Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I

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Abstract We have shown previously that low density lipoprotein (LDL) subjected to vortexing forms self-aggregates that are avidly phagocytosed by macrophages. That phagocytic uptake is mediated by the LDL receptor. We now show that LDL self-aggregation is strongly inhibited (80–95%) by the presence of high density lipoprotein (HDL) or apolipoprotein (apo) A-I. Another type of LDL aggregation, namely that induced by incubation of LDL with phospholipase C, was also markedly inhibited by HDL or apoA-I. The aggregation of LDL induced by vortexing was not inhibited by 2.5 M NaCl, and apoA-I was still able to block LDL aggregation at this high salt concentration, strongly suggesting hydrophobic interactions as the basis for the effect of apoA-I. The fact that apoA-I protected against LDL aggregation induced by two apparently quite different procedures suggests that the aggregation in these two cases has common features. We propose that these forms of LDL aggregation result from the exposure of hydrophobic domains normally masked in LDL and that the LDL-LDL association occurs when these domains interact. ApoA-I, because of its amphipathic character, is able to interact with the exposed hydrophobic domains of LDL and thus block the intermolecular interactions that cause aggregation. —**Khoo, J. C., E. Miller, P. McLoughlin, and D. Steinberg.** Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I. *J. Lipid Res.* 1990. **31:** 645–652.

Supplementary key words LDL self-aggregation • apoB

There is strong evidence that a high concentration of plasma high density lipoprotein (HDL) may inhibit the atherosclerotic process (1, 2). This is generally presumed to be due to its role in promoting reverse cholesterol transport (3, 4), whereby the HDL may prevent accumulation of cholesterol in the arterial wall. However, other hypotheses are possible. For example, HDL is derived in part from the catabolism of chylomicrons and very low density lipoproteins (VLDL), and thus HDL levels may simply reflect an increase in lipoprotein lipase activity (5). Certain classes of HDL may directly inhibit uptake of low density lipoprotein (LDL) (6, 7). Also, HDL can ameliorate the cytotoxic effect of oxidized LDL on vascular smooth muscle and endothelial cells (8), and can prevent the oxidative modification of LDL (9, 10). Apolipoprotein (apo) A-I has been shown to prevent the fusion of LDL with cholesteryl ester-phospholipid microemulsions (11).

We have previously shown that LDL readily formed aggregates with itself when subjected to brief vortexing. The aggregates were avidly ingested by macrophages via phagocytosis, transforming them into cholesteryl ester-rich foam cells. Furthermore, this rapid uptake of aggregates was linked to the LDL receptor (12). Suits et al. (13) reported that treatment of LDL with phospholipase C induced aggregation and that those aggregates also were taken up much more rapidly than native LDL. Again, as in the studies of Khoo et al. (12), the aggregates were taken up by phagocytosis and the process was dependent upon the LDL receptor. In the present studies we show that aggregation of LDL induced by either one of these two modalities can be inhibited by the addition of HDL or simply by the addition of apoA-I, the major apolipoprotein of HDL.

MATERIALS AND METHODS

Chemicals

Phospholipase C (Type XI, from *Bacillus cereus*) with a specific activity of 1,000 units/mg protein, human albumin (fraction V), and gentamicin were purchased from Sigma Chemical Co., St. Louis, MO. D-Phenylalanyl-L-prolyl-arginine chloromethyl ketone, chloramphenicol, and phenylmethylsulfonyl fluoride were obtained from Calbiochem Behring Corp., La Jolla, CA. Bio-Gel A-0.5m (10% agarose) was obtained from Bio-Rad, Richmond, CA. Carrier-free $Na^{125}I$ was purchased from Amersham, Arlington Heights, IL, and Iodogen from Pierce, Rockford, IL.

Abbreviations: apoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; apoC-III, apolipoprotein C-III; apoE, apolipoprotein E; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; VLDL, very low density lipoprotein.

