Effect of Oxidation on the Platelet-Activating Properties of Low-Density Lipoprotein

Suzanne J.A. Korporaal, Gertie Gorter, Herman J.M. van Rijn, Jan-Willem N. Akkerman

Objective—Because of the large variation in oxidizing procedures and susceptibility to oxidation of low-density lipoprotein (LDL) and the lack in quantification of LDL oxidation, the role of oxidation in LDL–platelet contact has remained elusive. This study aims to compare platelet activation by native LDL (nLDL) and oxidized LDL (oxLDL).

Methods and Results—After isolation, nLDL was dialyzed against FeSO₄ to obtain LDL oxidized to well-defined extents varying between 0% and >60%. The oxLDL preparations were characterized with respect to their platelet-activating properties. An increase in LDL oxidation enhances platelet activation via 2 independent pathways, 1 signaling via p38MAPK phosphorylation and 1 via Ca²⁺ mobilization. Between 0% and 15% oxidation, the p38MAPK route enhances fibrinogen binding induced by thrombin receptor (PAR-1)-activating peptide (TRAP), and signaling via Ca²⁺ is absent. At >30% oxidation, p38MAPK signaling increases further and is accompanied by Ca²⁺ mobilization and platelet aggregation in the absence of a second agonist. Despite the increase in p38MAPK signaling, synergism with TRAP disappears and oxLDL becomes an inhibitor of fibrinogen binding. Inhibition is accompanied by binding of oxLDL to the scavenger receptor CD36, which is associated with the fibrinogen receptor, α₁β₃.

Conclusion—At >30% oxidation, LDL interferes with ligand binding to integrin α₁β₃, thereby attenuating platelet functions. (Arterioscler Thromb Vasc Biol. 2005;25:867-872.)

Key Words: lipoproteins • platelets • oxidized LDL • scavenger receptor • lysophosphatidic acid

Patients with familial hypercholesterolemia (FH) show an increased incidence of premature coronary artery disease. These patients lack or have a defective receptor for low-density lipoprotein (LDL), the apolipoprotein (apo) B/E-receptor,¹ which results in an impaired uptake of LDL from the circulation. LDL accumulates and becomes oxidized in the vessel wall at sites of injured endothelium. Uptake of oxidized LDL (oxLDL) transforms macrophages into foam cells, which are characteristic for the fatty streak, the early atherosclerotic lesion.² Plasma levels of oxLDL are higher in atherosclerotic plaques than in normal intima.³ Platelets are key elements in the development of arterial thrombosis and atherosclerosis. They adhere to injured endothelium, to exposed collagen, and to macrophages. On activation, platelets secrete cytokines and growth factors that contribute to migration and proliferation of smooth muscle cells and monocytes. Platelets of FH patients are hyper-reactive and show hyperaggregability in vitro and enhanced activity in vivo as illustrated by increased plasma levels of the α-granule product β-thromboglobulin and an increased prostaglandin (PG) and thromboxane (TXA₂) metabolism.⁵ Moreover, activated platelets have been found in the circulation of FH patients⁶ and high concentrations of oxLDL stimulate platelet adhesion and aggregation via suppression of endothelial production of nitric oxide and stimulation of the synthesis of PG precursors and prostaglandins.⁷ These observations suggest that LDL enhances platelet responsiveness. Native LDL (nLDL) is a mild activator of platelets via TxA₂-dependent and TxA₂-independent pathways.⁸–¹¹ Activation is mediated via a specific LDL receptor, which differs from the classical apoB/E receptor because a similar response is observed after LDL stimulation of platelets from healthy subjects, platelets from FH patients, and platelets that were treated with apoB/E receptor–blocking antibodies.¹² We recently identified apolipoprotein E receptor 2' (apoER2') as a possible candidate for LDL binding to platelets.¹³ apoER2' is a splice variant of apoER2 that has been identified in platelets and megakaryocytic cell lines.¹⁴ At physiological concentrations (0.6 to 0.9 g/L), nLDL increases the sensitivity of platelets for α-thrombin, collagen, and ADP but fails to independently induce platelet functions.⁸,¹⁰,¹¹,¹⁵ At higher concentrations (3 g/L), nLDL becomes an independent initiator of platelet activation triggering aggregation and secretion.¹⁶ nLDL-induced platelet sensitization is mediated via the activation of p38 mitogen-activated protein kinase.
Lysophosphatidic acid (LPA), generated during oxidation. LPA has platelet-activating properties, and LPA receptors respond to LPA. At low concentrations (EC50 < 0.5 pmol/L), LPA induces platelet aggregation. The ability of LDL to function as a platelet activator increases with oxidation. CuSO4-oxidized LDL independently induces arachidonic acid release and TxA2 formation. TxA2 further activates platelets via stimulation of the TxA2 receptor, causing activation of integrin αIIbβ3 and ligand-induced outside-in signaling through αIIbβ3.

