measured for each ligand. Non-specific binding was determined in the presence of 500 μg/ml of unlabeled LDL. Each curve represents specific binding. Values are mean ± SEM (n = 3) for each experiment. Right panel: FITC-LDL and ^{125}I-labeled LDL (2 μg/ml) were incubated with immobilized Lp[a] in the presence of various concentrations (0–100 μg/ml) of unlabeled LDL for 1 h at 25°C. The amount of LDL bound was expressed as percentage of the LDL bound in the absence of unlabeled LDL. Values are mean ± SEM (n = 3) for each experiment. ^{125}I-labeled LDL (●), FITC-LDL (○).

in **Fig. 4**, lipoproteins containing apoB (LDL and VLDL) inhibited the binding of LDL to Lp[a] and apo[a]. However, the binding was minimally affected by HDL, which is devoid of apoB (data not shown). The estimated half maximal inhibition (IC_{50}) values for LDL and VLDL were of approximately 3 and 6 μg/ml, respectively. A similar competitive inhibition was observed when immobilized free apo[a] was used as the substrate for binding LDL with an estimated IC_{50} of 2 and 3 μg/ml for LDL and VLDL, respectively (Fig. 4).

To exclude the possibility that adsorption of the Lp[a] particle to the plastic could induce conformational changes that would either hinder or enhance its interaction with apoB, binding of FITC-LDL to immobilized Lp[a] was

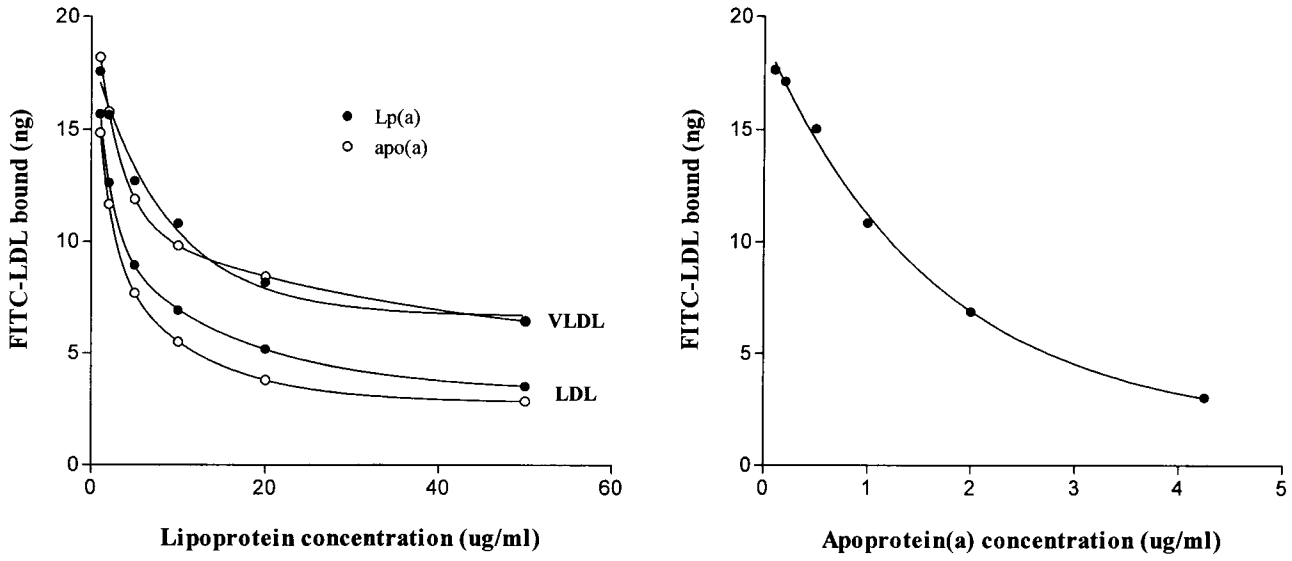

Fig. 4. Inhibition of binding of LDL to Lp[a] by apoB-containing lipoproteins and free apo[a]. Left panel: FITC-LDL (2 μg/ml) was incubated with immobilized Lp[a] (●) and immobilized apo[a] (○) in the presence of various concentrations (0–50 μg/ml) of unlabeled LDL or VLDL for 1 h at 25°C. Right panel: FITC-LDL (2 μg/ml) was incubated with immobilized Lp[a] in the presence of various concentrations (0–5 μg/ml) of soluble free apo[a] for 1 h at 25°C. Bound LDL was determined as described in Materials and Methods. Values are means of three determinations for each experiment.

performed in the presence of increasing concentrations of soluble free apo[a]. As shown in Fig. 4, free apo[a] inhibited the binding in a concentration-dependent manner with an estimated IC_{50} of 2 μg/ml.