Methods

LDL (d 1.019–1.063 g/ml), HDL (d 1.063–1.21 g/ml), and lipoprotein-deficient serum (LPDS) (d > 1.21 g/ml) were isolated by preparative ultracentrifugation from fresh human plasma (14). Blood was collected in 0.1% EDTA and 1 μM D-phenylalanyl-L-prolyl-arginine chloromethyl ketone. A mixture of gentamicin (100 μg/ml), chloramphenicol (50 μg/ml), and phenylmethylsulfonyl fluoride (0.5 mM) was added to the plasma and to the sodium bromide solutions during preparative ultracentrifugation. The lipoproteins were dialyzed extensively against phosphate-buffered saline (PBS) containing 0.01% EDTA (pH 7.4). LPDS was obtained by removal of the thrombin clot from the d > 1.21 g/ml fraction by extensive dialysis against PBS containing 0.9 mM Ca^{2+} and 2.5 mM Mg^{2+}. ApoA-I was purified from human HDL as described by Glass et al. (15) and radioiodinated by the method of Salacinski et al. (16). ApoE was purified from rat HDL as described for the purification of apoA-I, followed by further purification by heparin-Sepharose affinity chromatography (17). ApoC-III was purified from human VLDL as described by Brown, Levy, and Fredrickson (18).

LDL aggregates were prepared by vortexing LDL (0.5 mg protein/ml PBS) on a flat surface Thermolyne Maxi Mix (Thomas Scientific) for 60 sec. Aggregates were also prepared by incubating LDL (0.25 mg protein/ml PBS) with phospholipase C (1 unit/ml) at 37°C for 1 h. The molar ratios of apoA-I to apoB and of apoE to apoB were calculated based on the molecular weights of apoA-I = 28,100 (19), apoE = 34,200, (20), and apoB = 550,000 (21, 22).

Lipids were extracted by the method of Folch, Lees, and Sloane Stanley (23) and the phosphorus content of the phospholipids was determined by the method of Rouser, Siakotos, and Fleischer (24). Protein was determined by the method of Lowry et al. (25). Gel filtration chromatography was performed on a 10% agarose column (1.5 × 5.5 cm).

RESULTS

Anti-aggregatory effect of HDL and of apoA-I on LDL aggregation generated by vortexing

As previously reported (12), when human LDL (in PBS containing 0.01% EDTA) was subjected to vortexing for 60 sec, it formed aggregates with itself, the solution becoming frankly opaque. The aggregates, which were too large to pass through a 1 μm Millipore filter, were readily visible under light microscopy. The LDL aggregates appeared as long, complex strands with large numbers of expanded, bulbous segments having the appearance of external lipid droplets. During this brief period of vortexing, there was no fragmentation of the apoB-100 (judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and there was no production of thiobarbituric acid-reactive material (12).

As an index of aggregation, the turbidity generated by vortexing LDL (0.5 mg protein/ml) was measured spectrophotometrically in terms of absorbance at 680 nm. As shown in **Fig. 1**, the formation of LDL aggregates increases with vortexing time. When HDL (2 mg/ml) was present during the vortexing of LDL, the formation of aggregates was inhibited at each time interval with an overall inhibition of 83.5%. HDL was not only capable of blocking the formation of LDL aggregates when the two lipoproteins were vortexed simultaneously, but was also capable of interrupting the further formation of LDL aggregates when added after the process had been initiated (**Fig. 2**). When HDL (2 mg protein/ml) was added to LDL that had been vortexed alone for 15 sec (absorbance 0.71), the propagation of aggregates was almost completely arrested (final absorbance 0.81).

To determine whether the lipid or the protein moiety of the HDL was responsible for its anti-aggregatory effect on LDL, we tested the ability of apoA-I, the major apolipoprotein of human HDL, to block LDL aggregation. As shown in **Fig. 3**, when LDL (0.5 mg protein/ml) was vortexed in the presence of apoA-I (2 mg/ml), the formation of LDL aggregates was inhibited by 95%. Thus, apoA-I was slightly more potent than HDL in blocking the aggregation of LDL. The molar ratio of apoA-I to

