We investigated whether apolipoprotein B-containing lipoproteins could bind to the insoluble complexes of lipoprotein (a) (Lp(a)) induced by Ca\(^{2+}\). Lp(a), but not low density lipoprotein (LDL), very low density lipoprotein (VLDL), or high density lipoprotein (HDL) (HDL\(_a\) or HDL\(_b\)) for positive charge on LDL reduced this interaction. At higher Ca\(^{2+}\) concentrations, the binding of acetyl LDL to Lp(a) in the insoluble complexes was greater than that of LDL. Since more Ca\(^{2+}\) was required for concentration-dependent saturation of acetyl LDL binding, it is likely that Ca\(^{2+}\) cross-bridging was responsible for this binding. Thus, LDL, especially its modified forms, could contribute to the formation of insoluble complex of Lp(a) with Ca\(^{2+}\) in atherosclerotic lesions and help explain its preferential accumulation there.

Although Lp(a)\(^3\) is considered to be a risk factor for cardiovascular diseases (1–4), neither its potential atherogeneity nor its physiological function has been clearly elucidated. Lp(a) resembles LDL in composition except that it also possesses a unique protein, apolipoprotein (a) (apo(a)), which is covalently linked to apolipoprotein B (apoB) via a disulfide bond (5, 6). The presence of N- and O-linked glycosylation sites in each kringle allows apo(a) to bear a high carbohydrate content. This carbohydrate has been shown to contain a high content of sialic acid, which probably contributes to the high negative charge of Lp(a) at physiologic pH (7–10).

Apo(a) is characterized by possessing multiple kringle 4 repeats of plasminogen (7, 8), and Lp(a) was shown to compete directly with plasminogen for sites on fibrin (11, 12) and vascular cells (13, 14) under in vitro conditions. These results suggest that Lp(a) may possess antifibrinolytic properties. In addition, Lp(a) could also be atherogenic by accumulating in atherosclerotic lesions and then contributing in some yet undetermined manner to accelerate the atherosclerotic process. We and others have shown previously that Lp(a) accumulates in human atherosclerotic lesions to an even greater extent than LDL, when normalized for equivalent plasma concentrations (15–17). Moreover, plasma and tissue Lp(a) contents correlated significantly (15). Some of this accumulation could have resulted from particle aggregation after complexing with Ca\(^{2+}\). Although two reports show that Lp(a) undergoes aggregation in the presence of Ca\(^{2+}\) (18, 19), no data were provided giving any indication of the underlying mechanism(s). Therefore, one of the aims of this current study was to determine the underlying mechanism of Ca\(^{2+}\)-induced aggregation of Lp(a).

In this report we show that Lp(a) forms aggregates, even in the presence of physiologic Ca\(^{2+}\) concentrations, and that they result from Ca\(^{2+}\) cross-bridges formed between sialic acids on separate particles. Furthermore, we report that LDL or VLDL co-precipitate with Lp(a) in the presence of Ca\(^{2+}\), probably in the form of complexes, and that the putative LDL-Lp(a) interaction appears to be ionic in nature involving positive charges on apoB in LDL. Finally, we report that electronegative forms of modified LDL such as acetyl LDL interact more avidly than unmodified LDL with Lp(a) in the presence of Ca\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Materials**

