

Effect of fluid mechanical stresses and plasma constituents on aggregation of LDL

Roy M. Talbot, Jessica D. del Rio, and Peter D. Weinberg¹

School of Animal and Microbial Sciences, University of Reading, Reading, United Kingdom

Abstract LDL aggregates when exposed to even moderate fluid mechanical stresses in the laboratory, yet its half-life in the circulation is 2–3 days, implying that little aggregation occurs. LDL may be protected from aggregation in vivo by components of plasma, or by a qualitative difference in flows. Previous studies have shown that HDL and albumin inhibit the aggregation induced by vortexing. Using a more reproducible method of inducing aggregation and assessing aggregation both spectrophotometrically and by sedimentation techniques, we showed that at physiological concentrations, albumin is the more effective inhibitor, and that aggregation is substantially but not completely inhibited in plasma. Heat denatured and fatty-acid-stripped albumin were more effective inhibitors than normal albumin, supporting the idea that hydrophobic interactions are involved. Aggregation of LDL in a model reproducing several aspects of flow in the circulation was 200-fold slower, but was still inhibited by HDL and albumin, suggesting similar mechanisms are involved. Within the sensitivity of our technique, LDL aggregation did not occur in plasma exposed to these flows. Thus, as a result of the characteristics of blood flow and the inhibitory effects of plasma components, particularly albumin, LDL aggregation is unlikely to occur within the circulation.—Talbot, R. M., J. D. del Rio, and P. D. Weinberg. Effect of fluid mechanical stresses and plasma constituents on aggregation of LDL. *J. Lipid Res.* 2003. 44: 837–845.

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Aggregation or fusion of LDL particles can be induced by lipolysis (1–4), proteolysis (5–8), oxidation (9–13), and other chemical modifications (9, 14–16). The pioneering work of Khoo et al. (17, 18) showed that fluid mechanical stresses are particularly potent inducers of aggregation: substantial turbidity was seen in LDL solutions vortexed for only 5–10 s, and near maximal aggregation involving the production of particles sufficiently large to be resolved by light microscopy occurred within 1 min. Further evidence for the susceptibility of LDL to flow-induced aggregation is the care with which it must be stirred in the laboratory.

LDL is subject to substantial hemodynamic stresses within the bloodstream, particularly in the heart and large arteries. Rapid aggregation and removal from the blood would consequently be expected, yet its half-life in vivo is 2–3 days. An important question, therefore, is why aggregation does not occur at the anticipated rate. One possibility is that substances within blood inhibit it. Khoo et al. (18) found that lipoprotein-deficient serum, HDL, apolipoprotein A-I (apoA-I), and human serum albumin all conferred some protection, putatively through hydrophobic interactions, although aggregation was not entirely prevented by the concentrations used. It is also possible that the flows in vivo are qualitatively different from those used in vitro. The absence of air-water interfaces could be particularly significant. A second important question is whether aggregation of LDL still occurs in the bloodstream, albeit at a lower rate or for only a fraction of the total LDL pool. If it does occur, it could be a significant influence on the balance between LDL synthesis and degradation.

Both these questions have been investigated in the present study. Two types of in vitro flow were used. The first was similar in principle to vortexing but, because it involved parameters that can be accurately quantified, is repeatable; it could be reproduced precisely in other laboratories. The second was designed to replicate more accurately flows present in the circulation. The effect of both types of flow on the aggregation of plasma, or LDL solutions with and without various concentrations of plasma constituents, was investigated. Additionally, inhibitory influences of modified plasma constituents and other substances were investigated to determine the likely role of hydrophobic interactions.

METHODS

Isolation of lipoprotein fractions

Blood was collected from healthy volunteers into EDTA (3 mmol/l final concentration), and plasma was obtained from it by centrifugation. Alternatively, plasma was obtained from the Oxford Regional Blood Transfusion Service, again after collection

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¹ To whom correspondence should be addressed.
e-mail: p.d.weinberg@reading.ac.uk

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from healthy volunteers. The anticoagulant in this case was citrate acid dextrose. No difference was observed in the behavior of lipoproteins obtained from the two sources. Institutional approval and informed consent of the blood donors were obtained.

Lipoproteins were isolated by sequential ultracentrifugation. Plasma from several donors was pooled in each preparation, all fractions contained 0.3 mmol/l EDTA, and spins were carried out at 4°C. The methods of Havel et al. (19) were used, with minor modifications. Briefly, the plasma was spun at 150,000 *g* for 18 h after its density had been adjusted to 1.019 g/ml. The lower band was adjusted to 1.063 g/ml and recentrifuged in the same way. To obtain LDL, the upper fraction was readjusted to 1.063 g/ml, respun, and the upper fraction was retained. To obtain HDL, the lower fraction of the second spin was adjusted to 1.210 g/ml and spun at 150,000 *g* for 48 h. The upper fraction was readjusted to 1.210 g/ml, overlain with a solution of density 1.063 g/ml, and spun at 246,000 *g* for 48 h. The upper fraction was discarded.

LDL and HDL were extensively dialysed against “dialysis buffer” [154 mmol/l NaCl, 21.1 mmol/l Na₂HPO₄, 16.7 mmol/l NaH₂PO₄, 0.1 mmol/l EDTA (pH 7.4)] and filter sterilized. Their protein content was determined by the modified Lowry assay of Schacterle and Pollack (20) using BSA (Applied Protein Products) as a standard.

