Platelet Activation by Oxidized Low Density Lipoprotein Is Mediated by Cd36 and Scavenger Receptor-A

Suzanne J.A. Korporaal, Miranda Van Eck, Jelle Adelmeijer, Martin Ijsseldijk, Ruud Out, Ton Lisman, Peter J. Lenting, Theo J.C. Van Berkel, Jan-Willem N. Akkerman

Objective—The interaction of platelets with low density lipoprotein (LDL) contributes to the development of cardiovascular disease. Platelets are activated by native LDL (nLDL) through apoE Receptor 2′ (apoER2′)-mediated signaling to p38MAPK and by oxidized LDL (oxLDL) through lysophosphatidic acid (LPA) signaling to Rho A and Ca2+. Here we report a new mechanism for platelet activation by oxLDL.

Methods and Results—Oxidation of nLDL increases p38MAPK activation through a mechanism that is (1) independent of LPA, and (2) unlike nLDL-signaling not desensitized by prolonged platelet-LDL contact or inhibited by receptor-associated protein or chondroitinase ABC. Antibodies against scavenger receptors CD36 and SR-A alone fail to block p38MAPK activation by oxLDL but combined blockade inhibits p38MAPK by >40% and platelet adhesion to fibrinogen under flow by >60%. Mouse platelets deficient in either CD36 or SR-A show normal p38MAPK activation by oxLDL but combined deficiency of CD36 and SR-A disrupts oxLDL-induced activation of p38MAPK by >70%.

Conclusion—These findings reveal a novel platelet-activating pathway stimulated by oxLDL that is initiated by the combined action of CD36 and SR-A. (Arterioscler Thromb Vasc Biol. 2007;27:2476-2483.)

Key Words: platelets ■ LDL ■ oxidized LDL ■ CD36 ■ scavenger receptor-A

An elevated level of native low density lipoprotein (nLDL) is a risk factor for arterial thrombosis and atherosclerosis as demonstrated in familial hypercholesterolemia, where defective apoB/E receptors fail to remove nLDL from the circulation. Atherogenesis starts when nLDL accumulates in the vessel wall at sites of injury and is oxidized by products from macrophages, smooth muscle cells, and endothelial cells.1 Oxidized LDL (oxLDL) accumulates in monocytes that have infiltrated the subendothelium and differentiated into macrophages. The resulting foam cells are characteristic for the early atherosclerotic lesion.2 OxLDL further contributes to atherosclerosis because it contains lysophosphatidic acid (LPA), which starts platelet shape change and aggregation.3

In healthy individuals, the concentration of oxLDL is low. The normal intima contains little oxLDL (1.86 ± 0.59 ng/µg apolipoprotein B100 [apoB100]) but levels increase 6-fold in atherosclerotic lesions (11.9 ± 1.7 ng/µg apoB100).4 Blood from atherosclerotic patients contains autoantibodies that react with oxidation-specific epitopes in both the lipid and protein moiety of oxLDL,5-6 indicating that oxLDL is also present in the circulation. Hence, in the circulation, platelets can come into contact with oxLDL and become activated, thereby contributing to thrombotic occlusion.

The oxidation of nLDL in vivo can be mimicked in vitro by treatment of nLDL with FeSO4. These oxLDL preparations resemble in vivo oxidized LDL with respect to electrophoretic mobility, density, LPA content, fragmentation of apoB100, chemotactic activity for monocytes, and susceptibility to degradation by macrophages.5-7-9 LPA makes oxLDL a potent platelet activating agent through its LPA1 and LPA3 receptors,10 which are members of the endothelial differentiation gene receptor family. At low concentrations, LPA stimulates Rho, Rho-kinase, and myosin light chain phosphorylation resulting in platelet shape change caused by changes in the actin cytoskeleton.11 At high concentrations, LPA stimulates Ca2+ mobilization and the tyrosine kinases Syk and Src resulting in integrin αmβ3 activation and aggregation.12

Recently, our laboratory identified the signaling receptor through which nLDL changes the behavior of platelets: the apolipoprotein E Receptor 2′ (apoER2′).13 It is a 130-kDa splice variant of apoER2, a member of the LDL receptor family, also known as LRP8. The ligand-binding domain of full-length apoER2 contains 7 complement type A binding repeats14 and the apoER2′ variant lacks binding repeats 4 to 6.15 ApoER2′ is activated by contact with the receptor-specific domain within apoB100 of LDL, called the B-site,
with amino acid sequence RLTRKRLGLKA. Receptor activation starts signaling through p38MAPK and cytosolic phospholipase A2 leading to formation of thromboxane A2, a potent platelet activating agent. The result is an increase in responsiveness to thrombin, collagen, and ADP, leading to enhanced aggregation and secretion on contact with nLDL.