Carrier-free Na\(^{125}\)I was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Aprotinin, leupeptin, pepstatin, Na\(_2\)EDTA, NaBr, NaNO\(_3\), acetic anhydride, sodium acetate, sodium borate, sodium bo-
isolation of the degree of insoluble complex formation—purified Lp(a) and other lipoproteins in stock solutions were dialyzed against 200-fold volumes of 5 mM Tris-HCl (pH 7.2) using Sephacryl 2 dialysis tubing prior to experiments of insoluble complex formation. Lp(a) (final concentration, 100 µg of cholesterol/ml) was mixed with varying concentrations of CaCl₂ in the presence or absence of appropriate amounts of VLDL, LDL, or HDL (final volume, 50 µl) and kept for 10 min at room temperature in microtiter plates (96 wells). The amount of insoluble complex formed was then estimated by turbidity at 600 nm using a microplate reader (Bio-Rad model 3550). The identity of lipoprotein solutions containing CaCl₂, was measured as a blank. The amount of Lp(a) in the form of insoluble complexes was also determined by measuring the amount of Lp(a) cholesterol precipitated. Insoluble complex formation was carried out in microcentrifuge tubes (0.5 ml) instead of a microplate (final volume, 50 µl), and tubes were subjected to centrifugation (10,000 rpm, 10 min) to precipitate insoluble complexes. The precipitates were then washed twice with 5 mM of Tris-HCl (pH 7.2) containing the same concentration of CaCl₂. Cholesterol contents in the precipitates were measured after resolubilizing with equivalent volumes of 0.5 M NaCl and 1 mM Na₂EDTA. To determine the amount of Lp(a) and LDL (or modified LDL) in the insoluble complexes expressed as cholesterol content, 125I-Lp(a) or 125I-LDL (modified LDL) was used in separate experiments. After precipitating the insoluble complexes, the tips of tubes containing the precipitates were cut off with a blade after aspirating the supernatants. Subsequently, the radioactive precipitates of supernatants were individually counted and converted into cholesterol content.

Electrophoresis—The electrophoretic mobility of the lipoprotein was determined on prenatem 1% agarose gels following the manufacturer's instructions except that electrophoresis was performed at 50 volts for 90 min. Gels were stained for lipid using 0.025% Fat Red 7B in 60% methanol. The protein components of reduced and nonreduced lipoproteins were analyzed by SDS-PAGE. Lipoprotein samples were solubilized in the buffer containing 2% SDS, 60 mM Tris, 10% glycerol, and 0.01% bromphenol blue. Reduced samples were prepared by boiling at 100 °C for 7 min in the presence of 5% 2-mercaptoethanol, and appropriate amounts of samples were applied on precasted 15% gradient acrylamide gels and electrophoresed at 12.5 mA for 30 min using the Phast Gel automated system (Pharmacia). After electrophoresis, proteins were visualized by silver staining.

Chemical Analysis—Protein was measured by the bicinchoninic acid assay as described by Smith et al. (31) except that a 1-h 60 °C heating step was used. Bovine serum albumin was used as a standard. Cholesterol were determined by the procedure of Roeschlaub et al. (32).

RESULTS

Insoluble Complex Formation of Lp(a) with Ca²⁺—To determine the dependence of the insoluble complex formation of Lp(a) on Ca²⁺ concentration, we mixed Lp(a) (100 µg of cholesterol/ml) with Ca²⁺ at concentrations ranging from 0 to 40 mM and estimated the degree of insoluble complex formation by solution turbidity. As low a concentration as 1.25 mM caused significant increases in solution turbidity, which reached a maximum at 20 mM and then decreased at higher Ca²⁺ concentrations (Fig. 2). The same result was obtained with separate Lp(a) samples derived from different donors (not shown). To assess the specificity of such Ca²⁺-induced aggregation of lipoproteins, we also mixed VLDL, LDL, or HDL (100 µg of cholesterol/ml) with Ca²⁺ under the same conditions as performed with Lp(a). However, none of these lipoproteins induced significant increases in solution turbidity.
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**FIG. 1. Insoluble complex formation of Lp(a) with Ca\(^{2+}\).** Lp(a), VLDL, LDL, and HDL\(_{3}\) cholesterol (final concentration, 100 \(\mu\)g/ml) were separately mixed with increasing concentrations of CaCl\(_2\) for 10 min at 25 °C. The degree of insoluble complex formation (panel a) was then determined by measuring turbidity at 600 nm, and the cholesterol content of the insoluble complex of Lp(a) (panel b) was measured as described under "Experimental Procedures."

(Fig. 1a), thus demonstrating the specificity of Lp(a) for such aggregation. To verify that the increase in turbidity reflected insoluble complex formation of Lp(a), we also determined the amount of Lp(a) cholesterol precipitated by low speed centrifugation. This index of particle aggregation closely mimicked solution turbidity, except that maximum precipitation (86%) occurred at lower Ca\(^{2+}\) concentrations (2.5 mM) (Fig. 1b) than did turbidity. Given that physiologic concentrations of Ca\(^{2+}\) range from 2.5 to 5 mM (18, 19), these results suggest that Lp(a) specifically forms insoluble complexes at physiologic Ca\(^{2+}\) concentrations under these experimental conditions.