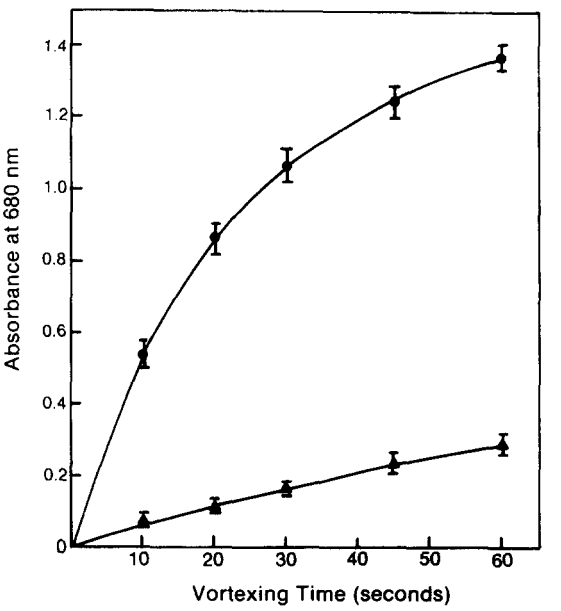

Fig. 1. LDL aggregates generated by vortexing in the absence or in the presence of HDL. LDL (0.5 mg protein/ml) was vortexed alone (●) or in the presence of HDL (2 mg protein/ml) (▲). At the indicated times, the absorbance was measured at 680 nm. Each point represents the mean ± SE of 26 experiments.

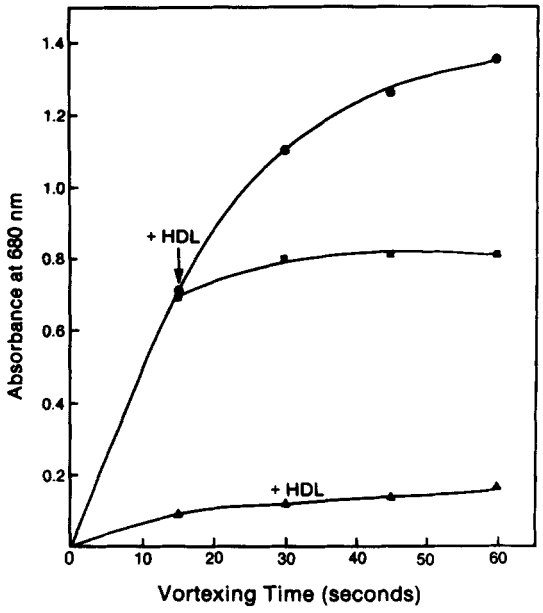

Fig. 2. The inhibitory effect of HDL on the generation of LDL aggregates by vortexing. LDL (0.5 mg protein/ml) was vortexed either alone (●), in the presence of HDL (2 mg protein/ml) (▲), or alone for 15 sec, followed by the addition of HDL (2 mg protein/ml) (■). The absorbance was measured at 680 nm at the indicated times.

apoB was 78:1. As with holo-HDL, apoA-I arrested further aggregation of LDL once the process had been initiated (**Fig. 4**). In addition, apoC-III and apoE, with physicochemical properties similar to those of apoA-I, were also effective in preventing LDL aggregation. However, it is unlikely that apoE and apoC of the HDL used in these studies played a significant role in preventing LDL aggregation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the HDL fraction, before and after vortexing and reisolation by ultracentrifugation at 1.21 g/ml, revealed that apoA-I accounted for nearly all of the protein and there were only trace amounts of apoE and apoC. Furthermore, there was virtually no change in the apoprotein composition of the HDL after vortexing.

When NaCl (1 M or 2.5 M) was added to the LDL (0.5 mg protein/ml) and vortexed for up to 60 sec, the aggregation proceeded normally (**Fig. 5**). The addition of apoA-I (2 mg/ml) blocked this aggregation. These data strongly suggested that the LDL aggregation was not due to ionic interactions but rather to van der Waals forces. The fact that apoA-I was just as effective in the presence of the high salt also suggested that its ability to protect rested on its ability to interact with the exposed hydrophobic surfaces of the LDL during vortexing. HDL and apoA-I were much more potent than other proteins in inhibiting LDL aggregation. As shown in **Fig. 6**, human albumin at 2 mg/ml inhibited aggregation by only 22.5% whereas HDL and apoA-I, at the same protein concentration, inhibited by 85% and 96%, respectively. Even at 10 mg/ml, human albumin inhibited LDL aggregation by less than 50%. Other proteins tested (LPDS, gamma globulin, and thyroglobulin) at 2 mg/ml inhibited LDL aggregation by less than 20% (data not shown).