To understand how oxidation enhances the platelet-activating properties of nLDL, signaling through p38MAPK has been compared with LPA-dependent platelet activation at different degrees of LDL oxidation. Both pathways were mutually independent because oxLDL signaling to p38MAPK was unaffected by L-NASPA, an inhibitor of LPA receptors, and LPA was incapable of activating p38MAPK. Below 30% oxidation, activation occurred primarily through the p38MAPK pathway resulting in sensitization, whereas at higher oxidation levels also the LPA pathway was initiated resulting in aggregation.

In an attempt to understand platelet activation by oxLDL in more detail, we compared the activation of the p38MAPK pathway by oxLDL with that induced by nLDL. We found that oxidation led to a strong increase in p38MAPK signaling caused by loss of apoER2 activation and appearance of the combined activity of CD36 and scavenger receptor-A.

Methods
To elucidate the receptors mediating platelet activation by oxLDL, human and murine platelets from wild-type C57Bl/6 mice and mice deficient in either CD36, SR-A, or both were incubated with 1.0 g/L native or partially oxidized LDL and phosphorylation of apoER2 and/or p38MAPK was determined as a measure of activation. In these experiments, nLDL and oxLDL signaling to p38MAPK was separated by prolonged incubation of platelets with nLDL before treatment with nLDL or partially oxidized LDL. To investigate functional consequences of oxLDL signaling through CD36 and SR-A, human platelets were incubated with partially oxidized LDL (31% to 60% oxidation) and adhesion to immobilized fibrinogen was measured at a shear rate of 300 s⁻¹ in the absence and presence of inhibitors.

A full description of the preparation of native and modified LDL, the isolation of human and murine platelets, measurement of phosphorylation of apoER2 and p38MAPK, and adhesion to immobilized fibrinogen is available in the detailed Methods section at http://atvb.ahajournals.org.

Results
P38MAPK Signaling by oxLDL and nLDL
To understand how oxidation changes the regulation of the p38MAPK pathway, LDL was oxidized for 30% to 60% and compared with nLDL. At saturating concentrations (1.0 g/L), partially oxidized LDL induced about 2-fold more p38MAPK phosphorylation than nLDL without affecting the transient kinetics of this activation. (Figure 1A and 1B). This agrees with earlier observations showing that oxidation between 0 and >60% led to a proportional increase in p38MAPK activation. Thus, oxidation increases activation of the p38MAPK pathway. Recently, we described that nLDL activates the p38MAPK pathway through apoER2 thereby increasing the sensitivity of platelets to agonist stimulation. To determine whether the enhanced activation of p38MAPK by partially oxidized LDL was the result of better activation of apoER2, platelets were incubated with both types of LDL and tyrosine phosphorylation of apoER2 was measured. Both LDL preparations induced a similar activation of apoER2 reaching a maximum after 30 seconds and returning to prestimulation values after 10 minutes (Figure 1C). Thus, the extra activation of p38MAPK by partially oxidized LDL could not be attributed to stronger apoER2 activation.

Oxidation of nLDL Introduces ApoER2-Independent Platelet Signaling
To identify the pathway through which oxLDL activates p38MAPK, platelets were first treated with 1.0 g/L nLDL for 1 minute to saturate apoER2 signaling to p38MAPK and thereafter treated with a second dose of either nLDL or partially oxidized LDL. A second addition of nLDL did not further increase p38MAPK phosphorylation, confirming maximal activation by the first dose. In contrast, a second addition of a saturating concentration of partially oxidized LDL increased p38MAPK activity 4-fold to the range found with a single dose of oxLDL (supplemental Figure IA). A second addition of nLDL or oxLDL after a first treatment with oxLDL did not change p38MAPK activation by oxLDL. These data fit to the concept that oxidation of LDL introduces a second property that activates p38MAPK and is independent of the activation induced by nLDL.