To determine whether the highly electronegative surface charge on Lp(a) was responsible for inducing particle aggregation in the presence of Ca\(^{2+}\) by forming ionic cross-bridges, we asked whether the reduction of the negative surface charge would inhibit particle aggregation. Since most of the electronegativity of Lp(a) is caused by the high sialic acid content of apo(a) (9), we studied neuraminidase digestion of sialic acids in Lp(a) would prevent insoluble complex formation. When Lp(a) was digested with neuraminidase, a time-dependent decrease in the electrophoretic charge of Lp(a) was observed by agarose electrophoresis (Fig. 2). The sialic acid content of Lp(a) decreased from 85.4 to 59.4 \(\mu\)g/mg lipoprotein protein after a 6-h incubation, representing a 30% decrease. The sialic acid content of unmodified Lp(a) was similar to values reported previously (33). No degradation of apo(a) or apoB could be observed by SDS-PAGE (not shown), which would have indicated the presence of proteases in the neuraminidase samples being used or associated with Lp(a) particles. Neuraminidase treatment of Lp(a) for at least 6 h resulted in an inhibition of insoluble complex formation, especially at higher (5–10 mM) Ca\(^{2+}\) concentrations (Table I).

When excess sialic acid was added to the mixture of Lp(a) and Ca\(^{2+}\), the insoluble complex formation was also inhibited (not shown). These results strongly suggest that the mechanism of aggregation of Lp(a) is via Ca\(^{2+}\) cross-bridges involving sialic acids on different Lp(a) particles. However, since a reduction in sialic acid contents of 30% by neuraminidase was sufficient to inhibit Ca\(^{2+}\)-induced aggregation, it would appear that only a smaller percentage of the total sialic acids in Lp(a) forms cross-bridges with Ca\(^{2+}\).

**Involvement of Other Lipoproteins in The Insoluble Complex Formation of Lp(a) with Ca\(^{2+}\)**—Since Lp(a) has been recently shown to demonstrate specific binding with LDL and VLDL (20–22), we asked whether these lipoproteins might contribute to the bulk of aggregated Lp(a) by directly interacting with Lp(a) if present during Ca\(^{2+}\)-induced aggregation. We therefore mixed Lp(a) with LDL, VLDL, or HDL\(_{3}\) (all at 100 \(\mu\)g of cholesterol/ml) in the presence of increasing concentrations of Ca\(^{2+}\) and estimated the degree of insoluble complex formation by turbidity. The addition of both LDL and VLDL increased the turbidity relative to Lp(a) alone at increasing Ca\(^{2+}\) concentrations (Fig. 3a). This increase was also found for individual LDL and VLDL samples derived from different donors (not shown). By contrast, the addition of HDL\(_{3}\) actually decreased the turbidity relative to Lp(a) alone at lower Ca\(^{2+}\) concentrations (Fig. 3a). We also measured the amount of cholesterol in the insoluble complexes formed in the presence of both LDL and Lp(a) with increasing Ca\(^{2+}\) concentrations (Fig. 3b) and compared these amounts with those obtained for Lp(a) alone (Fig. 1b). The addition of LDL led to a substantial increase in cholesterol precipitated over the range of Ca\(^{2+}\) concentrations (Fig. 3b) relative to Lp(a) alone (Fig. 1b). In particular, at physiologic Ca\(^{2+}\) concentrations, the precipitated cholesterol was twice as much as that of
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FIG. 3. VLDL and LDL involvement in the insoluble complex formation of Lp(a) with Ca2+. Lp(a) (final concentration, 100 μg/ml) and equivalent amounts of VLDL, LDL, or HDL cholesterol were individually mixed with increasing concentrations of CaCl2. The degree of insoluble complex formation (panel a) was determined by measuring turbidity at 600 nm, and the cholesterol content in the insoluble complexes (panel b) was measured as described under “Experimental Procedures.”