Radioiodination of LDL

LDL was iodinated by the iodine monochloride method of McFarlane (21) as modified by Bilheimer et al. (22). Briefly, Na¹²⁵I (1 mCi, Amersham) was gently mixed with LDL (2 mg protein/ml) in sodium glycine buffer (0.1 mol/l) for 1 min. The LDL was then dialyzed for 48 h against large volumes of “dialysis buffer” to remove free iodide, filter sterilized, and diluted with unlabeled LDL to give a specific activity of 20–40 cpm/ng protein.

Preparation of lipid-deficient plasma

Plasma was delipidated by using PHM-L Liposorb (Calbiochem). Liposorb (1 g) was added to plasma (20 ml), which was then gently stirred, allowed to stand for 10 min at 4°C, and centrifuged for 3 min at 950 *g*. This treatment decolorised the plasma, while the Liposorb changed from white to orange. In order to validate the technique, a solution of LDL at approximately physiological concentration was treated with Liposorb in the same way. A protein assay, conducted as described above, showed that between 98–100% (*n* = 3) of the apolipoprotein was removed. Previous work (23) has shown an absence of lipoprotein bands after electrophoresis of similarly treated serum on agarose gels, and minimal removal of other protein from the serum.

Removal of albumin from delipidated plasma

In order to prepare plasma deficient in lipoproteins and in albumin, plasma was delipidated as described above and then passed through an antialbumin affinity chromatography column (HiTrap Blue, Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Measurement of viscosity

Viscosities were assessed using a glass capillary viscometer that had been coated with silicone to prevent adhesion of LDL. Because this coating altered the bore of the capillary through which the movement of solutions was being timed, only relative values were obtained, but this was sufficient for obtaining solutions having equal viscosities, as required by the protocol.

Induction of aggregation

In some experiments, a vortexing method similar to that of Khoo et al. (18) was used. LDL-containing solutions (3.6 ml) were placed in a 50 ml centrifuge tube (diameter 28 mm, height 113

mm, including a conical bottom 15 mm high, Greiner) and aggregated using a bench-top vortexor (Fisons) at three quarters maximum speed. Vortexing was interrupted at intervals of 30 s or 120 s so that samples could be withdrawn, examined, and replaced.

A rotation method (“inverting”) was developed to produce similar flows to those induced by vortexing, but in a better-defined way. LDL-containing solutions (3.6 ml) or a similar volume of plasma were placed in the 50 ml centrifuge tubes and continuously rotated about an axis perpendicular to the long axis of the tube at 55 rpm. This value was chosen after preliminary experiments showed that substantially lower or higher speeds resulted in much less aggregation, the higher speeds doing so by keeping the solution at the ends of the tube. Rotation was interrupted at intervals of 30 s or 120 s so that samples could be withdrawn, examined, and replaced.

More physiologically realistic flows were obtained by using a peristaltic pump (Watson-Marlow type MHRE200) to induce flows around a closed loop of silicone-rubber tubing. The tubing was formed into a seamless loop by an external cuff. Although physically simple, this apparatus mimicked several essential features of arterial geometry and mechanics. The diameter of the loop (90 mm) and the internal diameter of the tube (6 mm), the frequency of oscillation (4 Hz, determined from the rate at which rollers contacted the tubing), and the mean flow rate (180 ml/min) were all realistic for the aorta of a medium-sized mammal. The velocity waveform was determined by examining flow when the loop was not closed; peristaltic pumps are insensitive to backpressure, and hence similar flows are expected through the closed loop. A frame-by-frame analysis of a video recording of the flow showed a pulsatile waveform with considerable backflow over approximately one third of each cycle (Fig. 1). The backflow occurred as each roller of the pump disengaged from the tube; similar periods of reverse flow are seen in the arterial system. The mean Reynolds number was 640 and the Womersley parameter, a dimensionless index of pulsatility, was 15. Again, these properties are typical of *in vivo* flows. Furthermore, the tube was distensible, the fluid was impelled by a squeezing motion as in the heart, and there was no air-liquid interface.

The tube was filled with 9.25 ml of plasma or solutions containing LDL. These fluids had been degassed for 1 h using a Bunsen pump. Flow was interrupted at intervals of 5 min so that samples could be withdrawn, examined, and returned. Withdrawals were made via a hypodermic needle inserted through the wall of the tube. Control values, obtained by withdrawing samples from a tube through which flow was not occurring, were subtracted from experimental data in order to account for any aggregation caused by the withdrawal process itself.

In some experiments, the tube was occluded by an external clamp just downstream of the pump so that the influence of the

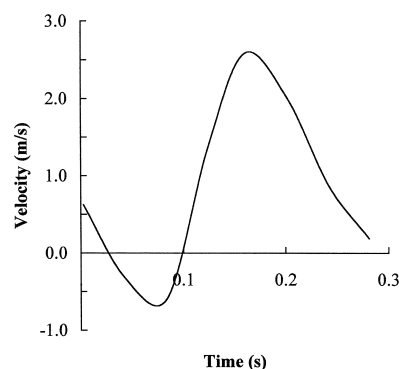


Fig. 1. Velocity waveform in the model of physiological-type flows.

turbulent flow induced locally by the rollers squeezing the tube could be assessed in the absence of the flow around the entire loop, which would have been laminar at this combination of Reynolds number and Womersley parameter (24). The clamp was removed for 30 s before and after each sample was taken to allow mixing of the fluid influenced by the rollers with that elsewhere in the tube. This permitted direct comparison with the unclamped experiments where such mixing took place.