ApoA-I does not ordinarily bind to LDL to any significant extent; the two can be separated completely using gel filtration chromatography. We considered the possibility, however, that apoA-I might bind to LDL because of changes induced in the structure of LDL during vortexing. To test this, ^{125}I-labeled apoA-I was vortexed with LDL for 60 sec at a concentration of apoA-I sufficient to prevent LDL aggregation. The LDL was precipitated by addition of phosphotungstic acid at the end of the vortexing; no radioactivity was found with the precipitated LDL. As a further test, LDL was vortexed with ^{125}I-labeled apoA-I and then subjected to gel filtration on a short 10% agarose column. Only 1.6% of the ^{125}I-labeled apoA-I was recovered in the LDL fraction, a value not different from that found in the control when the vortexing step was omitted (data not shown). These results show that if there are any interactions between the hydrophobic domains of apoA-I and of LDL during vortexing, they must be transient.

Anti-aggregatory effect of HDL and of apoA-I on LDL aggregation induced by the action of phospholipase C

Suits et al. (13) reported that when native LDL is incubated with phospholipase C, the LDL particles fuse to

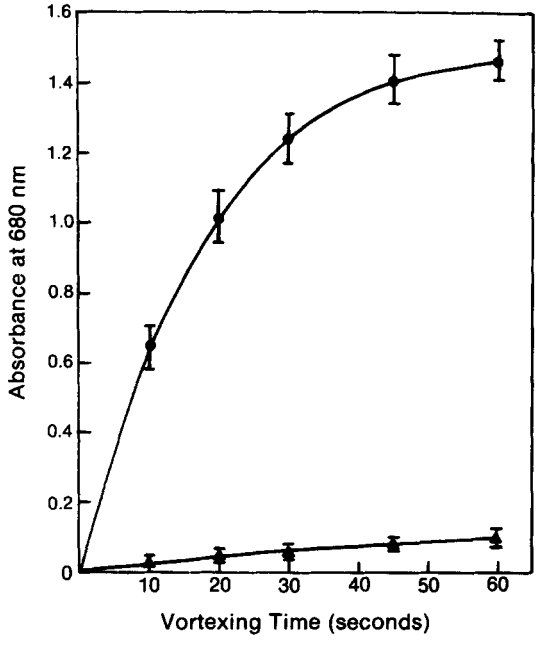

Fig. 3. LDL aggregates generated by vortexing in the absence and in the presence of apoA-I. LDL (0.5 mg protein/ml) was vortexed alone (●) or in the presence of apoA-I (2 mg/ml) (▲). At the indicated times, the absorbance was measured at 680 nm. Each point represents the mean ± SE of 10 experiments.

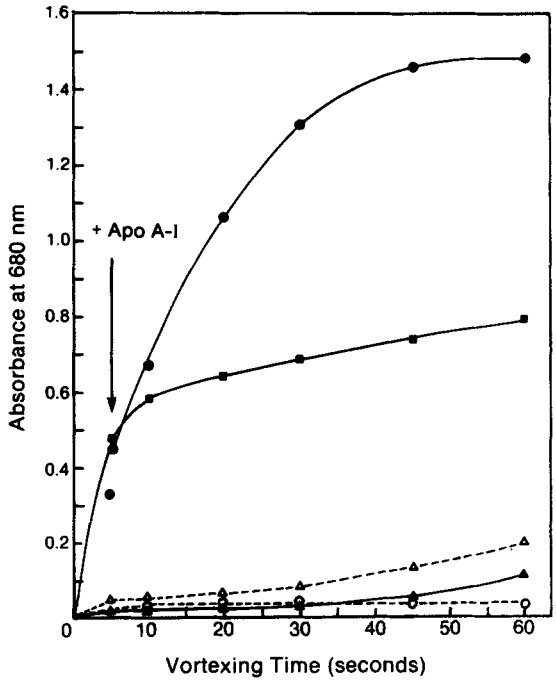

Fig. 4. The inhibitory effect of apoA-I, apoE, and apoC-III on the generation of LDL aggregates of vortexing. LDL (0.5 mg protein/ml) was vortexed either alone (●), in the presence of apoA-I (2 mg/ml) (▲), or alone for 5 sec, followed by the addition of apoA-I (2 mg/ml) (■). LDL was also vortexed in the presence of apoE (2 mg/ml) (○) or apoC-III (2 mg/ml) (△). The absorbance was measured at 680 nm at the indicated times.