Receptor-associated protein (RAP) is a specific blocker of ligand binding to LDL receptor family members. RAP induced a dose-dependent inhibition of p38MAPK phosphorylation induced by nLDL, which is in agreement with involvement of apoER2, an LDL receptor family member (supplemental Figure IB). In contrast, p38MAPK phosphorylation induced by oxLDL was hardly affected by RAP, the minor decrease probably reflecting residual nLDL in this partially oxidized preparation. This conclusion was supported by the observation that apoER2 tyrosine phosphorylation induced by nLDL and oxLDL was equally inhibited by RAP (supplemental Figure IC). Chondroitinase ABC removes chondroitin and dermatan sulfate side chains from proteoglycans, which are important for nLDL binding to its receptor. Chondroitinase ABC completely abolished p38MAPK phosphorylation by nLDL, but hardly interfered with oxLDL-induced phosphorylation, the minor decrease again probably resulting from residual nLDL (supplemental Figure ID). Treatment with L-NASPA to block LPA binding to its receptors did not affect p38MAPK activation by oxLDL (data not shown). Thus, the receptor that mediates the extra p38MAPK activation by oxLDL is therefore not a member of the LDL receptor or LPA receptor families.

Desensitization of nLDL-Induced Signaling
A property of many receptors is the ability to become desensitized after prolonged ligand contact. To assess whether receptors mediating nLDL and oxLDL signaling to p38MAPK could be desensitized, platelets were incubated with nLDL (1.0 g/L) for 30 minutes and thereafter stimulated with a second dose of nLDL or with partially oxidized LDL. Indeed, a first treatment with nLDL almost completely abolished p38MAPK activation by a second dose of nLDL, indicating that this treatment arrested nLDL signaling (Figure 2A). In contrast, p38MAPK activation by partially oxidized LDL remained mostly intact after a first treatment with
nLDL, the minor decrease reflecting residual nLDL (Figure 2A). Figure 2B illustrates the desensitization of nLDL signaling by preincubation with nLDL and residual nLDL present in 2 partially oxidized LDL preparations. OxLDL signaling was not desensitized. Thus, prolonged contact with nLDL desensitized nLDL signaling but not oxLDL signaling to p38MAPK.

The possibility to separate nLDL and oxLDL signaling to p38MAPK by prolonged incubation with nLDL was used to quantify the contribution of oxidation to the signaling properties of LDL. nLDL preparations were oxidized to different extents, and p38MAPK was activated before and after the desensitization phase induced by nLDL (Figure 2C). nLDL contained <15% oxidized LDL and was responsible for >90% of total p38MAPK phosphorylation through the nLDL pathway. As more LDL was oxidized, the contribution of nLDL signaling to p38MAPK decreased and was replaced by oxLDL signaling. LDL preparations of >60% oxidation activated the platelets almost exclusively through the oxLDL receptor.

The loss of nLDL signaling after prolonged nLDL-platelet contact appears a logical consequence of desensitization at the level of the receptor. To confirm this concept, apoER2′ phosphorylation by nLDL was analyzed before and after 30 minutes incubation with nLDL (Figure 2D). Unexpectedly, a similar receptor activation was observed, illustrating that ligand activation of apoER2′ is fully reversible. Apparently, the cause of desensitization of p38MAPK signaling is not at the level of the receptor and must be sought downstream of apoER2′.

**CD36 and Scavenger Receptor-A Mediate oxLDL-Induced Activation of p38MAPK in Human and Murine Platelets**

Scavenger receptors (SRs) are membrane glycoproteins that bind oxLDL on cells like macrophages, smooth muscle cells,
and platelets. To investigate whether SRs play a role in p38MAPK activation, platelets were incubated with antibody FA6.152 against CD36, which is a class-B SR and also known as glycoprotein IV, and with fucoidan, which inhibits oxLDL binding to class A SRs. Subsequent stimulation with nLDL as a control and with partially oxidized LDL and p38MAPK phosphorylation was determined. Combined incubation with both inhibitors sharply decreased p38MAPK phosphorylation by oxLDL by 40% to the level found with nLDL (Figure 3A). Thus, the combined blockade of CD36 and SR-A leads to complete inhibition of oxLDL signaling to p38MAPK leaving nLDL signaling to p38MAPK undisturbed. The specificity of this inhibition was confirmed by the inability of RAP to interfere with oxLDL-induced signaling and of fucoidan with nLDL-induced signaling (Figure 3B and 3C).