For all three types of flow, the behavior of plasma was compared with that of solutions containing 0.5 mg LDL protein/ml, a concentration slightly lower than the average for plasma in Western societies (0.7 mg protein/ml). This reduction was made so that the LDL concentration in the solutions was unlikely to exceed that in any batch of plasma obtained from the normal volunteers, and hence to avoid an overestimate of the inhibitory effect of plasma on LDL aggregation. Additionally, this concentration of LDL was used by Khoo et al. (17, 18), and thus permitted direct comparison with their data. Solutions of LDL were made up in PBS (0.15 M, pH 7.4) containing 100 μ mol/l EDTA when comparisons were made with plasma containing EDTA, or in PBS containing citrate acid dextrose (7.48 mmol/l sodium citrate, 3.8 mmol/l citric acid, 13.6 mmol/l dextrose) when the plasma contained this. No differences in the behavior of lipoprotein or plasma were apparent when using these different solutions.

Measurement of aggregation

No attempt was made to discriminate between aggregation and fusion (8); the term aggregation is used to include both processes. The index of aggregation used in most experiments was the attenuation in a spectrophotometer at 680 nm (18). The term attenuation is used in preference to absorbance since aggregates scatter light rather than absorb it. Readings obtained before the induction of aggregation (generally around zero except for plasma) were subtracted from all experimental data.

To confirm that these measurements did reflect aggregation of LDL, additional experiments were conducted in which aggregation of 125 I-labeled LDL was assessed from its sedimentation characteristics using a modification of the methods of Guyton et al. (25) and Herrman and Gmeiner (26). Aggregated LDL forms a pellet when centrifuged at 10,000 *g* for 10 min, whereas monomeric LDL does not. Consequently, aggregation was assessed by measuring the γ activity of aliquots of solutions containing 125 I-labeled LDL before and after they had been centrifuged in this way. The fractional drop in activity indicated the fraction of the original LDL that could be sedimented.

Data presentation and statistics

Each experiment was carried out in triplicate, a different batch of LDL being used each time. For each one of these triplicates, six independent replicates were generally performed. The graphs presented below show one typical triplicate of the three conducted for each experiment. Points on the graphs show the mean \pm SD of the replicates. Aggregation in each replicate at each timepoint was assessed by using triplicate measures of attenuation, which were averaged, or by using single measures of sedimentation.

Attenuance and sedimentation increased with time in a linear fashion, except that the gradient was consistently lower between the first (i.e., zero timepoint) and second readings than at subsequent times. This discrepancy presumably arose because the initial aggregation resulted in particles that were too small to scatter light of this wavelength or to sediment significantly within 10 min at 10,000 *g*. Consequently, rates of aggregation were calculated by linear regression, ignoring data from the zero timepoint. Differences in the regression coefficients were assessed by Student's unpaired *t*-test (27).

Aggregation of LDL by vortexing and by continuous rotation

Figure 2A shows the increase in attenuation with time of LDL solutions that were vortexed or continuously rotated ("inverted") for 2 min. Attenuance rapidly increased during vortexing, as shown by Khoo et al. (17, 18), and the solution became visibly turbid. Attenuance increased more slowly during inverting. The apparently lower initial rate of aggregation, described above, is clearly visible in the latter data. Measurements of the sedimentation of 125 I-labeled LDL that had been subjected to vortexing indicated, as did the data of Khoo et al. (17), that $\geq 70\%$ of the LDL formed aggregates during the procedure. Furthermore, a linear relation between the sedimentation and attenuation indices was obtained during vortexing and inversion (data not shown; see below), supporting the view that attenuation is a reliable index of aggregation.

Figure 2B shows data from an experiment of longer duration. From this it is clear that the maximum attenuation is broadly the same for the vortexing and inverting methods, although it takes longer to reach with the latter. It is also apparent that attenuation decreases from this maximum value with further inverting. A similar though smaller effect is detectable with vortexing. We speculate that fluid mechanical stresses can cause the breakdown of aggregates as well as their formation, and that at this time the breakdown is occurring more rapidly.

Comparison of LDL and plasma

Figure 3A shows that the increase in attenuation during inversion occurred at a much slower rate for plasma than for LDL on its own. The inhibition was 93% ($P < 0.001$), and was assumed to represent the effect on LDL aggregation of substances within plasma. In order to determine whether the substances were macromolecules or not, the rate of increase of attenuation for plasma was compared with that for plasma that had been dialysed against PBS using tubing with a 14 kDa cut-off. There was no signifi-

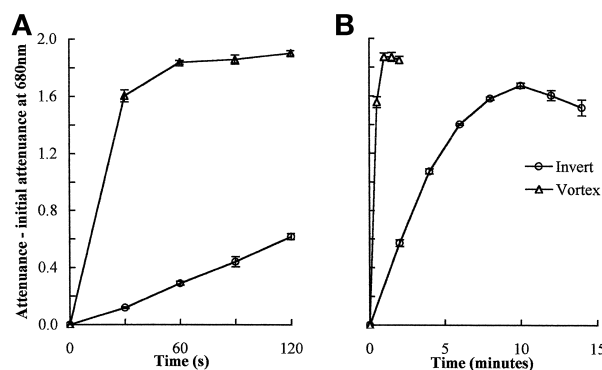


Fig. 2. Changes in the turbidity of LDL solutions subjected to vortexing or repeated inversion in a centrifuge tube. Inverting resulted in a slower initial rate of increase (A) but a similar maximum (B). A net breakdown of aggregates is apparent at longer times. Points show the mean \pm SD of six replicates. The graphs are representative of three independent experiments.

