LDLs Increase the Exposure of Fibrinogen Binding Sites on Platelets and Secretion of Dense Granules

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Abstract Because previous studies show that lipoproteins affect platelet aggregation, we studied the effect of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) on the binding of fibrinogen, which mediates platelet-platelet contact. Neither LDL nor HDL induced fibrinogen binding at concentrations up to 2 g protein/L. In contrast, platelets stimulated with 10 μmol/L ADP bound 63 734±2453 molecules of fibrinogen per platelet. A 5-minute preincubation with LDL (0.5 to 2 g/L protein) induced a dose-dependent increase to 91 307±2164 molecules of fibrinogen per platelet at 1.5 g/L, which is in the range found after optimal stimulation with α-thrombin. The increased fibrinogen binding in the presence of LDL resulted in faster aggregation with a 16% increase in single platelet disappearance and a faster optical aggregation at 5 μmol/L ADP and 1.5 g protein/L LDL. Inhibition of plasma membrane, it is unable to bind soluble fibrinogen to the glycoprotein (GP) IIB/IIIA complex. Also, when normal platelets are incubated with isolated LDL, the cells show an increased sensitivity to aggregating agents than platelets from normal subjects.

Epidemiological studies indicate that the incidence of thromboarteriosclerotic diseases correlates closely with the concentration of plasma cholesterol. A key step in the induction of thrombosis/artherosclerosis is the adhesion and aggregation of platelets to the damaged vessel wall. Recent data indicate that lipoproteins, which are the main carriers of cholesterol, affect platelet function. Platelets of patients with an increased level of low-density lipoproteins (LDLs), such as in type II hyperlipoproteinemia and familial hypercholesterolemia, are more sensitive to aggregating agents than platelets from normal subjects. Also, when normal platelets are incubated with isolated LDL, the cells show an increased sensitivity to platelet agonists. Studies on a possible interaction between high-density lipoproteins (HDLs) and platelets have led to conflicting results. Some studies reported inhibition of thrombin-induced aggregation and secretion by HDL, whereas others described no effect of HDL or even an enhanced sensitivity to activating agents.

Platelet aggregation is mediated by binding of fibrinogen to the glycoprotein (GP) IIB/IIIa complex. Although GPIIb/IIIa is present on the outer leaflet of the plasma membrane, it is unable to bind soluble fibrinogen unless the cells are stimulated. On addition of platelet-activating agents such as ADP and α-thrombin, there is a change in conformation of the GP complex that makes it accessible to fibrinogen, fibrinectin, von Willebrand factor, and vitronectin. Fibrinogen forms bridges between platelets leading to the formation of aggregates.

In the present study, we investigated the effect of isolated lipoproteins on the ADP-induced exposure of fibrinogen binding sites on the GPIIb/IIIa complex. Our data showed that LDL but not HDL enhanced the exposure of fibrinogen binding sites via a mechanism that is different from the effect of LDL on platelet secretion.

Methods

Indomethacin, phenylmethylsulfonyl fluoride, imipramine, and α-thrombin were purchased from Sigma, St Louis, Mo, and ADP and the “Monotest cholesterol” were from Boehringer Mannheim, Mannheim, FRG. Fibrinogen (grade L) was from Kabi, Stockholm, Sweden, and NaI (specific activity, 629 GBq/mg) and [14C]serotonin (specific activity, 1.85 to 2.20 GBq/mmol) were from Amersham International, Amersham, Buckinghamshire, UK. D-Phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (PPACK) was purchased from Bachem, Bubendorf, Switzerland, and thimerosal from Merck, Darmstadt, FRG. Bovine serum albumin (degeminalized) was from Organon Teknika, Turnhout, Belgium. All other chemicals were of analytical grade.