To confirm these observations by targeted interference with receptor expression, incubations with oxLDL were repeated with murine platelets deficient in either CD36 (CD36<sup>−/−</sup>), SR-A (SR-A<sup>−/−</sup>), or both (CD36<sup>−/−</sup>SR-A double knockout [dKO]). Murine wild-type (w.t.) platelets showed...

Figure 2. Desensitization of nLDL-induced signaling. A, Platelets were treated with vehicle (left panel) or nLDL (1.0 g/L, 30 minutes, right panel), stimulated with 1.0 g/L nLDL or partially oxidized LDL and p38MAPK phosphorylation was determined. B, Platelets were treated with vehicle, 1.0 g/L nLDL, or 2 preparations of oxidized LDL (31% to 60% and >60% oxidation) for 30 minutes, stimulated with vehicle, 1.0 g/L nLDL, or partially oxidized LDL and p38MAPK phosphorylation was determined. C, Platelets were treated with vehicle or 1.0 g/L nLDL for 30 minutes, then stimulated with 1.0 g/L LDL oxidized to the indicated extents (1 minute) and phosphorylation of p38MAPK was determined. D, Platelets were incubated with vehicle (left panel) or nLDL (1.0 g/L, 30 minutes, right panel), again stimulated with 1.0 g/L nLDL (1 minute), and tyrosine phosphorylation of apoER2<sup>+</sup> was measured. A and D, Data were expressed as percentage of the density of incubations with 1 minute nLDL before desensitization. C, Data were expressed as percentage of the density of incubations with oxLDL before desensitization (white bars=100%). Means±SEM, n=3.
the same p38MAPK activation by nLDL and oxLDL as human platelets and again only the combined addition of FA6.152 and fucoidan brought oxLDL activation by >70% back to levels found with nLDL (Figure 4). A similar blockade of oxLDL signaling was induced in CD36+/− platelets incubated with fucoidan and in SR-A+/− platelets incubated with FA6.152. The combined deletion of CD36 and SR-A expression sharply reduced oxLDL signaling even in the absence of additions. These results support the findings in human platelets and show that interference with both CD36 and SR-A is required for full inhibition of oxLDL signaling to p38MAPK.

CD36 and SR-A Mediate oxLDL-Induced Platelet Adhesion to Immobilized Fibrinogen

To investigate functional consequences of oxLDL signaling through CD36 and SR-A, human platelets were incubated with oxLDL (31% to 60% oxidation) and adhesion to immobilized fibrinogen was measured at a shear rate of 300 s⁻¹. OxLDL increased adhesion with about 35%, probably as a result of activation through the p38MAPK pathway because the p38MAPK inhibitor SB203580 strongly reduced adhesion (Figure 5B). At a higher shear rate (1200 s⁻¹), resembling the shear rates encountered in the arterial circulation, oxLDL induced a similar increase (24%) in platelet adhesion to fibrinogen (data not shown). Although the anti-CD36 antibody FA6.152 alone had no effect on oxLDL-induced p38MAPK activation, it inhibited platelet adhesion to fibrinogen by 37% to the level found in the absence of oxLDL. The presence of fucoidan alone had no effect, but in combination with FA6.152 there was a reduction of 63% (Figure 5B). Thus, adhesion to fibrinogen under flow is enhanced by oxLDL signaling in which both CD36 and SR-A participate and which appears to be a direct result of increased p38MAPK activation through these receptors.