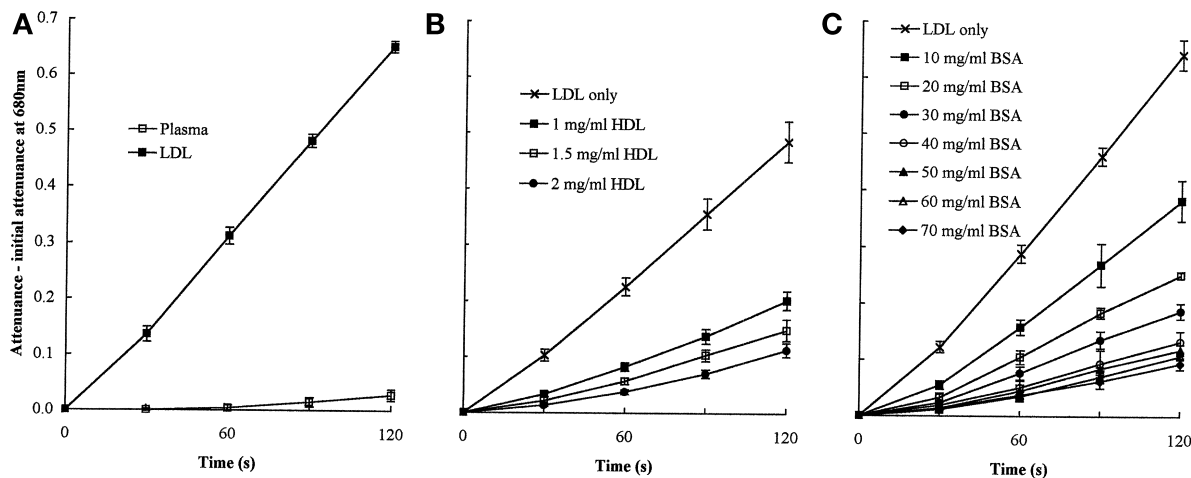


Fig. 3. A: The turbidity of plasma increased much more slowly than that of LDL solutions during repeated inversion ($P < 0.001$). The increase in turbidity of LDL solutions was reduced by HDL (B) and by BSA (C) in a concentration-dependent manner ($P < 0.001$ for all concentrations). Points show the mean \pm SD of six replicates. The graphs are representative of three independent experiments.

cant difference in the rate of increase of attenuance observed for the two fluids ($P > 0.3$, data not shown), suggesting that the main inhibitors are macromolecules.

Inhibition by constituents of plasma

The most likely antiaggregatory macromolecules within plasma are HDL and albumin (18). Figures 3B and C show, respectively, that HDL (obtained from the same plasma as the LDL) and BSA inhibited the increase in attenuance of inverted LDL solutions in a concentration-dependent manner. Values of 58%, 69%, and 76% inhibition were obtained for 1.0, 1.5, and 2.0 mg protein/ml HDL (all $P < 0.001$), the middle concentration being closest to the average plasma value. The highest concentration of BSA (70 mg/ml) inhibited by >95% and the physiological concentration (40 mg/dl) by about 80% in the experiment shown; the latter is somewhat below the typical value, >90% inhibition being obtained in several other experiments. The inhibition by BSA was significant at all concentrations ($P < 0.001$). There was no significant difference between human and BSA ($P < 0.001$); both inhibited by 92% when used at 40 mg/dl (data not shown).

Assessment by the sedimentation technique

The comparison of the attenuance and sedimentation methods for assessing LDL aggregation was extended by examining the inhibitory influence of HDL and BSA with the two techniques. Figures 4A and B show, respectively, the attenuance and sedimentation data obtained during one experiment. Both techniques gave a linear increase with time, apart from the first timepoint (as explained above). This initial discrepancy, thought to be due to the production of aggregates too small for detection, appeared somewhat larger with the sedimentation technique. Both techniques indicated an inhibition by 1.5 mg/dl HDL and a greater inhibition by 40 mg/dl BSA. The good agreement between the two techniques (e.g., $r = 0.995$ for the control data) confirms that attenuance is a satisfactory index of aggregation, as suggested above, and further shows that at least

most of the particles detected by the spectrophotometric method in the presence of HDL or albumin are aggregates of the LDL rather than of the additives.

Investigation of the role of lipoproteins and albumin within plasma

To further examine whether the inhibitory effect of plasma was attributable to the albumin and HDL within it, aggregation of LDL to which had been added a 1 in 10 dilution of normal plasma was compared with aggregation following the addition of diluted plasma that had been purified of lipoproteins and albumin. (The LDL concentration in all solutions was adjusted to 0.5 mg/ml by appropriate additions, and the plasma was diluted with PBS.) Figure 4C shows the increase in attenuance during repeated inversion of LDL in PBS in diluted normal plasma and in diluted lipid- and albumin-free plasma. As before, the plasma almost completely inhibited aggregation ($P < 0.001$ compared with LDL in PBS), but the modified plasma had a much smaller effect (21% inhibition instead of 91%) that was of borderline significance ($P \sim 0.05$). These results support the view that HDL and albumin are the main antiaggregatory constituents of plasma, although effects of lipoproteins other than HDL cannot be ruled out. They also suggest that there are other antiaggregatory plasma constituents, albeit less effective ones. The latter inference is supported by the observation that both the normal and modified plasma had to be diluted to obtain these results. In a pilot study where the modified and normal plasma were used at full strength, the difference in their inhibitory effects was much smaller ($P > 0.2$, data not shown); thus the antiaggregatory effects of plasma seem so powerful that even the minor components have a marked influence when it is used at full strength.