Platelet Isolation

Freshly drawn venous blood from healthy volunteers who claimed not to have taken any medication during the previous 10 days was collected into 0.1 vol 3.8% trisodium citrate. Platelet-rich plasma was prepared by centrifugation (200g for 10 minutes at 22°C). Platelets were isolated by gel filtration...
through a Sepharose 2B column equilibrated in calcium-free Tyrode's solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 1.7 mmol/L MgCl₂, and 11.9 mmol/L NaHCO₃, pH 7.25) containing 0.2% bovine serum albumin (demineralized) and 0.1% glucose.

**Preparation of ¹²⁵I-Labeled Fibrinogen**

Fibrinogen was made fibrin- and fibrinectin-free by passage through a gelatin-Sepharose 4B column. The isolated fibrinogen was radiolabeled with Na¹²⁵I by a modified Iodo-Gen method. Details of these procedures have been described.²⁷

**Isolation of LDL and HDL**

LDL (d, 1.019 to 1.063 g/mL) and HDL (d, 1.063 to 1.225 g/mL) were isolated from fresh citrate-phosphate-dextrose plasma from six random donors by sequential flotation in a Beckman L-80 ultracentrifuge. To prevent oxidation and bacterial contamination, 0.25 mmol/L phenylethylsulfonfluoride, 0.2 mmol/L thimerosal, 2 mmol/L NaN₄, and 0.4 mmol/L EDTA (all final concentrations) were added to the plasma. Subsequent centrifugations (20 hours at 175 000g at 18°C) were carried out in the presence of sodium azide and EDTA. Potassium bromide was used for density adjustment. Lipoproteins were dialyzed against buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.3 mmol/L NaN₄ and were subsequently filtered through a 0.45-μm filter. Isolated LDL and HDL were stored for not more than 14 days at 4°C, and before the incubation studies with platelets the lipoproteins were dialyzed a second time overnight against 150 mmol/L NaCl. The final preparation was devoid of appreciable amounts of oxidized LDL; compared with a sample concurrently dialyzed in the presence of 10 μmol/L EDTA, diene formation induced by 20 μmol/L CuSO₄ was within the experimental error of the control.²⁸ Contamination with endotoxins was below detection limits (less than 3 pg/mL).²⁹ The protein content of the lipoproteins was determined by using the procedure of Lowry et al.³⁰

**Lysine Modification of LDL**

Lysine residues in the cell-binding domain of LDL were modified by carbamoylation with cyanate. This treatment abolishes the specific binding of LDL to platelets.³¹ Details of this procedure are described by Weisgraber et al.³² Lipoproteins (2 to 5 g protein/mL) in 150 mmol/L NaCl and 0.27 mmol/L EDTA were diluted with 0.5 vol 0.3 mol/L sodium borate buffer pH 8.0. Potassium cyanate (20 g/L protein) was added to the lipoprotein solution, and the mixture was incubated at 35°C for 2 hours. Excess reagent was removed by dialysis at 4°C against 150 mmol/L NaCl and 0.27 mmol/L EDTA, pH 7.0, for 36 hours.

**Platelet Stimulation and Binding of ¹²⁵I-Fibrinogen**

Gel-filtered platelets (GFPs; 200 to 300×10⁶ platelets/μL) were stimulated at 22°C with 10 μmol/L ADP or 0.1 U/mL α-thrombin in the presence of 1 μmol/L ¹²⁵I-fibrinogen and without stirring. To prevent aggregation, 30 mmol/L PPACK (final concentration) was added 3 minutes after the addition of α-thrombin.

To investigate the effect of LDL, HDL, and lysine-modified LDL, GFPs were preincubated with different concentrations of these lipoproteins (2 g protein/L) for 5 minutes at 22°C without stirring. Subsequently, these lipoproteins were stimulated with 10 μmol/L ADP and incubated with 1 μmol/L radiolabeled fibrinogen for 5 to 60 minutes at 22°C without stirring.