The large variation in oxidizing procedures, the interindividual variation in susceptibility to oxidation of LDL and the lack in quantification of the degree of LDL oxidation have caused difficulties in comparing studies. Therefore, we prepared LDL preparations oxidized to well-defined extents and characterized their platelet-activating properties.

**Methods**

**Lipoprotein Modification**

To investigate the contribution of LPA in oxLDL-induced platelet activation, the mobilization of intracellular Ca2+ was measured by Fura-2/AM fluorescence. A similar p38MAPK phosphorylation was found in the presence of L-NASPA (Figure 1C), which blocks binding of LPA to its receptor, thereby antagonizing platelet functions induced by LPA, such as shape change and aggregation. These results indicate that LPA formed during LDL oxidation did not contribute to p38MAPK phosphorylation. As expected, oxLDL-induced p38MAPK phosphorylation was inhibited in the presence of the p38MAPK inhibitor SB203580 (Figure 1C).

To investigate the contribution of LPA in oxLDL-induced platelet activation, the mobilization of intracellular Ca2+ was measured by Fura-2/AM fluorescence. A similar p38MAPK phosphorylation was found in the presence of L-NASPA (Figure 1C), which blocks binding of LPA to its receptor, thereby antagonizing platelet functions induced by LPA, such as shape change and aggregation. These results indicate that LPA formed during LDL oxidation did not contribute to p38MAPK phosphorylation. As expected, oxLDL-induced p38MAPK phosphorylation was inhibited in the presence of the p38MAPK inhibitor SB203580 (Figure 1C).
significant change in [Ca$^{2+}$], but further oxidation strongly increased Ca$^{2+}$ mobilization to a 2-fold increase at $>60\%$ oxidation (Figure 1B). The increase in [Ca$^{2+}$], was completely blocked by l-NASPA (Figure 1C), indicating that LPA caused the Ca$^{2+}$ mobilization by oxLDL. OxLDL-induced Ca$^{2+}$ mobilization was not inhibited by SB203580 (Figure 1C). Thus, both p38 MAPK phosphorylation and Ca$^{2+}$ mobilization increased at increasing oxidation of LDL with a threshold of 15% to 30% oxidation, below which there was little difference with nLDL. In addition, the findings with l-NASPA and SB203580 suggest that oxLDL activates 2 independent pathways.

**OxLDL-Dependent Regulation of cAMP**
Platelet agonists initiate aggregation and secretion via G$_{i}$-mediated pathways while concurrently suppressing cAMP formation via ADP release and P2Y$_{12}$-mediated activation of the inhibitory G-protein, G$_{i}$.