Lp(a) alone, suggesting that LDL indeed co-precipitated with Lp(a) at these Ca2+ concentrations. Maximum co-precipitation of Lp(a) and LDL (∼84% of total cholesterol added) occurred at 2.5 mM Ca2+ and then decreased at higher Ca2+ concentrations.

To determine the actual amounts of Lp(a) and LDL co-precipitated in such insoluble complexes and also to understand the complexing mechanism, we performed the complexing interactions with a fixed amount of Lp(a) and three different concentrations of LDL in the presence of increasing concentrations of Ca2+. The amounts of Lp(a) and LDL co-precipitated were determined using 125I-Lp(a) or 125I-LDL in separate experiments. The maximum precipitation of Lp(a) (>95%) was achieved between Ca2+ concentrations ranging from 2.5 to 15 mM (Fig. 4a). The amount of Lp(a) precipitated was independent of the amounts of LDL added. A gradual decrease in Lp(a) precipitated was observed at concentrations of Ca2+ greater than 15 mM. When the amount of LDL co-precipitated with Lp(a) was assessed (Fig. 4b), the maximum was found at the same Ca2+ concentration (2.5 mM) and was independent of amounts of LDL added. The amount of LDL co-precipitated then sharply decreased at higher Ca2+ concentrations as we showed previously for total cholesterol in the precipitate (Fig. 3b).

To determine the molar ratio of LDL to Lp(a) in the insoluble complexes at saturation, we carried out the complexing interactions with a fixed amount of Lp(a) and increasing concentrations of LDL in the presence of 2.5 mM Ca2+ as described previously. As we increased the amount of LDL added up to 600 μg of cholesterol/ml, the amount of Lp(a) precipitated remained the same (95 μg) (Fig. 5). By contrast, LDL showed a dose-dependent increase in incorporation into the precipitate, which reached saturation (126 μg) at 600 μg/ml of LDL added. This gave a molar ratio of 5:4 (LDL to Lp(a)).

Possible Mechanisms of Interaction Between LDL and Lp(a) in Ca2+-induced Insoluble Complexes Formation—If the binding of LDL to Lp(a) in Ca2+-induced aggregates was ionic, and the positive charge on LDL was responsible, as suggested by the inhibition of LDL binding with increasing Ca2+ concentrations (Fig. 4b), increasing the positive charge on LDL should increase the interactions with Lp(a). By contrast, decreasing the positive charge should decrease this interaction. To verify this mechanism, we studied the effect of chemically modifying LDL on the formation of insoluble complexes. When LDL was digested with neuraminidase to remove sialic acids, thereby increasing the net positive charge as described previously for Lp(a), more than a twofold increase was found in the binding of desialylated LDL relative to unmodified LDL (Table II). We then asked whether decreasing the positive charge of such basic amino groups as lysines on apoB in LDL would decrease the interaction with Lp(a). When lysine residues on apoB were modified by acetylation to neutralize the positive charge, the binding of acetyl LDL was reduced relative to unmodified LDL. However, since the
TABLE II

Effect of LDL modifications on the formation of Ca\(^{2+}\)-induced insoluble complexes

| LDL type                  | Precipitated cholesterol |
|---------------------------|--------------------------|
|                           | Lp(a)    | LDL  |
| Native LDL                | 95 ± 3.1 | 126 ± 3.3 |
| Neuraminidase-treated LDL | 98 ± 3.0 | 302 ± 22.8 |
| Acetyl-LDL                | 48 ± 2.3 | 62 ± 5.0  |
| Methylated LDL            | 97 ± 2.1 | 130 ± 6.0  |

The amount of Lp(a) precipitated was also reduced when acetyl LDL was added (Table II), we cannot conclude whether the reduced acetyl LDL present in insoluble complexes was related to a reduced interaction with Lp(a) or to reduced precipitation of Lp(a). When the positive charge on LDL was further reduced by blocking arginine residues on apoB with cyclohexadione, no insoluble complex formation occurred with Lp(a) and 2.5 mM Ca\(^{2+}\) in the presence of increasing concentrations of free L-proline (from 0 to 0.1 M). Lp(a) and LDL cholesterol in the insoluble complexes were then individually determined from the amount of radioactivity precipitated as described under "Experimental Procedures."