form aggregates. We confirmed and extended these findings. When LDL (250 μg protein/ml) was incubated with phospholipase C (1 unit/ml) for 1 h at 37°C, 77% of the phospholipid was hydrolyzed and the preparation became highly turbid. The morphology of such aggregates under light microscopy appeared to be somewhat different from that of the LDL aggregates generated by vortexing in that they consisted of much smaller amorphous clumps and shorter strands. When HDL (100 μg protein/ml) was incubated along with the LDL (250 μg protein/ml) and phospholipase C (1 unit/ml), the aggregation of LDL was inhibited 90%; 50% inhibition was observed at 22 μg protein/ml of HDL (**Fig. 7**). This inhibition of LDL aggregation could in principle reflect competition between the phospholipid moieties of HDL and of LDL for the enzyme. However, this is made unlikely by the fact that purified apoA-I and apoE, both completely devoid of phospholipids, were, if anything, more potent than holo-HDL in inhibiting LDL aggregation. Thus, apoA-I (50 μg/ml) inhibited the aggregation of LDL (250 μg protein/ml) (molar ratio of apoA-I to apoB = 4:1) by 97%, with 50% inhibition observed at 7μg/ml. ApoE (50 μg/ml) (molar ratio of apoE to apoB = 3.3:1) inhibited LDL aggregation by 99%, with 50% inhibition at 6 μg/ml. In contrast, LPDS and albumin did not prevent the aggregation of LDL under the identical conditions; if anything, there was even a slight stimulation of aggregation (11% and 9%, respectively) (Fig. 7).

To investigate whether apoA-I became bound to LDL during treatment with phospholipase C, LDL (60 μg protein/ml) was incubated at 37°C for 1 h with phospholipase C (1 unit/ml) and ^{125}I-labeled apoA-I (100 μg/ml). This amount of ^{125}I-labeled apoA-I was sufficient to prevent any aggregation of LDL. At the end of the incubation, the mixture was subjected to chromatography on a short 10% agarose column. As shown in **Fig. 8A**, 29% of the added ^{125}I-labeled apoA-I co-eluted with the LDL peak emerging at the front; a control chromatogram of a mixture of untreated LDL plus the same amount of added ^{125}I-labeled apoA-I showed only 2% of the ^{125}I co-eluting with LDL (Fig. 8B).

DISCUSSION

The fact that HDL or isolated apoA-I can inhibit aggregation of LDL induced by two rather different treatments—vortexing and phospholipase C treatment—suggests that the mechanisms of aggregation may be related. Moreover the effect of the HDL appears to be largely accounted for by its content of apoA-I. ApoA-I is an amphipathic molecule, having a highly hydrophilic face on one side and a highly hydrophobic face on the