Because p38 MAPK activation and Ca$^{2+}$ mobilization sense changes in cAMP, we investigated whether the higher activation observed at more oxidation resulted from suppression of cAMP. Platelets had a basal cAMP concentration of $3.87\pm1.41$ ng/10$^{11}$ cells, which was not disturbed by nLDL or oxLDL (data not shown). Prostacyclin (PGI$_{2}$) induced a 2.6-fold increase in cAMP, which was not changed by LDL preparations oxidized up to 30%. At $>30\%$ oxidation, oxLDL reduced the increase in cAMP by 30% to 40% (Table II, available online at see http://atvb.ahajournals.org). Similar results were observed in the presence of l-NASPA, indicating that the reduction was independent of LPA. As expected, α-thrombin reduced the PGI$_{2}$-induced cAMP accumulation amounting to a decline of 70%, an effect that was independent of LPA. Assuming that the inhibition of PGI$_{2}$-induced cAMP accumulation by oxLDL reflects a similar effect on the basal level of cAMP, which is difficult to measure, the platelet-activating properties of oxLDL were enhanced by suppression of cAMP in an LPA-independent manner.

**The Effects of oxLDL on Aggregation**

nLDL-induced p38 MAPK phosphorylation is an early and rapid step in a slow process that after 5 minutes or more synergistically increases agonist-induced fibrinogen binding, aggregation, and secretion. In contrast, LPA independently raises [Ca$^{2+}$], inducing shape change, aggregation, and secretion within seconds. Because oxLDL was a more potent activator of p38 MAPK phosphorylation than nLDL, and LPA-mediated Ca$^{2+}$ signaling is especially evident at high stages of oxidation, we investigated the functional responses initiated by the 2 pathways. After 5 minutes of pre-incubation, nLDL enhanced thrombin receptor (PAR-1) activating peptide (TRAP)-induced aggregation (Figure 2A). Surprisingly, this property disappeared on oxidation and at high oxidation oxLDL became an inhibitor of TRAP-induced platelet aggregation (Figure 2A). The inhibition was independent of LPA, because similar results were observed in the presence of l-NASPA (data not shown). In contrast, oxLDL induced aggregation within seconds (Figure 2B). This finding was in agreement with the rapid LPA-dependent mobilization of Ca$^{2+}$ by oxLDL (Figure 1). Aggregation was inhibited in the presence of l-NASPA, indicating that LPA was responsible (data not shown).

We further investigated the inhibition of aggregation by oxLDL via the p38 MAPK pathway and measured TRAP-induced fibrinogen binding to integrin $\alpha_{IIb}\beta_{3}$ in the presence of oxLDL. In unstimmed platelet suspensions, LDL alone failed to induce fibrinogen binding at any oxidation stage (data not shown). In contrast, after 5 minutes of pre-incubation, nLDL enhanced TRAP-induced fibrinogen binding (Figure 2C). Oxidation to $<15\%$ preserved the synergistic properties of nLDL, but further oxidation reduced this property and, at high oxidation, oxLDL inhibited TRAP-induced fibrinogen binding (Figure 2C). Inhibition was already observed at a concentrations of 750 mg/L oxLDL ($P=0.0329$) and increased at higher concentrations of oxLDL (1.0 g/L; $P=0.0220$) (Figure 2D). nLDL enhances TRAP-induced fibrinogen binding at this concentration, which indicates that the inhibition by oxLDL was not caused by changes in lipid composition of the medium. The inhibition of TRAP-induced fibrinogen binding was independent of LPA (data not shown).

**Inhibition of Platelet Functions by oxLDL**
CD36 is a scavenger receptor that is present on platelets and binds oxLDL with high affinity. To determine whether...
binding of oxLDL to CD36 is involved in oxLDL-induced inhibition of platelet aggregation, platelets were treated with the antibody FA6.152 to block binding of oxLDL to CD36, before addition of oxLDL and TRAP. Inhibition of oxLDL binding to CD36 by FA6.152 abolished the reduction of TRAP-induced fibrinogen binding by oxLDL in a dose-dependent manner (Figure 3A).