The binding of fibrinogen was measured by placing 200-μL samples of cell suspension (in triplicate) on top of 100 μL 25% (wt/vol) sucrose in calcium-free Tyrode's solution in microsedimentation tubes (Sarstedt, Vienna, Austria) and separating the cells from the medium by centrifugation (12 000g for 2 minutes at 22°C) in a Beckman Microfuge E. The tip of the tube (pellet fraction) was cut off and the pellet and supernatant were counted in a gamma counter. The number of molecules bound per platelet was calculated from the radioactivity in the pellet fraction compared with the total activity in the pellet plus supernatant. The data were corrected for nonspecific binding, which was defined as the binding of ¹²⁵I-fibrinogen to unstimulated platelets. The nonspecific binding measured with a 100-fold excess of nonlabeled fibrinogen is identical to the binding of fibrinogen to unstimulated platelets.³⁷ Details have been described elsewhere.²⁷,³³,³⁴

**Single Platelet Disappearance and Optical Aggregation**

GFPs (approximately 200×10⁶ platelets/μL) were incubated with different concentrations of LDL (0 to 2 g protein/L) for 5 minutes at 22°C. The platelets were preheated at 37°C for 1 minute and subsequently stimulated with ADP (1 to 10 μmol/L) in the presence of 1 μmol/L fibrinogen at 37°C in a Chrono-Log lumiaagrégometer (Chrono-Log Corporation, Havertown, Pa) at a stirring speed of 900 rpm. Aggregation was measured as single platelet disappearance.³⁵ Samples were collected 15 seconds after addition of the agonist and were immediately fixed with 9 vol ice-cold 0.5% glutaraldehyde in 0.9% NaCl. The platelets were counted in a platelet analyzer (model 810, Baker Instruments, Allentown, Pa) with apertures set at 3.2 and 16 μm.⁶ Optical aggregation was monitored in a Chrono-Log lumiaagrégometer at 37°C at a stirring speed of 900 rpm. Changes of light transmission in the platelet suspension were recorded.

**Measurement of Dense Granule Secretion**

For the measurement of dense granule secretion, platelet-rich plasma was incubated with 1 μmol/L [³⁵S]serotonin for 30 minutes at 37°C followed by gel filtration on Sepharose 2B (see above). Secretion studies were carried out in the presence of 2.5 μmol/L imipramine to prevent reuptake of serotonin into the platelets. GFPs (150 to 200×10⁶ platelets/μL) were preincubated with purified LDL (2 g protein/L) for 5 or 30 minutes at 37°C and stimulated with 0.1 to 0.2 U/mL α-thrombin with stirring (900 rpm). At 5 minutes after stimulation samples were collected in 0.15 vol 1.035 mol/L formaldehyde in 150 mmol/L NaCl (0°C, freshly prepared) to stop secretion. After centrifugation (10 000g for 1 minute at 4°C), the supernatants were collected and used for analysis of [³⁵S]serotonin. The data were corrected for liberation of [³⁵S]serotonin from unstimulated suspensions after a 30-minute incubation without LDL (less than 3%) and with LDL (6±2%) and were expressed as a percentage of maximal secretable [³⁵S]serotonin, which was measured by incubating samples for 5 minutes at 37°C with 5 U/mL α-thrombin as defined elsewhere.³⁶

**Statistics**

All data are presented as mean±SD from at least three determinations. Differences were considered significant at a probability value of less than .05 by Student's t test.

**Results**

**Effect of LDL and HDL on the Exposure of Fibrinogen Binding Sites**

Stimulation of GFPs with ADP (10 μmol/L) in the presence of 1 μmol/L ¹²⁵I-fibrinogen induced a maximal binding of 63 734±2435 (n=9) fibrinogen molecules per platelet (Fig 1, top). When isolated platelets were first preincubated for 5 minutes with purified LDL (2 g protein/L, 22°C) and subsequently stimulated with ADP (10 μmol/L), a binding of 90 363±2461 (n=3) fibrinogen molecules per platelet was found. This number is in the range induced with α-thrombin (98 754±4375 [n=3]; Fig 1, top) and represents the maximal number of inducible fibrinogen binding sites. Essentially the
van Willigen et al  Effect of LDL on GPIIB/IIIa Exposure

same effect was seen after a 30-minute preincubation with 2 g protein/L LDL (10 μmol/L ADP, 69 520±2997; ADP+LDL, 103 277±3777 molecules of fibrinogen per platelet; mean±SD, n=3), indicating that a relatively short interaction between LDL and the platelet was sufficient for stimulation of fibrinogen binding.