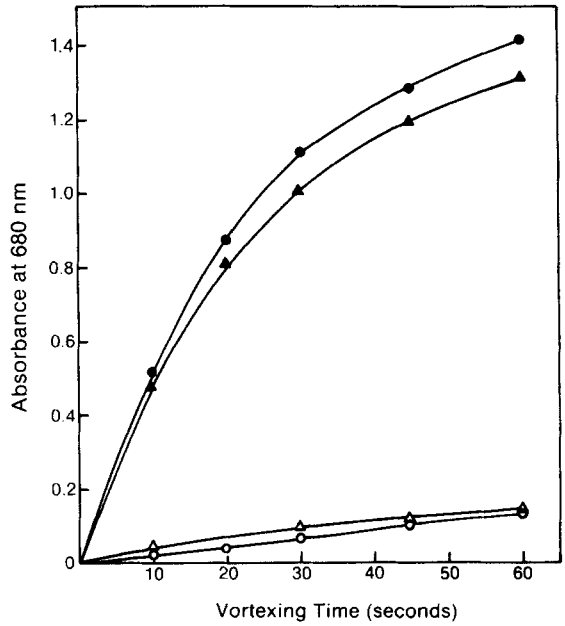

Fig. 5. The effect of NaCl on the generation of LDL aggregates induced by vortexing and on the anti-aggregatory action of apoA-I. LDL (0.5 mg protein/ml) was vortexed in the presence of 1 M NaCl (●) and 2.5 M NaCl (▲), or in the presence of 1 M NaCl plus apoA-I (2 mg/ml) (○) or in the presence of 2.5 M NaCl plus apoA-I (2 mg/ml) (△). The absorbance was measured at 680 nm at the indicated times.

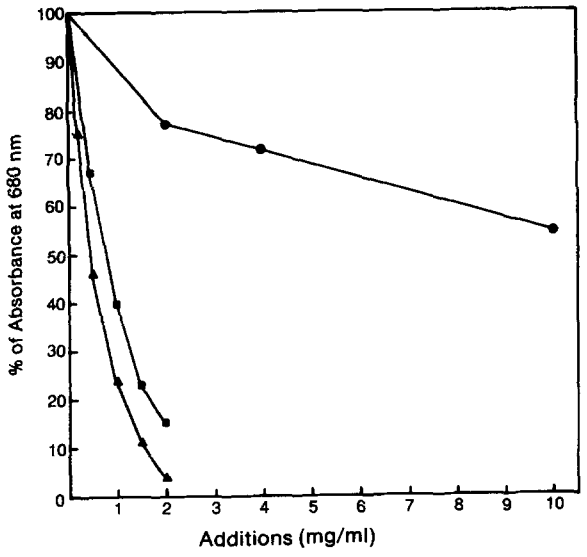

Fig. 6. The effect of human albumin, HDL, and apoA-I on LDL aggregation generated by vortexing. LDL (0.5 mg protein/ml) was vortexed for 60 sec in the presence of the indicated concentrations of human albumin (●), HDL (■), or apoA-I (▲). The results were expressed as percentage of the absorbance at 680 nm in the absence of any additions.

other (26). The well-documented self-association of apoA-I presumably reflects interaction between the hydrophobic faces of apoA-I, which are normally bound to lipids in the HDL particle (27, 28). This structure of apoA-I is shared to some extent by other apoproteins and presumably accounts for the ability of these apoproteins to solublize multilamellar phospholipid vesicles and to stabilize lipoproteins in solution (29–31).

The fact that the self-aggregation of LDL during vortexing was unaffected by high salt concentrations (up to 2.5 M NaCl), and that apoA-I was able to inhibit the self-aggregation equally well in the presence of this high salt concentration, speaks against a hydrophilic interaction and in favor of a hydrophobic interaction to account for the self-aggregation and for the ability of apoA-I to prevent it. In order for apoA-I to block LDL aggregation, it must presumably somehow interact with the LDL particle. Yet there is little or no association between apoA-I and native LDL. We propose that the association occurs only when the LDL structure is perturbed such that there is a greater exposure of hydrophobic domains (of lipids or/and of apoB). In the absence of added apoA-I, the number of LDL particles with these domains exposed may be such that the hydrophobic domains on different LDL particles interact with each other with sufficient frequency to lead to the observed aggregation. On the other hand, when apoA-I is present during the perturbation (be it vortexing or treatment with phospholipase C), the exposed hydrophobic domains of LDL interact not with each other but with the hydrophobic face of apoA-I. This masks the hydrophobic domains of LDL that may be responsible for aggregation. Surprisingly, the effectiveness of free apoA-I was only slightly greater than that of intact HDL at the same protein concentration. One might have expected that the free apoA-I would more readily be available to interact with LDL if the hypothesis presented is correct. Two possibilities should be considered: *1*) during vortexing, the association of apoA-I with the HDL particle may be disturbed; *2*) because of the extremely rapid mixing that occurs during vortexing, HDL-LDL interactions may occur with a frequency comparable to that of apoA-I-LDL interactions and the interactions may dissociate the apoA-I from the HDL particle.