CD36 is known to associate with α<sub>IIb</sub>β<sub>3</sub> on the plasma membrane of resting platelets. Binding of oxLDL to CD36 might therefore inhibit ligand binding to α<sub>IIb</sub>β<sub>3</sub> or inhibit α<sub>IIb</sub>β<sub>3</sub> activation. We investigated the association of CD36 with CD61 (the β<sub>3</sub> subunit of α<sub>IIb</sub>β<sub>3</sub>) in the presence of LDL. CD36 associated with CD61 on resting platelets confirming earlier observations. No change in the association was observed on incubation up to 30 minutes with either nLDL or oxLDL (Figure 3B). Immunoprecipitation experiments with a nonspecific antibody failed to immunoprecipitate both CD61 and CD36 (Figure 3B).

To determine whether binding of oxLDL to CD36 might block ligand-binding to or activation of α<sub>IIb</sub>β<sub>3</sub>, platelets were treated with nLDL or oxLDL, apoB100 was immunoprecipitated, and the coimmunoprecipitation with CD36 was determined. Immunoprecipitation of the lipoproteins was associated with the precipitation of the 88-kDa scavenger receptor, CD36 (Figure 3C). Binding of nLDL to CD36 was transient, leading to dissociation after 5 minutes. In contrast, in platelet lysates stimulated with apoB100 and CD36 was persistent, indicating that oxLDL was still bound to CD36 after 5 minutes of oxLDL–platelet interaction. Collectively, these results indicate that the persistent binding of oxLDL to CD36 but not of nLDL is sufficient to block ligand binding to or activation of α<sub>IIb</sub>β<sub>3</sub> induced by TRAP and thereby to block TRAP-induced fibrinogen binding and aggregation (Figure 2).

nLDL sensitizes platelets to stimulation by collagen and TRAP via ligand-induced outside-in signaling through α<sub>IIb</sub>β<sub>3</sub>. Inhibition of ligand-binding to α<sub>IIb</sub>β<sub>3</sub> caused by binding of oxLDL to CD36 might impede outside-in signaling through α<sub>IIb</sub>β<sub>3</sub> and thus inhibit platelet function. To investigate whether outside-in signaling through α<sub>IIb</sub>β<sub>3</sub> was inhibited, platelets were incubated with LDL and TRAP-induced P-selectin expression was determined as a marker for α-granule secretion. Pre-incubation with nLDL for 5 minutes did not influence TRAP-induced P-selectin expression. In contrast, oxLDL inhibited TRAP-induced α-granule secretion (Figure 4). This observation indicates that on oxidation, LDL becomes an inhibitor of platelet functions by inhibition of ligand-induced outside-in signaling through α<sub>IIb</sub>β<sub>3</sub>.
Discussion

nLDL increases the responsiveness of platelets to activating agents, resulting in faster aggregation and secretion after stimulation with thrombin, ADP, and collagen. This sensitization process is slow, requiring 5 minutes or more (37°C), and starts with the rapid activation of p38MAPK, which via cPLA2-mediated arachidonic acid release triggers the formation of TxA2. TxA2 further activates platelets by stimulating the TxA2 receptor, leading to activation of integrin αIIbβ3 and αIIbβ3-mediated ligand-induced outside-in signaling. Blockade of TxA2 formation by indomethacin sharply reduces secretion.

Oxidation increases the platelet-activating properties of nLDL via 2 mechanisms, which depend on the degree of oxidation and the duration of platelet–LDL contact. The first mechanism involves p38MAPK activation. In this mechanism, a further increase in nLDL-induced p38MAPK activation on LDL oxidation leads to a 6-fold increase at >60% oxidation. Despite this increase in the activating pathway, concurrent fibrinogen binding remains constant and even decreases at 16% oxidation or more. The second mechanism is LPA-mediated platelet activation, which is insignificant at low oxidation but becomes a potent activation pathway at >30% oxidation, inducing Ca2+ mobilization and aggregation.

The observations that LPA is unable to activate p38MAPK (Figure 1C)27 and that p38MAPK-mediated functions are insensitive to the LPA receptor antagonist 1-NASPA illustrate that both pathways are mutually exclusive.