When LDL was replaced by purified HDL, no effect on ADP-induced fibrinogen binding was observed (Fig 1, bottom). Furthermore, in the absence of ADP neither LDL nor HDL induced fibrinogen binding (Fig 1).

When isolated platelets were preincubated with various concentrations of LDL (0.5 to 2 g protein/L) for 5 minutes at 22°C and subsequently stimulated with ADP (10 μmol/L), a concentration-dependent increase in fibrinogen binding was seen (Fig 2). When platelets were stimulated with lower concentrations of ADP (1 μmol/L, 2.5 μmol/L, and 5 μmol/L) there was also an enhancement of fibrinogen binding by LDL that was again dependent on the concentration of LDL. After stimulation with 10 μmol/L ADP a maximum effect was reached with an LDL concentration of 1.5 g protein/L.

To investigate whether the effect of LDL on the exposure of fibrinogen binding sites was mediated via formation of prostaglandin (PG) G2/H2 and thromboxane A2 (TXA2), platelets were preincubated with indomethacin (30 μmol/L for 30 minutes at 37°C) before the addition of LDL. This treatment resulted in binding data that were similar to those of untreated platelets (93 618±3672 fibrinogen molecules per platelet, mean±SD, n=3, P>.05), indicating that LDL enhanced the fibrinogen binding independently of PGG2/H2 or TXA2 production.

Fig 1. Line graphs showing effects of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) on exposure of fibrinogen binding sites. Top, Gel-filtered platelets were preincubated with 2 g protein/L LDL (■,■) or with an equal volume of calcium-free Tyrode’s solution (●). Subsequently the suspensions were stimulated with 10 μmol/L ADP (closed symbols) in the presence of 1 μmol/L 125I-fibrinogen, or an equal volume of Tyrode’s was added (open symbols). In control studies, platelets were stimulated with 0.1 U/mL α-thrombin (▲; coagulation was prevented by 30 nmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone [PPACK] added 3 minutes after α-thrombin stimulation) to measure maximal fibrinogen binding. At different incubation times samples were analyzed for specific binding of fibrinogen. n=3 except for the ADP-stimulated suspension without LDL, for which n=9. Bottom, Gel-filtered platelets were preincubated with 2 g protein/L HDL (▲,▲) or with an equal volume of calcium-free Tyrode’s solution (●). Subsequently the suspensions were stimulated with 10 μmol/L ADP (closed symbols) in the presence of 1 μmol/L 125I-fibrinogen, or an equal volume of Tyrode’s was added (open symbols). In control studies, platelets were stimulated with 0.1 U/mL α-thrombin (▲; coagulation was prevented by 30 nmol/L PPACK added 3 minutes after α-thrombin stimulation) to measure maximal fibrinogen binding. At different incubation times samples were analyzed for specific binding of fibrinogen. n=3 except for the ADP-stimulated suspension without HDL, for which n=9. Data are mean±SD for both graphs. *Significantly different from control by Student’s t test at P<.05.

Fig 2. Line graph showing effect of different concentrations of low-density lipoprotein (LDL) on ADP-induced fibrinogen binding. Gel-filtered platelets were preincubated with 0 to 2 g protein/L LDL for 5 minutes at 22°C. Subsequently, the suspensions were stimulated with 1 μmol/L (▲), 2.5 μmol/L (○), 5 μmol/L (▲), and 10 μmol/L ADP (●) in the presence of 1 μmol/L 125I-fibrinogen. At 60 minutes after stimulation samples were analyzed for specific binding of fibrinogen. The results are expressed as mean±SD, n=3.
times samples were analyzed for specific fibrinogen binding. These platelets were stimulated with 10 mM ADP in the presence of 1 mM [125I]fibrinogen. After different incubation times samples were analyzed for specific fibrinogen binding. Data are mean±SD, n=3.