If this is the explanation, however, why do we fail to find apoA-I associated with the LDL at the end of a vortexing experiment? During vortexing, the perturbation in LDL structure may relate to the marked increase in air/water interface. The LDL that is at the interface may have a distorted structure (with exposure of hydrophobic domains) but immediately after the vortexing has been discontinued the particles may instantly revert to their normal, more stable configuration. Thus, apoA-I may bind to the distorted LDL *during* the vortexing, but as soon as the vortexing stops the LDL may resume its more stable configuration and the apoA-I will leave the LDL particle very promptly. This is consistent with our finding that when the LDL vortexed in the presence of apoA-I was

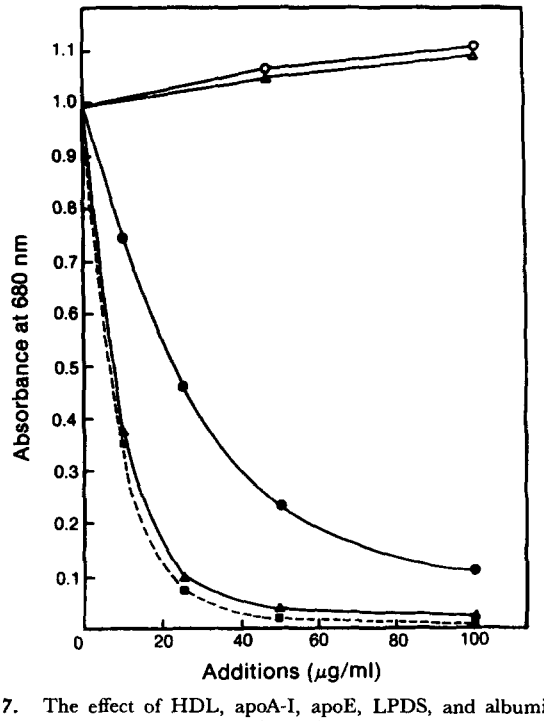

Fig. 7. The effect of HDL, apoA-I, apoE, LPDS, and albumin on LDL aggregation generated by the action of phospholipase C. LDL (0.25 mg protein/ml) was incubated at 37°C for 1 h in the presence of phospholipase C (1 unit/ml) and increasing protein concentrations of either HDL (●), apoA-I (▲), apoE (■), LPDS (△), or albumin (○). The absorbance was measured at 680 nm.