Unexpectedly, at >15% oxidation, synergism through the p38MAPK pathway does not result in faster platelet functions. Above this threshold, further oxidation decreases the sensitization of TRAP-induced fibrinogen binding and aggregation and inhibits ligand-induced outside-in signaling through αIIbβ3. A similar inhibition is seen with and without 1-NASPA, indicating that LPA is incapable of inducing fibrinogen binding after 5 minutes of pre-incubation with oxLDL. Importantly, the inhibition is absent when LDL oxidized at >30% makes immediate contact with stirred platelet suspensions. Apparently, the induction of platelet inhibition by oxLDL is a slow process because it does not interfere with the rapid induction of Ca2+ mobilization and platelet aggregation mediated via the LPA pathway. Possibly, the p38MAPK pathway becomes important at later stages of platelet–LDL contact when LPA receptors become desensitized.22,27

The cause of the inhibition of platelet aggregation by oxLDL has remained elusive.7 Mechanisms known to attenuate platelet functions are an increase in cAMP, which inhibits platelet activation via protein kinase A, the activation of platelet endothelial cell adhesion molecule-1 (PECAM-1), which generates inhibitory signals, and inhibition of ligand binding to integrin αIIbβ3, which interferes with outside-in signaling through αIIbβ3, thereby attenuating agonist-induced platelet responses.

The basal level of cAMP was unchanged during incubation with oxLDL, but oxLDL reduced the PGI2-induced accumulation of cAMP. 1-NASPA did not abolish this effect, illustrating that oxLDL suppressed the formation of cAMP independent of LPA. Hence, cAMP may be responsible for the inhibition of platelet function through the p38MAPK pathway by oxLDL.

PECAM-1 is a receptor that on phosphorylation of its immuno-receptor tyrosine-based inhibitory motifs generates inhibitory signals, thereby suppressing platelet activation induced by collagen39 and von Willebrand factor.40 Interestingly, nLDL activates both p38MAPK and PECAM-1, albeit with different time courses, thereby regulating initiation and termination of signal generation.31 Oxidation increases the capacity of LDL to activate PECAM-1, making it a candidate inhibitor of platelet function at extensive oxidation stages (data not shown). However, when PECAM-1 activation was mimicked by treatment with the antibody PECAM-1.3 and cross-linking with F(ab’)2-fragments, there was little interference with TRAP-induced fibrinogen binding, suggesting that PECAM-1 does not mediate platelet inhibition by oxLDL (data not shown).

Treatment of platelets with nLDL or oxLDL and immunoprecipitation with an antibody directed against the apoB100-moiet of LDL showed a clear association of an 88-kDa protein that was identified as CD36, a scavenger receptor that binds nLDL and oxLDL.44 The association of CD36 with nLDL was transient and disappeared after 5 minutes, but with oxLDL a persistent association between apoB100 and CD36 was found. CD36 associates with αIIbβ3 on intact platelets and plasma membrane preparations36,42 and colocalizes with fibrinogen and αIIbβ3 on immuno-electron micrographs.43 We demonstrated a strong association of CD36 with CD61 on resting platelets, which did not change in the presence of nLDL and oxLDL. Inhibition of oxLDL binding to CD36 abolished the inhibition of TRAP-induced aggregation by oxLDL. Hence, binding of oxLDL to CD36 blocks ligand-binding to αIIbβ3, thereby interfering with outside-in signaling and further platelet activation in a similar way as antibodies directed against αIIbβ3.18 A similar inhibition is observed with peptides that block ligand binding to αIIbβ3 and thereby block the stimulation of secretion by nLDL.18 The inhibition of TRAP-induced α-granule secretion by oxLDL supports this conclusion. Whether the inhibition of fibrinogen binding, aggregation, and αIIbβ3-mediated outside-in signaling was caused by steric hindrance by oxLDL or inhibited activation of αIIbβ3 remains to be clarified. Collectively, these data suggest that oxLDL directly interferes with fibrinogen binding to αIIbβ3, thereby abolishing outside-in signaling and the stimulation of aggregation and secretion observed with nLDL and LDL preparations oxidized at <15%.