Investigation of the role of hydrophobicity

To investigate whether the aggregation of LDL and its inhibition by BSA are hydrophobic in nature, as suggested by Khoo et al. (18), effects of BSA were compared with

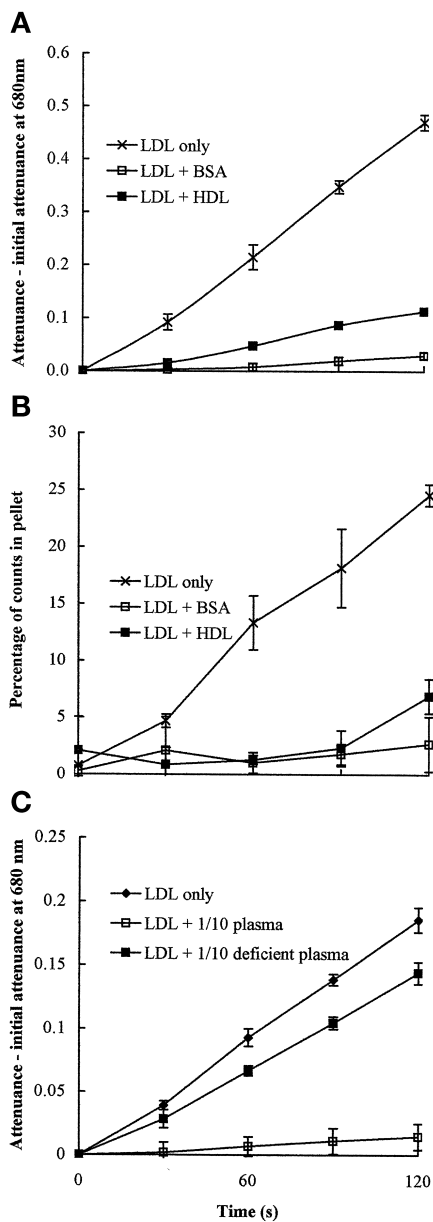


Fig. 4. Inhibitory influences of physiological concentrations of HDL and BSA on LDL aggregation were demonstrated equally by measurements of attenuation (A) or of the sedimentation of ^{125}I -LDL (B), indicating that the particles causing turbidity were aggregates of LDL rather than the inhibitors. C: Support for the inhibitory influences also derived from the finding that LDL aggregation was strongly inhibited by plasma diluted 1:10 with PBS ($P < 0.001$), but only marginally by diluted plasma from which lipoproteins and albumin had been removed ($P \approx 0.05$). Points show the mean \pm SD of three (A, B) or six (C) replicates. All graphs are representative of three independent experiments.

those of heat-denatured BSA [heating leads to unfolding and the exposure of hydrophobic domains (28)]. After BSA (5 mg/ml) had been heated to 85°C for 20 min, it was a significantly more effective inhibitor than normal BSA at the same concentration, reducing aggregation during inversion by 79% rather than 60% (Fig. 5A; $P < 0.001$).

To determine how much of the increased attenuation seen in the presence of these additives was caused by aggre-

gation of LDL rather than aggregation of the normal or heated BSA, the inhibitors were subjected to the same inversion procedure in the absence of LDL. Both gave only a small increase in attenuation (Fig. 5A), supporting the view, as with the sedimentation data in Fig. 4, that the increase seen in the presence of LDL was largely due to the aggregation of LDL itself. There was no significant difference in self-aggregation between normal and heated BSA ($P > 0.1$).

To further investigate the role of hydrophobicity, inhibition by normal BSA was compared with inhibition by BSA

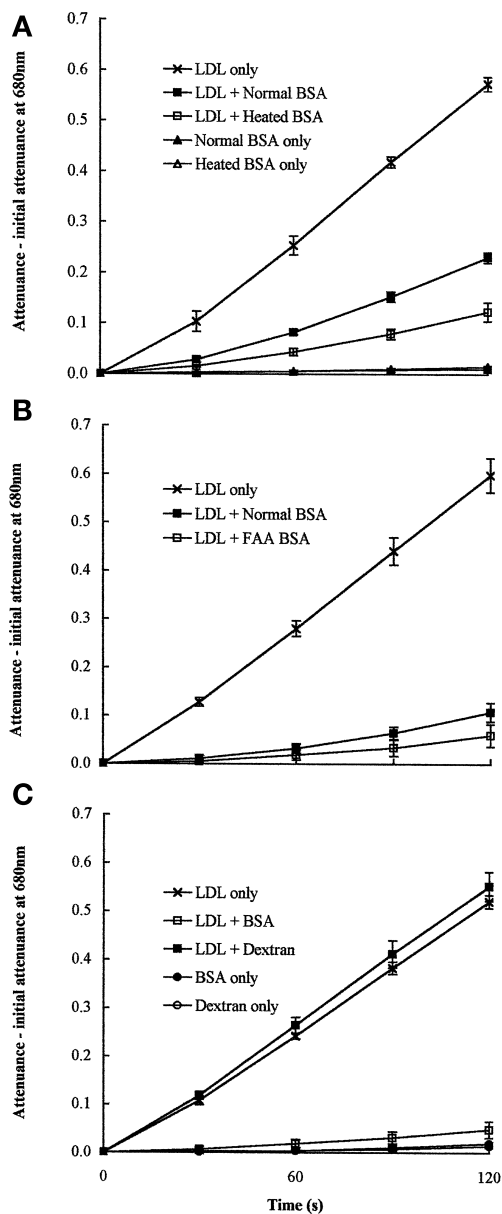


Fig. 5. A: Heat-denatured BSA and (B) BSA from which fatty acids had been absorbed (fatty acid-absorbed BSA) both inhibited LDL aggregation significantly more than normal BSA (both $P < 0.001$). The increase in turbidity observed for the inhibitors subjected to repeated inversion on their own was too small to have affected this conclusion. C: Dextran at a concentration giving the same viscosity as BSA had no influence on aggregation ($P > 0.1$). Points show the mean \pm SD of six replicates. The graphs are representative of three independent experiments.