LDL was different from the one occurring in Ca\(^{2+}\)-induced Lp(a) precipitates.

**Enhanced Binding of Acetyl LDL to Lp(a) in Insoluble Complexes**—We initially employed acetylation of LDL to illustrate the importance of positive charges on LDL in the interaction between LDL and Lp(a). However, acetyl LDL inhibited the insoluble complex formation of Lp(a) at 2.5 mM Ca\(^{2+}\) (Table II), suggesting competitive binding with Lp(a) for Ca\(^{2+}\). This result led us to hypothesize that the binding of acetyl LDL may be enhanced if the Ca\(^{2+}\) concentration was sufficiently high. To test this hypothesis, we determined the amounts of acetyl LDL and Lp(a) present in the insoluble complexes with increasing concentrations of Ca\(^{2+}\) (Fig. 7, a and b). Acetyl LDL inhibited Lp(a) insoluble complex formation in a concentration-dependent manner at low Ca\(^{2+}\) concentrations (1.25 and 2.5 mM) (Fig. 7a). However, at higher Ca\(^{2+}\) concentrations (5–20 mM) the amount of Lp(a) precipitated was independent of the amount of LDL (Fig. 4a) or acetyl LDL (Fig. 7a) added.

When the amount of acetyl LDL in the precipitate was determined, major differences relative to LDL were found (Figs. 4b and 7b). LDL showed a maximum co-precipitation at 2.5 mM Ca\(^{2+}\) which remained the same regardless of the LDL concentrations added (Fig. 4b). By contrast, the Ca\(^{2+}\) concentration at which co-precipitation of acetyl LDL reached saturation, increased with increasing acetyl LDL added (Fig. 7b). It is also of note that when 600 μg/ml acetyl LDL was added at the upper level of physiologic Ca\(^{2+}\) concentrations, e.g. 5 mM, 250 μg of acetyl LDL was present in the insoluble complex as compared with only 60 μg of LDL being present. At 15 mM Ca\(^{2+}\) the difference was even more dramatic, e.g. 380 versus 50 μg, respectively. In the absence of Lp(a), acetyl LDL did not form insoluble complexes with Ca\(^{2+}\) (not shown).

![FIG. 6. Effect of L-proline on the binding of LDL to Lp(a) in the insoluble complexes.](image)

![FIG. 7. Effect of Ca\(^{2+}\) concentrations on the binding of acetyl LDL to Lp(a) in the insoluble complexes.](image)
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In this study we have confirmed and extended previous reports (18, 19) demonstrating that Lp(a) undergoes insoluble complex formation in the presence of Ca\(^{2+}\). Dahlen et al. (19) had previously reported aggregation of Lp(a) under similar conditions, but only 30% of the Lp(a) particles became insoluble, as contrasted to this study in which about 80% were aggregated. This discrepancy may be because of methodological differences in the two studies. We propose that insoluble complex formation of Lp(a) is induced by Ca\(^{2+}\) cross-bridging between different Lp(a) particles, mediated by sialic acids on apo(a). This is based on the fact that Lp(a) has a large number of sialic acids in apo(a) (9), and sialic acids have been shown to demonstrate a high binding affinity for Ca\(^{2+}\) (34). This interpretation is consistent with the observation that desialylation of Lp(a) with neuraminidase prevented insoluble complex formation. Even though other lipoproteins also possess sialic acids, they did not form insoluble complexes with Ca\(^{2+}\) (Fig. 1). One of the reasons for this Lp(a) specificity could be the higher sialic acid content of Lp(a) compared with other lipoproteins (9, 33). A unique flexible extended open conformation of apo(a) (9) as contrasted to other apoproteins may be critical for this interaction. It is of note that only 30% of the sialic acids on Lp(a) appeared to be critical for Ca\(^{2+}\)-induced cross-bridging of Lp(a) particles, since 70% of the total sialic acid content still remained on neuraminidase-treated Lp(a) that did not undergo Ca\(^{2+}\)-induced complex formation. The observations that highly electronegative molecules such as acetyl LDL or HDL\(_3\) inhibited Lp(a) insoluble complex formation at physiologic Ca\(^{2+}\) concentrations could be because of direct competition between sialic acids on apo(a) and the other highly electronegative lipoproteins for the limited amount of Ca\(^{2+}\). A decrease in Lp(a) insoluble complex formation at higher Ca\(^{2+}\) concentrations (Fig. 1b) could be because of the complete blockage of sialic acids on individual Lp(a) particles by Ca\(^{2+}\), thereby preventing Ca\(^{2+}\) from cross-bridging.