In conclusion, the present study describes distinct mechanisms by which LDL modulates platelets. Between 0% and 15% oxidation, LDL sensitizes platelets to TRAP-induced fibrinogen binding and aggregation through activation of a slow sensitization process mediated by p38MAPK. At >30% oxidation, the LPA-mediated pathway induces the rapid activation of Ca2+ mobilization, leading to immediate aggregation. At >15% oxidation, platelet sensitization via p38MAPK is abolished and oxLDL inhibits TRAP-induced aggregation and secretion. This mechanism is independent of activation of PECAM-1 but is caused by binding to CD36 and interference with αIIbβ3-mediated ligand-induced outside-in signaling.
Acknowledgments

We thank Dr H.A.M. Voorbij (Department of Clinical Chemistry, University Medical Center Utrecht, Utrecht, the Netherlands) for his assistance. This study was supported in part by a grant from the Netherlands Thrombosis Foundation.

References

Effect of Oxidation on the Platelet-Activating Properties of Low-Density Lipoprotein
Suzanne J.A. Korporaal, Gertie Gorter, Herman J.M. van Rijn and Jan-Willem N. Akkerman

Arterioscler Thromb Vasc Biol. 2005;25:867-872; originally published online February 3, 2005;
doi: 10.1161/01.ATV.0000158381.02640.4b
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/25/4/867

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/04/03/01.ATV.0000158381.02640.4b.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/
THE EFFECT OF OXIDATION ON THE PLATELET-ACTIVATING PROPERTIES OF LOW-DENSITY LIPOPROTEIN

Suzanne J.A. Korporaal§*, Gertie Gorter§*, Herman J.M. van Rijn‡ and Jan-Willem N. Akkerman§,*‡

§Thrombosis and Haemostasis Laboratory, Department of Haematology, ‡Department of Clinical Chemistry, University Medical Center Utrecht, and *Institute for Biomembranes, University of Utrecht, the Netherlands

‡Correspondence to: Prof. Dr. J.W.N. Akkerman
Thrombosis and Haemostasis Laboratory, G.03.647, Department of Haematology, University Medical Center Utrecht
P.O. Box 85500, 3508 GA Utrecht, the Netherlands
Phone: +31-30-2506512,
Fax: +31-30-2511893,
E-mail: j.w.n.akkerman@azu.nl
Methods

Materials
The following agents were used: 1-oleoyl-L-α-lysophosphatidic acid (LPA, dissolved in the presence of BSA) and Brij-35 (Sigma, St.Louis, MO, USA); human α-thrombin (Kordia Life Sciences, Leiden, the Netherlands); N-palmitoyl-L-serine-phosphoric acid (L-NASPA) (Biomol, Plymouth, PA, USA); prostacyclin (PGI₂) (Cayman Chemical, Ann Arbor, MI, USA); FITC-conjugated anti-human fibrinogen (WAK-Chemie, Bad Soden, Germany); RPE-conjugated anti-human P-selectin (DAKO, Glostrup, Denmark); monoclonal antibody 1D2 directed against apoB100 (Yamasa Corporation, Tokyo, Japan); monoclonal antibody SZ21 directed against the β3-subunit of αmβ3 (CD61); anti-CD36 antibody FA6.152 (Immunotech, Marseille, France); and anti-CD11b clone ICRF44 (BD Pharmingen, San Diego, CA, USA) Thrombin receptor (PAR1)– activating peptide (TRAP) was synthesized with a semi-automatic peptide synthesizer (Labortec AG SP650, Switzerland). Antibody 131.2 against CD36 was a gift from Dr. N.N. Tandon (Otsuka American Pharmaceuticals, Inc, Rockville, USA). All other chemicals were as defined in the cited articles.