from which fatty acids had been removed in order to free their hydrophobic stabilization sites. The fatty acid-absorbed BSA (40 mg/ml), prepared by the method of Chen (29), was a better inhibitor. It reduced aggregation by 90%, which was significantly higher than the 82% obtained with normal BSA (Fig. 5B; $P < 0.001$). In the absence of LDL, the increase in attenuation produced by fatty acid-absorbed BSA alone was too small to have influenced this result to a significant extent, and was not significantly different from that obtained with normal BSA ($P > 0.7$, data not shown).

Investigation of the role of viscosity

Although the influences of BSA, HDL, and heating can be explained in terms of hydrophobicity, they might also result from increases in viscosity. Heating in particular produced a large increase in the viscosity of the BSA solution. (It was for this reason that heated and unheated BSA had to be compared at subphysiological concentrations.) To investigate this possibility, the attenuation of LDL solutions inverted in the presence or absence of dextran was measured. Dextran (71 kDa; Sigma) was used at 6.5 mg/ml to give the same viscosity as BSA at 40 mg/ml. The albumin inhibited aggregation by 95% ($P < 0.001$), but the dextran had no influence (Fig. 5C; $P > 0.1$). Neither the BSA nor the dextran self-aggregated to an extent that could affect the interpretation of this result (Fig. 5C).

Aggregation by physiological-type flows

Aggregation of LDL solutions did occur in the model of physiological flow but at a 200-fold lower rate than during inversion (Fig. 6A). The difference was highly significant ($P < 0.001$). The increase in attenuation of plasma subjected to this flow was 91% lower than that of LDL on its own (Fig. 6B; $P < 0.001$). This figure appears close to the 93% obtained during repeated inversion (Fig. 3A), but may be an underestimate. When plasma was compared with lipid-deficient plasma (Fig. 6C), there was no significant difference between the two ($P > 0.3$). Therefore, LDL aggregation is unlikely to be contributing to the increase in attenuation seen with normal plasma; the inhibition of LDL aggregation in plasma, within the sensitivity of our techniques, appears to be 100%.

Further experiments were conducted to determine the likely roles of HDL and albumin in this inhibition. Figure 7A shows that aggregation of LDL was inhibited 63% ($P < 0.001$) by 1.5 mg/ml HDL, a very similar figure to the 69% obtained during inversion (Fig. 3B). The increase in attenuation seen with HDL alone was too small to have affected this result (Fig. 7A). Inhibition of aggregation by BSA at 40 mg/ml appeared to be 66% (Fig. 7B; $P < 0.01$), a much lower figure than the values (typically 90%) observed during inversion. However, the increase in attenuation observed for LDL and BSA together was not significantly different from that observed for BSA alone (Fig. 7B; $P > 0.8$). Hence, the true inhibition of LDL aggregation by BSA during arterial-type flows is likely to be closer to the figure observed during inverting, and could be as high as 100%. It is also plausible that the self-aggregation of albumin may explain some of the increase in attenu-

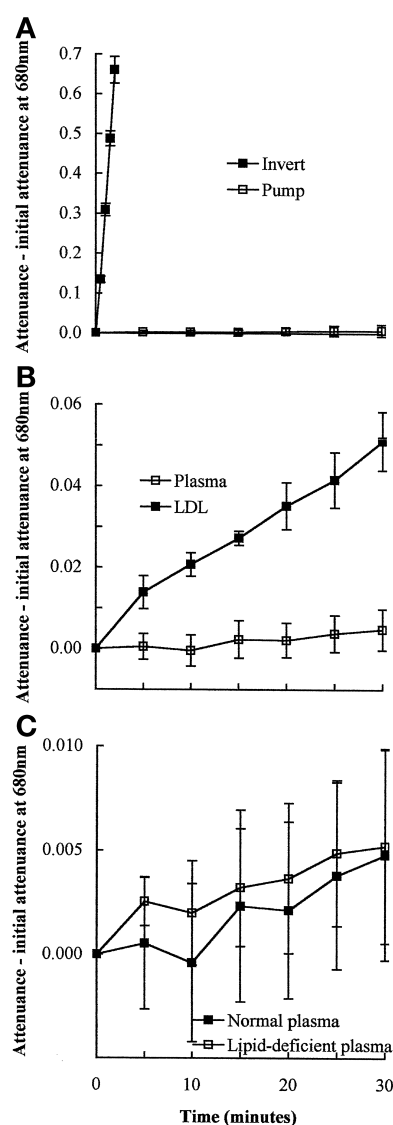


Fig. 6. A: Aggregation of LDL solutions subjected to physiological-type flows (“Pump”) was 200-fold slower than aggregation during repeated inversion ($P < 0.001$). B: The increase in turbidity of plasma exposed to physiological-type flows was <10% of the increase for LDL solutions ($P < 0.001$) and (C) was not different from that observed for lipid-deficient plasma ($P > 0.3$), suggesting that the increase in turbidity was not caused by LDL aggregation. Points show the mean \pm SD of six replicates, except for lipid-deficient plasma, where $n = 3$. All graphs are representative of three independent experiments.

ance seen in lipid-deficient plasma subject to this flow. (Since there are approximately 500 times more albumin particles than LDL particles in each volume of blood, this finding is unlikely to have any significance for the half-life of albumin in the circulation.)