Inhibition of LDL binding to Lp[a] by l-proline and lysine analogs

The interaction of apo[a]:apoB has been shown to be inhibited by proline and lysine analogs in various assay systems (19, 21, 23–26). Therefore, we evaluated the effect of l-proline and two lysine analogs (ε-ACA and tranexamic acid) in this assay. The results (**Fig. 5**) showed a concentration-dependent inhibition of LDL binding to Lp[a] with half-maximal inhibition at 94, 19, and 29 mm for ε-ACA, tranexamic acid, and proline, respectively. These agents were also equally effective in inhibiting the binding of LDL to immobilized apo[a] (Fig. 5) with an IC_{50} of 71, 15, and 27 mm for ε-ACA, tranexamic acid, and proline, respectively.

Identification of inhibitors of the binding of LDL to Lp[a]

The assay was also used to examine whether nicotinic acid, known to lower Lp[a] levels in humans, would affect the binding of LDL to Lp[a]. The results shown in Fig. 5 indicate that nicotinic acid inhibited LDL binding to Lp[a] and apo[a] in a concentration-dependent manner with an IC_{50} of 39 and 42 mm, respectively. Based on these results we performed a cursory evaluation of agents known to affect plasma lipids or the development of atherosclerosis and have found that captopril was also able to inhibit binding of the LDL to Lp[a] and apo[a] with an IC_{50} of 34 and 76 mm, respectively. The inhibition curves are shown in Fig. 5.

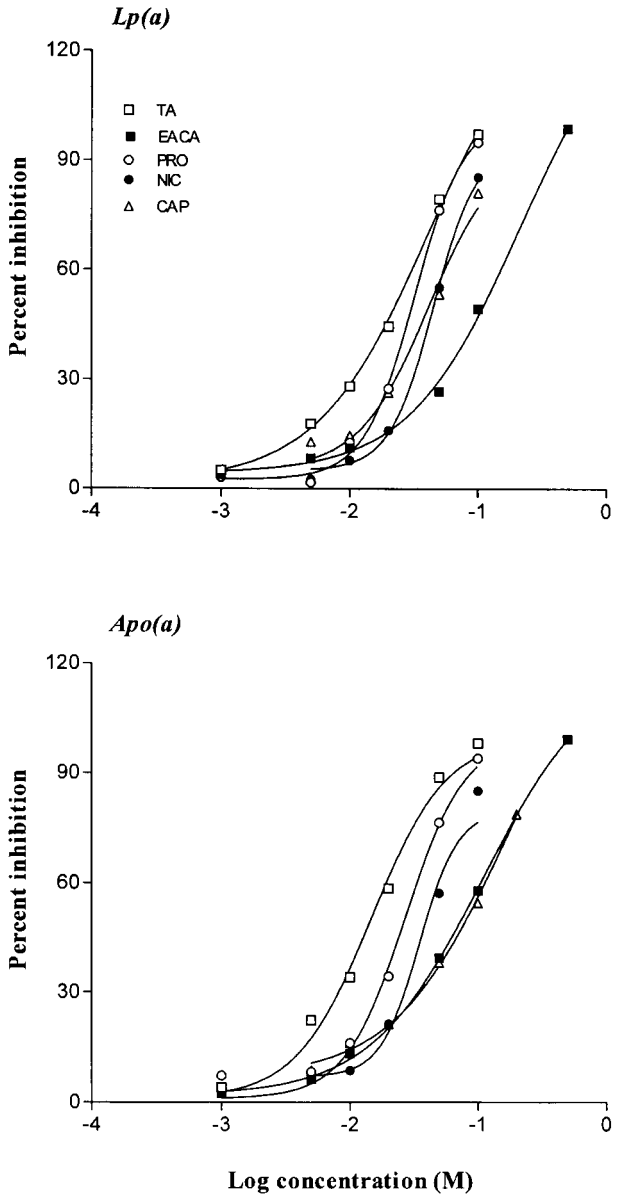

Fig. 5. Inhibition of binding of LDL to Lp[a] and apo[a] by proline, lysine analogs, nicotinic acid, and captopril. FITC-LDL (2 μg/ml) was incubated with immobilized Lp[a] (top panel) and immobilized apo[a] (bottom panel) in the presence of various concentrations (0–500 mm) of tranexamic acid (□), ε-ACA (■), l-proline (○), nicotinic acid (●), and captopril (△) for 1 h at 25°C. Bound LDL was determined as described in Materials and Methods. Values are means of three determinations for each experiment. Results are expressed as percent inhibition of LDL binding compared to the control binding measured in the absence of inhibitors.