To investigate the role of feedback activation by secreted ADP, the experiment was repeated in the presence of the P2Y₁₂ receptor antagonist AR-C69931MX. This treatment reduced adhesion from 100% to 50%, confirming earlier results.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** CD36 and SR-A mediate oxLDL-induced activation of p38MAPK in human platelets. A, Platelets were treated with vehicle, FA6.152 (anti-CD36; 4 μg/mL, 30 minutes), and fucoidan (anti-SR-A; 50 μg/mL, 30 minutes), stimulated with nLDL or partially oxidized LDL (1.0 g/L, 1 minute), and p38MAPK phosphorylation was determined. Data were expressed as percentage of the density of incubations with nLDL. Means±SEM, n=3. B and C, Platelets were treated with vehicle, (B) GST-RAP (50 μg/mL, 10 minutes), or (C) fucoidan, stimulated with the indicated concentrations (B) partially oxidized LDL or (C) nLDL (1 minute), and p38MAPK phosphorylation was determined.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** CD36 and SR-A mediate oxLDL-induced activation of p38MAPK in murine platelets. WT murine platelets, CD36−/−, SR-A−/−, and CD36×SR-A dKO platelets were treated with vehicle, FA6.152, or fucoidan before stimulation with 1.0 g/L nLDL or partially oxidized LDL, and p38MAPK phosphorylation was determined. Data were expressed as percentage of the density of incubations with nLDL. Means±SEM, n=3. *P<0.05, **P<0.01.
In the presence of oxLDL, platelet adhesion was reduced from 135% to 75%. Thus, also in platelets with blocked P2Y12 signaling, the stimulation of adhesion by oxLDL was preserved (Figure 5C).

**Discussion**

The present study shows that oxLDL activates p38MAPK through the combined stimulation of CD36 and SR-A thereby increasing platelet adhesion to fibrinogen under flow. This property functions concurrently with platelet activation by the LPA content of oxLDL because both pathways are mutually independent.\(^1\) Platelet activation by oxLDL is also independent of apoER2', which is the receptor through which nLDL increases the sensitivity to platelet activating agents.\(^1\) Because under physiological conditions LDL oxidation is probably far from complete, oxLDL particles will change platelet behavior through at least 3 independent signaling routes which are initiated by apoER2' (nLDL), LPA/LPA receptors (LPA in oxLDL), and CD36/SR-A (oxLDL).

Earlier studies showed that the ability of LDL to act as a platelet agonist increases on oxidation,\(^2\) and involves LPA-dependent\(^3\),\(^11\),\(^12\),\(^17\) and LPA-independent\(^17\) signaling. LPA-independent signaling was mediated through p38MAPK activation, which increased with increasing oxidation of LDL. Because p38MAPK is also an intermediate in nLDL-induced platelet activation, a simple explanation emerged in which nLDL activates a receptor and oxidation enhances this process. The discovery of apoER2' as the exclusive receptor for platelet activation by nLDL made it possible to study whether this receptor recognizes oxLDL. The present data showed that nLDL and partially oxidized LDL shared a similar activation of apoER2'. In contrast, both preparations
induced different degrees of p38MAPK activation with partially oxidized LDL inducing more activation at lower concentrations than nLDL. These data are best explained by assuming that LDL oxidized for <60% contains an nLDL fraction responsible for apoER2 activation with concurrent p38MAPK phosphorylation and an oxidized fraction that triggers more p38MAPK activation through a different receptor. This second pathway is unaffected by RAP which blocks ligand binding to LDL receptor family members, chondroitinase ABC which interferes with the binding of nLDL to apoER2 mediated by proteoglycans, and L-NASPA, which blocks ligand binding to LPA receptors. It is therefore independent of LDL receptor family members or LPA receptors. In addition, this pathway remains intact after prolonged incubation with nLDL offering a means to clarify oxLDL signaling without interference of the apoER2 pathway.