**Effect of Lysine-Modified LDL on the Exposure of Fibrinogen Binding Sites**

Modification of lysine residues in LDL is known to inhibit specific binding to platelets. When GFFs were incubated with lysine-modified LDL and subsequently stimulated with ADP (10 μmol/L), 74 928±230 (n=3) molecules of fibrinogen were bound per platelet (Fig 3). This number was in the same range as observed without LDL (71 631±2896, n=3), indicating that LDL stimulated the exposure of fibrinogen binding sites via specific binding to the platelet.

**Effect of LDL on Platelet Aggregation**

To investigate whether an increased fibrinogen binding in the presence of LDL also resulted in an increased platelet aggregation, we measured the disappearance of single platelets in platelet suspensions stimulated with ADP. A 5-minute preincubation with increasing concentrations of LDL (0 to 2 g protein/L) resulted in a faster disappearance of single platelets at all ADP concentrations tested (Table 1). With low ADP concentrations (1 and 2.5 μmol/L), 0.5 g protein/L LDL had no effect. The maximal stimulation was observed at an LDL concentration of 1.5 g protein/L. As expected, LDL also enhanced the optical aggregation of platelets. Fig 4 shows a typical example of a 5-minute preincubation with 1.5 g protein/L LDL on optical aggregation. Again the velocity of aggregation increased and the extent of the aggregation curve was higher in the presence of LDL. These results indicated that the stimulation of LDL on exposure of fibrinogen binding sites results in faster aggregation and formation of larger aggregates.

**Effect of LDL on [14C]Serotonin Secretion**

To investigate whether the increase in fibrinogen binding by LDL was accompanied by a similar effect on the secretion response, the effect of a 5- or 30-minute preincubation with 2 g protein/L LDL on the secretion of [14C]serotonin was investigated. Platelets from different donors were stimulated with different concentrations of α-thrombin to find suspensions that released approximately 40% to 60% of their dense granule contents. As shown in Table 2, the secretion induced by a-thrombin was not affected by a 5-minute preincubation with LDL, which is in contrast with the effect seen on fibrinogen binding. However, a 30-minute preincubation almost doubled the a-thrombin-induced secretion. Note worthy is the observation that the effect of LDL on secretion was unchanged after lysine modification of LDL, indicating that LDL increased the secretion response without binding to specific binding sites on the platelets.

**Discussion**

Several studies report that LDL enhances platelet functions induced by ADP, thrombin, and other plate-
let-activating agents. After a 10- to 30-minute preincubation with LDL (one- to twofold the normal concentration of about 0.7 g protein/L), LDL enhances the rate and extent of ADP-induced aggregation and stimulates the secretion of serotonin and TXA₂. The effect of LDL is explained by facilitating the phospholipase A₂-mediated liberation of arachidonic acid resulting in more PGG₃/H₂ and TXA₂ production. Indeed, inhibitors of cyclooxygenase or antagonists of PGG₃/H₂/TXA₂ receptors block the stimulation by LDL, but since the effect was incomplete, a second, indomethacin-insensitive mechanism had to be involved. The nature of this second mechanism is still obscure.  

Our present data showed that a 5-minute preincubation with purified LDL induced a dose-dependent increase in ADP-induced fibrinogen binding. At 1.5 g LDL protein/L the increase was maximal and reached the extent seen after optimal stimulation with thrombin. Landolfi et al. and Peerschke et al. show that the rate and extent of ADP-induced aggregation are determined by the number of bound fibrinogen molecules. Similar results were obtained by measuring surface-bound fibrinogen with an anti-fibrinogen antibody. Thus, the increased binding seen in the presence of LDL in our present experiments explains the increased ADP aggregation observed in earlier studies. The stimulation of fibrinogen binding by LDL also provides an explanation for the increased aggregation tendency seen in patients with increased levels of LDL, such as type II hyperlipoproteinemia and familial hypercholesterolemia.  