Isolation of LDL and oxidation
Fresh plasma from 3 donors, each containing less than 100 mg lipoprotein(a)/L, was pooled and LDL was isolated by sequential flotation. The concentration of LDL was expressed as g apoB100/L as determined on a Behring Nephelometer 100 (Dade Behring, Marburg, Germany). The quality of these preparations has been described. Prior to each experiment, nLDL was dialyzed overnight against 10⁴ volumes of 150 mmol/L NaCl. LDL (5 g/L) was oxidized to different extents by dialysis against 5 µmol/L FeSO₄·7H₂O in phosphate-buffered saline (PBS) and 150 mmol/L NaCl containing 1 mmol/L NaN₃, pH 7.2, for 24, 48 or 72 hrs (22°C). After modification, oxLDL was dialyzed against 10³ volumes of 150 mmol/L NaCl containing 1 mmol/L EDTA and 1 mmol/L NaN₃. The degree of lipid modification was inferred from the formation of conjugated dienes at 234 nm and expressed as % oxidation compared to nLDL. Protein modification was assessed by agarose gel electrophoresis (Beckman Coulter, Fullerton, CA, USA).

Platelet isolation and incubations
Washed human platelets were prepared as described and resuspended in HEPES-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 10 mmol/L HEPES, 5 mmol/L D-glucose, pH 7.2). Platelet count was adjusted to 2.0 x 10¹¹ platelets/L. Platelets were incubated with LDL (1.0 g/L, 5 minutes, 37°C) oxidized to different extents, unless stated otherwise. To address the role of LPA-signaling, experiments were performed without and with a pre-incubation with L-NASPA (10 µmol/L, 5 minutes, 37°C), which is an inhibitor of LPA binding to the LPA-receptors. Ca²⁺ mobilization and aggregation were measured in stirred platelet suspensions (900 rev.p.m.). The other measurements were performed in unstirred suspensions to avoid pre-activation of signaling molecules.
Phosphorylation and immunoprecipitation studies
The phosphorylation of p38\textsuperscript{MAPK} was measured as reported\textsuperscript{7,8}. For co-immunoprecipitation studies of apoB100, CD36 and α\textsubscript{IIb}β\textsubscript{3}, platelets were lysed with ice-cold lysis buffer (1% (v/v) Brij-35, 250 mmol/L NaCl, 25 mmol/L Tris-HCl, 5 mmol/L EDTA), supplemented with 1% (v/v) protease inhibitor cocktail and 1 mmol/L NaVO\textsubscript{3}. ApoB100 and CD36 were immunoprecipitated (16 hours, 4°C) with antibody 1D2 (1 µg/mL) and FA6.152 (2.5 µg/mL), respectively, and protein G-sepharose (60 minutes, 20°C). An antibody against CD11b, which is not expressed on platelets, was used as a non-specific control. After washing, samples were taken up in Laemmli sample buffer and proteins were analyzed by SDS-PAGE and Western blotting. After blocking, membranes were incubated with the appropriate antibody (16 hours, 4°C). Antibody binding was detected using peroxidase-linked secondary antibodies, and visualized by the enhanced chemiluminescence reaction. For semi-quantitative determination, the bands were analyzed using ImageQuant software (Molecular Dynamics).

Ca\textsuperscript{2+} mobilization and measurement of cAMP
Ca\textsuperscript{2+} mobilization was measured in Fura-2/AM-labeled platelets upon addition of a low concentration of LDL (0.2 g/L) to avoid quenching of the fluorescent signal\textsuperscript{9}. To investigate whether LDL interfered with the accumulation of cAMP, platelets were incubated with LDL and PGI\textsubscript{2} (10 ng/mL, 5 minutes, 22°C). Incubation with α-thrombin (0.5 U/mL, 5 minutes, 22°C) was used as a control as it is known to suppress the formation of cAMP through the P2Y\textsubscript{12} receptor\textsuperscript{10}. Samples on ice were lysed with perchloric acid, centrifuged (11,000 x g, 10 minutes, 4°C) and cAMP was measured in the supernatant with a [$\textsuperscript{3}H$]-based immuno-assay (Amersham Pharmacia Biotech, Buckinghamshire, England).