DISCUSSION

This study describes the development of a simple, rapid, and high throughput assay to measure the non-covalent association between apo[a] and apoB that occurs during assembly of the Lp[a] particle. This assay has several advantages. First, it consists of three simple and robust steps (plate coating, binding, and detection) that can be performed in a single microtiter plate. Second, plates can be prepared in large quantities and stored for more than 3 months at −70°C. This feature reduces the need for fresh Lp[a] preparations and the difficulties that arise upon storage of soluble Lp[a] such as instability and self-association. Third, either Lp[a] or apo[a] can be used as the substrate. Fourth, LDL can be labeled with different probes thereby making the assay amenable to use with several detection systems (colorimetric, fluorescent, radioactive). Finally, this method is compatible with automation and can be applied to a high throughput screening.

An advantage used in the development of this method was the feasibility of using the whole Lp[a] particle as a surrogate apo[a] substrate. This alternative was selected based on the studies of Trieu et al. (26, 32) who established that both Lp[a] and recombinant apo[a] exhibited similar binding constants when complexed with LDL. Their findings suggested that formation of the non-covalent Lp[a]:LDL and apo[a]:LDL complexes are mediated by the same site(s) on apo[a]. Accordingly, we compared the binding of LDL to immobilized Lp[a] and free apo[a] and confirmed the observation that LDL binds to apo[a] and Lp[a] with similar affinity. The K_d values obtained in our studies (1.5–3.3 nm) were lower than the values (10–20 nm) reported by Trieu et al. (26, 32) but were in agreement with the K_d reported by Phillips et al. (19).

Although the binding affinity of LDL to Lp[a] and free apo[a] was the same, the amount of LDL bound to Lp[a] and apo[a] was different. Because of differences in the coating efficiency of Lp[a] and apo[a], this assay was not designed to compare the total binding (B_{max}) of apoB to Lp[a] and apo[a] but rather to determine the affinity of the binding. In this context it should be emphasized that similar K_ds were obtained with different Lp[a]/apo[a] preparations and, also, that the same K_ds were observed despite the dissimilar amounts of LDL that bound to Lp[a] and apo[a]. The effect of apo[a] size was not investigated in this assay. However, several studies using recombinant apo[a] fragments have shown that kringle IV-2, which determines the size of apo[a], is not directly involved in the binding of apo[a] to apoB (21–23, 25).

The ability of free apo[a] and apoB-containing lipoproteins to competitively inhibit the binding of LDL to Lp[a] and apo[a] demonstrated the specificity of the apo[a]:apoB interaction. Moreover, the inhibitory effect of soluble free apo[a] confirmed that the assay indeed measured a genuine apo[a]:apoB interaction and not a condition peculiar to immobilized Lp[a].

FITC labeling of apoB did not affect the LDL affinity to Lp[a] as evidenced by the binding kinetics that revealed a K_d identical to that obtained with ^{125}I-labeled LDL. This observation was further supported by the ability of both ligands to compete equally with unlabeled LDL.

Inhibition of LDL binding to Lp[a] and apo[a] was observed with l-proline and lysine analogs, agents known to interfere with the non-covalent interaction between apo[a] and apoB (19, 21, 23–26). In our studies, l-proline was a more effective inhibitor than ε-ACA. The IC_{50} values for l-proline were similar to those previously reported by other investigators (19, 23, 26), whereas the IC_{50} values for ε-ACA were more variable and tended to be higher than those reported by Frank et al. (33) and Koschinsky et al. (23) who used recombinant apo[a] in their assembly studies.

A novel observation emerging from our studies was the inhibitory effect of nicotinic acid. This finding suggests that the lowering of Lp[a] in vivo by this agent may, at least in part, be related to the ability of nicotinic acid to interfere with the apo[a]:apoB interaction during assembly of the Lp[a] particle.

In addition, we observed that the ACE inhibitor captopril also interferes with the association between apo[a] and apoB in vitro. The inhibitory effect of this agent may be due to the proline content or other features of the molecule. However, the concentrations of nicotinic acid and captopril required to inhibit the apo[a]:apoB complex formation in vitro are higher than their respective plasma concentrations (NA: 126 μm; CAP: 1 μm) observed in human studies at therapeutic doses of the drugs (34, 35). Therefore, further studies are needed to better understand these effects, but to our knowledge this is the first report describing an inhibitory effect of nicotinic acid and captopril on the assembly of Lp[a].

In conclusion, the results of the studies reported here suggest that this method could provide a useful tool to identify novel inhibitors of Lp[a] formation. These selective compounds could be used to modulate plasma levels of Lp[a] and would be helpful in establishing the efficacy of reducing Lp[a] levels in the prevention and treatment of coronary heart disease.

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