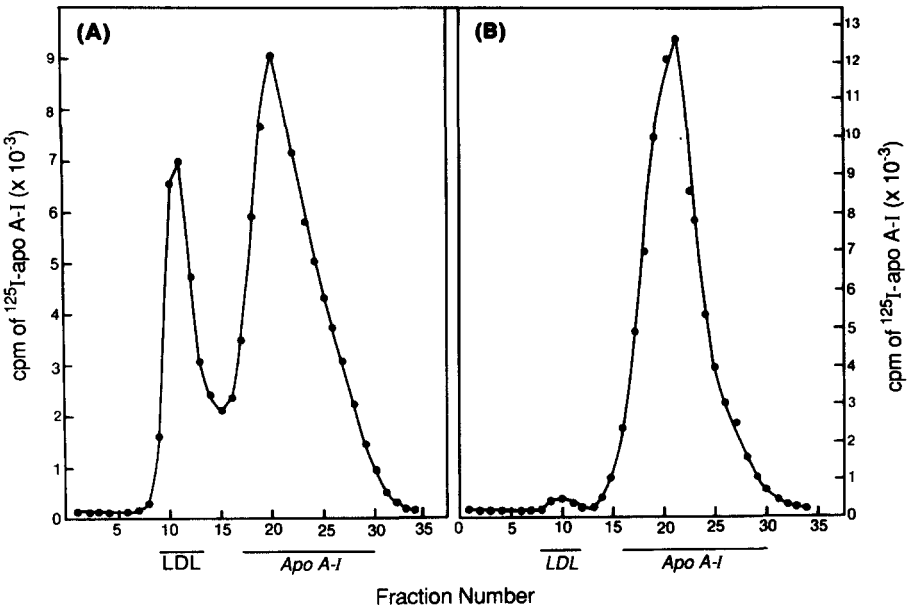

Fig. 8. Association of ^{125}I-labeled apoA-I with phospholipase C-treated LDL. (A) LDL (60 μg protein/ml) was incubated at 37°C for 1 h with phospholipase C (1 unit/ml) and ^{125}I-labeled apoA-I (100 μg/ml) in PBS with a final volume of 1 ml. The incubation mixture was centrifuged at 10,000 *g* for 10 min and the supernatant was chromatographed on a 10% agarose column (Bio-Gel A-0.5 m, 1.5 × 5.5 cm) at a flow rate of 4 ml/h. The 0.4-ml fractions were counted for ^{125}I-labeled apoA-I in a gamma counter. The total cpm of ^{125}I-labeled apoA-I loaded on the column was 98,000. (B) Control for panel A without the addition of phospholipase C. The total cpm loaded on the column was 90,000.

re-isolated by ultracentrifugation at a density of 1.063 g/ml, the re-isolated LDL remained soluble, with particle size similar to that of native LDL. The ability of that re-isolated LDL to undergo self-aggregation induced by a second vortexing was identical to that of the original native LDL (data not shown). An alternative explanation is that during vortexing the air/water interface is increased, causing surface denaturation of the LDL, but the addition of HDL or apoA-I reduces surface tension and thereby protects the LDL particles. However, this explanation would not be relevant to LDL aggregation induced by phospholipase C.

The treatment of LDL with phospholipase C represents quite a different situation. Here ^{125}I-labeled apoA-I did remain bound to the LDL particles that it was protecting from self-aggregation, in contrast to the situation with aggregation induced by vortexing, where apoA-I did not remain bound. This implies that the change induced by phospholipase C treatment is essentially irreversible while the change induced by vortexing is not. This is understandable since the treatment with phospholipase C removes the polar head groups and leaves a particle with a greater exposure of hydrophobic domains. Our hypothesis that apoA-I masks the exposed hydrophobic domains of LDL particles is consonant with all the experimental data.

HDL has been shown to prevent the interaction of LDL with a number of other macromolecules. It can inhibit the binding of LDL to proteoglycans (32), to glycosaminoglycans (33), and to elastin (34). The molecular basis for these additional examples in which HDL is protective against the binding of LDL remains to be established. It has been suggested that the binding of LDL to proteoglycans and to glycosaminoglycans is likely an ionic interaction; the nature of the binding to elastin is not clear but might involve hydrophobic interactions analogous to the interactions proposed here to explain LDL self-aggregation and the prevention of it by HDL. Elastase treatment of LDL induces dimerization (35) but the effect of HDL has not been reported.

The procedures used in the present study to induce LDL aggregation are quite aggressive. Conditions in the arterial intima are unlikely to be as violent and it is difficult to predict whether or not the protective effect of HDL or apoA-I would be operative in vivo. It has been reported that the molar ratio of apoB to apoA-I in the intima is approximately 5:1 (36, 37), and the concentration of the albumin in the intima is about 25% of its concentration in plasma (38). The concentration of LDL within the intima may be at least as high and probably higher than its concentration in circulating plasma (39, 40) and the concentration in lesions may be still higher (39). At these high concentrations, aggregation of LDL to itself or adherance of LDL to matrix elements is more likely to occur and some evidence for such aggregation has been presented. Frank and Fogelman (41) have recently examined

the ultrastructure of the intima in WHHL rabbit aortas prepared by ultra-rapid freezing and freeze-etching. They demonstrated large lipid particles (70–169 nm in diameter) that might represent aggregates of LDL. Mora, Lupu, and Simionescu (42) have shown the colocalization of apoB and large extracellular liposomes in the lesion-prone area of the aorta of cholesterol-fed rabbits. Self-aggregates of LDL, as previously reported (12, 13), are more readily taken up by macrophages, generating cholesteryl ester-rich foam cells. The protective effect of HDL against LDL aggregation could then be a component of its ability to "protect" against clinically significant atherosclerosis.

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