Dissociation of the effects of laminar and turbulent flow

In some experiments, aggregation of LDL in PBS was examined in the model circulation after the tube had been clamped just downstream of the peristaltic pump. Thus, the LDL was subject to the squeezing effect of the rollers and to local flows induced by the pump, but not to flow around the loop of tubing (except for short periods

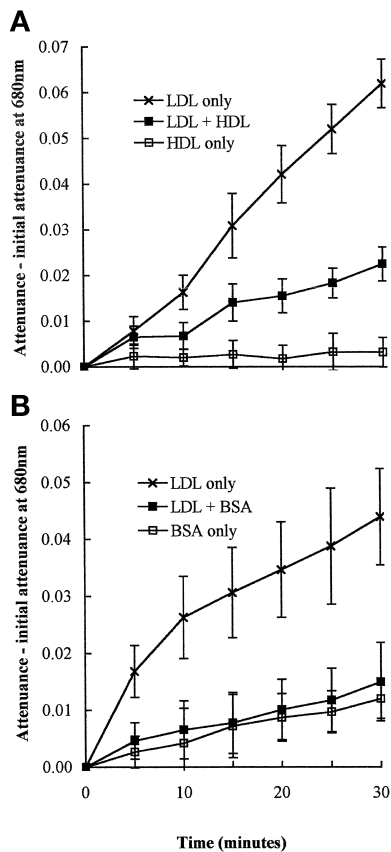


Fig. 7. A: Aggregation of LDL solutions subjected to physiological-type flows was inhibited by physiological concentrations of HDL ($P < 0.001$); self-aggregation of HDL was too low to have influenced this result. B: BSA at physiological concentrations also inhibited LDL aggregation ($P < 0.01$). There was no significant difference from the result obtained for BSA alone ($P > 0.8$), suggesting that the inhibition by BSA was complete or near complete. Points show the mean \pm SD of six replicates. The graphs are representative of three independent experiments.

before and after each sample was withdrawn, when such flow was used to mix the tube contents). Attenuance in the presence of the clamp reached a value $80\% \pm 5\%$ (mean \pm SD for three independent experiments) of that reached in control experiments conducted without the clamp, consistent with the pump-induced squeezing and local flows being responsible for the majority of the aggregation occurring in the unclamped model. Results from a typical experiment are shown in **Fig. 8**. As in all three individual experiments, the difference in slopes between clamped and control experiments was not significant ($0.2 > P > 0.1$). However, there was a significant effect when the mean of all three experiments was considered ($P < 0.05$), consistent with a real proaggregatory influence of laminar flow around the loop of tubing.

DISCUSSION

This study was motivated by recipes for lipoprotein preparation that warn that LDL is prone to aggregation

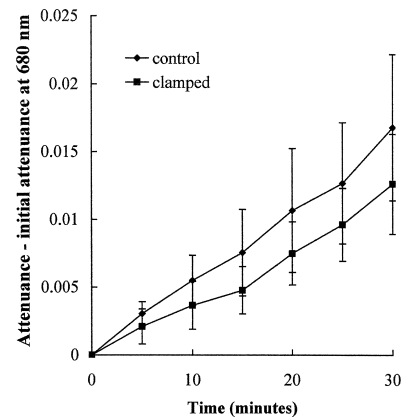


Fig. 8. Aggregation of LDL solutions in the model of physiological-type flows was reduced if the loop of tubing representing the arterial system was clamped. The difference in aggregation rate was not large ($0.2 > P > 0.1$), but when all three experiments were considered it was significant ($P < 0.05$), suggesting that the pumping action and, to a lesser extent, the laminar flow around the loop both contributed to the aggregation. Points show the mean \pm SD of six replicates. The graph is representative of three independent experiments.

when stirred even at moderate rates in the laboratory. More severe fluid mechanical stresses are present in the circulation and would result in the rapid loss of all circulating monomeric LDL unless aggregation were reduced in some way. Two obvious possibilities are that substances within plasma inhibit aggregation, and that there is a qualitative difference between in vitro and in vivo flows. Khoo et al. (18) showed that HDL and low concentrations of lipoprotein-deficient serum or albumin can partially inhibit the aggregation of LDL induced by vortexing, but aggregation of LDL in plasma or in solutions containing physiological concentrations of albumin, and the effects of the types of flow found in vivo, have not previously been investigated.

The first part of the present work investigated influences of plasma and its constituents, and the possible mechanisms involved. Aggregation was induced by repeatedly inverting a closed tube containing solutions of LDL, as well as by vortexing. Aggregation was assessed from the attenuance of light passing through the solution and, in several experiments, by the sedimentation of radioiodinated LDL. Inverting, like vortexing, resulted in a time-dependent increase in attenuance; the rate was somewhat slower, but the same maximum was attained. Unlike vortexing, however, the method is well defined and could be replicated precisely. With both methods, good agreement was obtained between the attenuance and sedimentation indices, supporting the view that the simpler attenuance measurement is a satisfactory measure of aggregation.