Essentially the same enhancement by LDL was observed in platelets treated with indomethacin. Thus, although formation of PGG₃/H₂ and TXA₂ is thought to mediate at least part of the activating properties of LDL, this pathway does not appear to affect the regulation of GPIIb/IIIa.  

So far it has been difficult to clarify how LDL modulates the activation mechanisms of the platelet. Binding studies with 1 g iodinated LDL protein/L show saturation of specific binding sites on platelets within 2 minutes at 22°C. Under our experimental conditions (a preincubation of 5 minutes with an LDL concentration of 0.5 to 2 g protein/L at 22°C), the LDL binding sites are probably fully occupied, which favors the concept that LDL enhances fibrinogen binding via a receptor-mediated event. The fact that LDL lost its activating properties after modification of lysine residues, which appear essential for specific binding, also points to a receptor-mediated mechanism that controls the exposure of binding sites on GPIIb/IIIa.  

Activation of protein kinase C (PKC) plays a major role in the exposure of GPIIb/IIIa. Andrews et al. show that LDL increases the phosphorylation of pleckstrin, a main substrate for PKC, induced by different agonists. This may explain the increased fibrinogen binding observed in this study. A second mechanism that regulates GPIIb/IIIa, a cyclic AMP-dependent step, closes the binding sites when cyclic AMP is increased slightly. Although in studies by Bruckdorfer et al. LDL attenuated the increase in cyclic AMP induced by prostacyclin, we found an equally rapid closure of binding sites by 1 to 10 nmol/L prostacyclin with and without LDL (1 g protein/L), indicating that the modulation of GPIIb/IIIa by LDL appears independent of this control mechanism (data not shown). Thus, the mechanism by which LDL affects exposure of fibrinogen binding sites must be sought in the effect of LDL on PKC.  

LDL potentiates the binding of MG-63 cells to immobilized fibronectin. The stimulation by LDL was the same after modification of the cell-binding domain on LDL by acetylation, indicating that this effect differs from stimulation of fibrinogen binding to platelets by LDL. This may be due to the fact that the LDL receptor on platelets differs from the LDL receptor on nucleated cells.  

Koller et al. report that LDL binds directly to the GPIIb/IIIa complex of platelets, providing a means to activate the complex independently of intracellular signaling processes. Their evidence is based on binding studies with biotinylated fibrinogen to GPIIb/IIIa blotted on nitrocellulose. Both LDL and HDL blocked the binding of fibrinogen to GPIIb/IIIa completely, suggesting that they share binding sites on GPIIb/IIIa with fibrinogen. In the present study no effect of HDL was found, whereas LDL increased the binding of fibrinogen instead of inducing inhibition. Blotting of GPIIb/IIIa on nitrocellulose probably causes the complex to assume a different conformation than that seen in intact platelets.  

Assuming that LDL changed the exposure of GPIIb/IIIa via intracellular mechanisms, one might expect a similar potentiation of the secretion responses. We investigated this possibility by using α-thrombin as a stimulus, since ADP induced little secretion in GPs. The incubation temperature was raised to 37°C to improve standardization of the secretion response. A 5-minute preincubation with LDL (2 g protein/L) had no effect on the [³⁵S]serotonin secretion induced by 0.1 U/mL α-thrombin. However, in accordance with earlier studies, a longer preincubation (30 minutes) with LDL increased the [³⁵S]serotonin secretion induced by 0.1 U/mL α-thrombin from 46 ± 7% to 90 ± 6% (n = 3). When the lysine residues in the cell-binding domain of LDL were modified, LDL did not lose its potentiating effect on secretion. Hence, the stimulation of [³⁵S]serotonin secretion by LDL differs in two aspects with the effect on fibrinogen binding. First, it requires a long LDL-platelet contact and second, it does not require interaction with specific binding sites for LDL on platelets. These observations raise the possibility that LDL

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<tr>
<th>TABLE 2. Effect of LDL on [³⁵S]Serotonin Secretion</th>
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<tr>
<td>Control (α-thrombin)</td>
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stimulates GPIb/IIIA exposure and secretion via different mechanisms.

References

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