Many receptors become inaccessible to ligand binding after prolonged ligand exposure. Examples are glycoprotein Ib and PAR-1 which are internalized after platelet activation and sorted to lysosomes rather than recycled to the plasma membrane.26,27 After 30 minutes incubation with nLDL, activation of apoER2 was fully reversible showing no signs of irreversible ligand binding, receptor inactivation, or internalization. Surprisingly, this treatment almost completely abolished a second activation of p38MAPK by nLDL revealing a block between apoER2 activation and p38MAPK activation generated on prolonged nLDL contact. The cause of this blockade is unclear but may involve mechanisms known to downregulate p38MAPK such as the inhibitory receptor PECAM-1.28

OxLDL binds to nucleated cells through SRs expressed on their cell surface. SRs are glycoproteins that recognize a broad variety of ligands, such as oxidized- and glycosylated lipoproteins, anionic phospholipids, apoptotic cells, and fatty acids.19 SRs identified on platelets so far include CD36,29 SR-BI,30 CD68,31 and LOX-1.32 Earlier reports described that CuSO4-oxidized LDL binds to platelets and that binding to CD36 accounted for about 75% of total binding.21,33 Binding was disturbed by maleylated human serum albumin, which inhibits ligand binding to SRs of classes A and B. Other receptors might also contribute to oxLDL binding to platelets such as LOX-1, the function of which appears to be restricted to activated platelets.32 The present study shows that p38MAPK phosphorylation induced by oxLDL was unaffected by separate addition of inhibitors of CD36 and SR-A. A combination of inhibitors to both scavenger receptors effectively reduced oxLDL-induced activation of p38MAPK to the level found with nLDL. Consistent with these findings were observations in mice deficient in either CD36 or SR-A that responded normally to oxLDL, but the absence of both receptors either by genetic targeting or addition of inhibitors blocked signaling by oxLDL. These findings indicate that the LPA-independent platelet activation by oxLDL is mediated through the combined involvement of CD36 and SR-A.

In platelet suspensions, oxLDL binding to CD36 interferes with fibrinogen binding to integrin αIIbβ3, thereby inhibiting TRAP-induced aggregation.17 The present study shows that oxLDL increases platelet adhesion to immobilized fibrinogen under flow and that this effect is independent of ADP secretion. Binding of soluble fibrinogen is known to depend on prior activation of αIIbβ3, but surface-coated fibrinogen binds to the closed conformation present on resting platelets.34 At the low shear rate used in the present study (300 s⁻¹), binding to immobilized fibrinogen exclusively involves αIIbβ3.35,36 Hence, binding of oxLDL to CD36 might interfere with the activation of αIIbβ3, thereby interfering with binding of soluble but not with surface-coated fibrinogen. There is a 63% reduction of the stimulation by oxLDL in the presence of inhibitors of CD36 and SR-A. A similar inhibition is seen at the level of p38MAPK activation. This suggests that the better adhesion induced by oxLDL is the result of combined activation of CD36 and SR-A and enhanced signaling to p38MAPK, which is an upstream regulator of thromboxane A₂ formation.37

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Disclosures
None

References


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PLATELET ACTIVATION BY OXIDIZED LOW DENSITY LIPOPROTEIN IS MEDIATED BY CD36 AND SCAVENGER RECEPTOR A

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Methods

Mice

C57Bl/6 mice were obtained from Charles River (Maastricht, the Netherlands). CD36\(^{-/-}\) mice were kindly provided by Dr. M. Febbraio (Department of Medicine, Weill Medical College of Cornell University, New York, USA),\(^1\) and Mex-4 SR-A\(^{-/-}\) mice by Dr. T. Kodama (Department of Molecular Biology and Medicine, University of Tokyo, Tokyo, Japan).\(^2\) CD36 x SR-A double knockout mice were generated by cross-breeding of the CD36\(^{-/-}\) animals with the Mex-4 SR-A\(^{-/-}\) mice. All mice were backcrossed at least 4 generations to the C57BL/6 background. Mice had unlimited access to water and regular chow diet, containing 4.3 \(\%\) (w/w) fat with no added cholesterol (RM3, Special Diet Services, Witham, UK). All experimental protocols were approved by the local ethics committees for animal experiments.

Materials

We obtained bovine serum albumin (acid free; BSA) from ICN Biomedicals (Aurora, Ohio, USA), chondroitinase ABC from Seikagaku America (Falmouth, MA, USA), Renaissance chemiluminescence Western blot reagent from PerkinElmer Life Sciences (Boston, MA, USA), fucoidan, protease inhibitor cocktail and sodium orthovanadate (NaVO\(_3\)) from Sigma (St. Louis, MO, USA), fibrinogen from Kordia (Leiden, the Netherlands), prostacyclin (PGI\(_2\)) from Cayman Chemical (Ann Arbor, MI, USA), and protein G-Sepharose from Amersham (Uppsala, Sweden). All other chemicals used were of analytical grade.