Using the inverting system, the increase in attenuance of plasma was found to be much lower than that of LDL solutions, but it was still measurable, suggesting that plasma constituents partially inhibit LDL aggregation under these conditions but cannot completely prevent it. To investigate the mechanisms underlying this effect, influ-

ences of HDL and albumin on the attenuation of LDL solutions during inverting were investigated. The highest concentrations of these plasma constituents studied by Khoo et al. (18) were 2.0 mg protein/ml for HDL and 10 mg/ml for albumin. These concentrations inhibited aggregation during vortexing by 84% and 45%, respectively, indicating that HDL is a more powerful inhibitor than albumin. These figures are close to the values (76% and 40%) for the same concentrations of HDL and albumin obtained during inverting in the present study. The agreement suggests that aggregation occurs by the same fundamental mechanism during inverting as during vortexing. We additionally examined the effects of albumin at concentrations up to and exceeding those in plasma. Its inhibitory influence was strongly dependent on concentrations up to 40 mg/ml, and then increased only slowly. This concentration is equivalent to about 600 albumin molecules per LDL particle. At physiological concentrations of albumin and HDL, it was albumin that had the larger inhibitory effect on attenuation (>80% vs. 69%).

By also measuring aggregation with the sedimentation technique, and by examining the behavior of the inhibitors on their own, it was confirmed that the increases in attenuation were attributable to LDL aggregation and not to the aggregation of albumin or HDL. Experiments in which lipoproteins (including HDL) and albumin were removed from plasma also supported the view that these are its main antiaggregatory constituents, although evidence was obtained for the existence of other components with a smaller influence. HDL had a much higher specific effect, but albumin, by virtue of its higher physiological concentration, is likely to be more important in vivo. This may be one reason why serum albumin concentrations are inversely correlated with cardiovascular mortality and coronary heart disease (30, 31).

Khoo et al. (17, 18) found that aggregation and its inhibition still occurred at high salt concentrations or after methylation of the LDL. They consequently inferred that both processes depend on hydrophobic interactions, and suggested that hydrophobic domains of LDL are exposed by a conformational change occurring at air-water interfaces, allowing these interactions to take place. The present study demonstrated that the inhibitory influence of albumin on LDL aggregation was increased if the albumin was first heated or stripped of fatty acids. Since these modifications increase the hydrophobicity of albumin, the data are consistent with the view of Khoo et al. that hydrophobic interactions are involved. The failure of high-molecular-weight dextran to inhibit aggregation eliminated the possibility that increases in viscosity are responsible.

The present study also investigated for the first time whether physiological flows cause less aggregation of LDL. A model was developed which mimicked several of the essential features of circulatory geometries and flows: the mean flow rate, Reynolds number, Womersley parameter, diameter, and curvature were appropriate for the aorta of a laboratory animal, such as the rabbit, or for medium-sized human arteries. Furthermore, flow was induced by a squeezing motion, as in the heart. Aggregation

of LDL solutions still occurred in this model but at a 200-fold lower rate than during repeated inverting. Thus, violent flows, such as those produced in a vortexor, are not required in order to induce aggregation, and the presence of air-water interfaces is also unnecessary. In this model, albumin and HDL again inhibited the aggregation of LDL solutions, suggesting that hydrophobic interactions may still be important. The majority of the aggregation appeared to be caused by the squeezing motion mimicking the action of the heart and by the local turbulent flows it caused, but shearing between laminae within the fluid circulating around the loop representing the arterial system seemed to cause ~20% of the LDL aggregation, possibly by unfolding it and exposing hydrophobic domains.

When plasma was examined in this model, its attenuation increased at a very low rate and, importantly, there was no tendency, significant or otherwise, for this rate to be higher than the rate observed for lipid-deficient plasma. Thus, within the sensitivity of our system, no evidence could be obtained that any LDL aggregation occurs in plasma exposed to physiological-type flows. Even if some undetected aggregation of LDL did occur, the rate could be still lower in vivo since the walls of the model artery were probably more hydrophobic than the surface of the arterial wall, and flow may have increased interaction of the LDL particles with them. The results appear to preclude any significant influence of arterial flows on LDL turnover within the circulation.

Maor and colleagues (32), using spectrophotometric and sedimentation techniques similar to those employed here, found no evidence that aggregated LDL particles circulate in human blood. This supports the observations of the present study, and appears to rule out the possibility that aggregates are formed by hemodynamic stresses other than those we investigated, for example, by the high shear rates present in the microcirculation (33). It seems unlikely that the negative result of Maor et al. could reflect the formation and then rapid removal of aggregates, because circulating aggregates were found in apoE-deficient mice aged ≥ 4 months (32). This observation suggests that unless there is a substantial difference between species, aggregates formed in the human circulation would remain there long enough to be detected. The fact that aggregates were absent in younger mice is consistent with the view that their formation depends in some way on arterial lesions, which are present in mature but not younger mice, and not solely on the flow of blood itself, which would be qualitatively similar at all ages. Aggregates could form from oxidized LDL leaching out of lesions, or could form in lesions and then enter the circulation, perhaps via adventitial lymphatics. There is substantial evidence that aggregates are present within lesions (34–39).

In summary, the main conclusion of the present study is that significant LDL aggregation is unlikely to occur within circulating blood: the hemodynamic stresses are insufficiently severe and the protective effect of albumin and HDL are too great. The increased evidence from the present study that LDL aggregation involves hydrophobic

interactions and the suggestion that albumin plays the largest antiaggregatory role may assist in elucidating the pathways by which such aggregation occurs within the arterial wall. **■**

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