This is the first study to report the binding of apoB-containing lipoproteins such as LDL and VLDL to Lp(a) in Ca\(^{2+}\)-induced insoluble complexes, as evidenced by their co-precipitation with Lp(a). The mechanism of binding appears to be ionic, and the positive charge on LDL could be responsible for the interaction. As such, it differs from the Lp(a)-LDL interaction reported by Trieu et al. (22), which requires the interaction of apo(a) with proline residues on LDL, rather than Ca\(^{2+}\). Our observation that excess L-proline failed to inhibit co-precipitation of Lp(a) and LDL in the presence of Ca\(^{2+}\) is consistent with the conclusion that the mechanisms differ in the two situations. It is unlikely that Ca\(^{2+}\) forms cross-bridges between Lp(a) and LDL, since increasing the net positive surface charge on LDL by desialylation increased its co-precipitation with Lp(a) at physiologic Ca\(^{2+}\) concentrations. The observation that the binding of LDL to Lp(a) in the insoluble complexes was inhibited by higher Ca\(^{2+}\) concentrations also supports the importance of the positive charge on LDL for this interaction. Excess Ca\(^{2+}\) could compete with electropositive sites on apoB in LDL for electronegative sites on Lp(a), e.g. saturate them, thereby inhibiting the LDL binding.

The studies on the comparison of the interaction between Lp(a) with LDL or with acetyl LDL yielded several interesting observations. Although acetyl LDL, unlike LDL, appeared to bind strongly to Ca\(^{2+}\), probably because of its increased surface negative charge, it did not, by itself, form insoluble complexes with Ca\(^{2+}\), but rather bound to Lp(a) in the insoluble complexes. However, the mechanism for binding of acetyl LDL seems to differ from that of LDL and appears to be mediated by Ca\(^{2+}\) cross-bridges between negatively charged sites on acetyl LDL and on Lp(a). This interpretation is based on the observation that the Ca\(^{2+}\) concentration at which saturated binding of acetyl LDL occurred increased with increasing amounts of acetyl LDL added, as was reported previously between acetyl LDL and dextran sulfate (35). It is of note that at physiologic concentrations of Ca\(^{2+}\) (5 mM), more acetyl LDL than LDL bound to Lp(a) in the insoluble complexes.

The greater binding of acetyl LDL than LDL to Lp(a) during Ca\(^{2+}\)-induced insoluble complex formation at physiologic Ca\(^{2+}\) concentrations would suggest that chemically modified forms of LDL such as oxidized LDL found in the arterial wall (36–39) might demonstrate enhanced interaction with Lp(a). In fact, we found that oxidized LDL induced by incubating with 10 \(\mu\)M Cu\(^{2+}\) for 24 h bound more avidly than LDL to Lp(a) in the insoluble complexes (not shown). We have demonstrated previously the accumulation of both Lp(a) and LDL in human atherosclerotic lesions (15). It is tempting to speculate that some of this accumulation might have been caused by Ca\(^{2+}\)-induced insoluble complex formation of Lp(a) and the co-precipitation of some of the accumulated LDL. Thus, such aggregation in the vessel wall could, in large part, be responsible for the accumulation in plaques of not only Lp(a) but a significant part of the LDL. It is of note that cardiovascular disease was shown to be particularly prevalent in individuals with high plasma Lp(a) and high LDL as in heterozygous familial hypercholesterolemia (3, 40), in which more Lp(a) and LDL would be expected to accumulate in the arterial intima. If these in vitro observations reported in this study also occurred in the arterial wall, they could help explain the apparent atherogenicity of Lp(a).

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