Flow cytometry
TRAP-induced fibrinogen binding to integrin α\textsubscript{IIb}β\textsubscript{3} after treatment with LDL was determined as reported\textsuperscript{5}. For P-selectin expression, platelets were incubated with LDL and thereafter stimulated with TRAP (15 µmol/L, 5 minutes, 22°C). After addition of an excess of HEPES-Tyrode buffer, samples were incubated with RPE-conjugated anti-human P-selectin (5 µg/mL), fixed in 1% formaldehyde and analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA, USA).

Platelet aggregation
Platelet suspensions were stimulated with LDL alone or a suboptimal concentration of TRAP (2 µmol/L) without or with pre-incubation with LDL in the presence of fibrinogen (1 µmol/L) at a stirring speed of 900 rev.p.min.. Optical aggregation was monitored in a Chrono-Log lumiaggregometer (Chrono-Log Corporation, Havertown, PA, USA).

Statistical analysis
Results are expressed as means±SEM with number of observations n, and analyzed with the Student's t-test for unpaired observations.
Table I. Assessment of oxidative modification of LDL
The degree of lipid modification was inferred from the amount of conjugated dienes of LDL. The electrophoretic mobility was assessed by agarose gel electrophoresis. REM is defined as $R_{f_{\text{oxLDL}}} / R_{f_{\text{nLDL}}}$. 

<table>
<thead>
<tr>
<th>Type of lipoprotein</th>
<th>Conjugated dienes (AU)</th>
<th>Lipid oxidation range (%)</th>
<th>Protein modification REM</th>
</tr>
</thead>
<tbody>
<tr>
<td>nLDL</td>
<td>0.338±0.005</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>oxLDL (24 hrs)</td>
<td>0.358±0.019</td>
<td>0-27</td>
<td>1.05±0.02</td>
</tr>
<tr>
<td>oxLDL (48 hrs)</td>
<td>0.485±0.029</td>
<td>0-72</td>
<td>1.17±0.04</td>
</tr>
<tr>
<td>oxLDL (72 hrs)</td>
<td>0.704±0.023</td>
<td>20-94</td>
<td>1.40±0.08</td>
</tr>
</tbody>
</table>
Table II. OxLDL-induced suppression of cAMP levels is LPA-independent

Platelets were pretreated at 37°C with vehicle or L-NASPA (10 µM, 5 minutes), incubated with LDL and treated with PGI₂ (10 ng/mL, 5 minutes, 22°C). As a positive control, PGI₂-induced cAMP accumulation was suppressed by α-thrombin (0.5 U/mL, 5 minutes, 22°C). After lysis, the amount of cAMP in the cAMP-rich supernatant was measured with a [³H]-based immuno-assay. (means±SEM, n=5, ‡ P<0.05 versus control)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Vehicle cAMP (% of control without L-NASPA)</th>
<th>L-NASPA cAMP (% of control without L-NASPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>105±16</td>
</tr>
<tr>
<td>nLDL</td>
<td>91±5</td>
<td>82±8</td>
</tr>
<tr>
<td>oxLDL (1-15%)</td>
<td>93±8</td>
<td>90±12</td>
</tr>
<tr>
<td>oxLDL (31-60%)</td>
<td>66±3‡</td>
<td>86±10</td>
</tr>
<tr>
<td>oxLDL (&gt;60%)</td>
<td>60±6‡</td>
<td>68±9‡</td>
</tr>
<tr>
<td>α-thrombin</td>
<td>30±9‡</td>
<td>29±6‡</td>
</tr>
</tbody>
</table>
References