Antibodies and Proteins

We obtained FA6.152, a monoclonal antibody directed against the oxLDL binding domain of human CD36 (amino acids 155-183)\(^3\) from Immunotech (Marseille, France), polyclonal antibodies against dual phosphorylated p38\(^{MAPK}\) (phosphoplus p38\(^{MAPK}\)) and p38\(^{MAPK}\), and horseradish peroxidase-labeled anti-rabbit IgG from New England Biolabs (Beverly, USA), the goat polyclonal antibody directed against the ectodomain of ApoER2 (D-18) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the anti-
Korporaal et al. Supplement Platelet receptors for oxLDL

phosphotyrosine monoclonal antibody 4G10 from Upstate Biotechnology (Bucks, UK), monoclonal anti-α-actinin from Sigma (St.Louis, MO, USA), and peroxidase-linked goat anti-mouse antibody from DAKO (Glostrup, Denmark). Receptor-associated protein (RAP) fused to glutathione S-transferase (GST) was prepared as described previously. The ADP receptor P2Y antagonist, the ATP analogue N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK).

Lipoprotein Isolation and Modification

Fresh, non-frozen plasma from 3 donors, each containing less than 150 mg lipoprotein(a)/L, was pooled and nLDL (1.019 - 1.063 kg/L) was isolated by sequential flotation. The concentration of nLDL was determined on the Behring Nephelometer 100 (Dade Behring, Marburg, Germany) and expressed as g apoB100/L. The quality of these preparations has been described. Prior to each experiment, nLDL was dialyzed overnight against 10^4 volumes of 150 mmol/L NaCl. nLDL was oxidized to different extents by dialysis of 5 g/L EDTA-free nLDL in 5 µmol/L FeSO_4\cdot H_2O in phosphate-buffered saline (PBS) and 150 mmol/L NaCl containing 1 mmol/L NaN_3 (pH 7.2, 24-72 hrs, 20°C). After modification, the preparations were dialyzed against 10^3 volumes of buffer containing 150 mmol/L NaCl, 1 mmol/L NaN_3 and 1 mmol/L EDTA. Prior to each experiment, oxLDL was dialyzed overnight against 10^4 volumes of 150 mmol/L NaCl. The degree of lipid modification was inferred from the formation of conjugated dienes at 234 nm and expressed as % oxidation.

Isolation of Platelets

Human platelets were isolated as previously described. In short, blood was collected from healthy volunteers (with informed consent) into 0.1 volume of 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication 10 days prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (156 x g, 15 min, 20°C). Then, 0.1 volume of ACD (2.5% trisodium citrate,
1.5% citric acid and 2% D-glucose) was added to lower the pH to 6.5. Platelets were further purified by centrifugation (330 x g, 15 min, 20°C). The platelet pellet was resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na$_2$HPO$_4$, 1 mmol/L MgSO$_4$, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5). Prostacyclin (PGI$_2$) was added to a final concentration of 10 ng/mL and the washing procedure was repeated once. The platelet pellet was resuspended in Hepes-Tyrode buffer, pH 7.2. Platelet count was adjusted to 2.0 x 10$^{11}$ platelets/L, unless stated otherwise.

For perfusion studies, reconstituted blood was prepared as described.$^{10}$ In short, PRP was acidified by addition of 0.1 volume of ACD, and the platelets were spun down. The platelet pellet was resuspended in Hepes-Tyrode buffer, pH 6.5 and PGI$_2$ (10 ng/mL) was added to prevent platelet activation during the subsequent washing step. Platelets were spun down and resuspended in a small volume of Hepes-Tyrode buffer and diluted in human albumin solution (HAS; 4% human albumin, 4 mmol/L KCl, 124 mmol/L NaCl, 20 mmol/L NaHCO$_3$, 2 mmol/L Na$_2$SO$_4$, 1.5 mmol/L MgCl$_2$, 5 mmol/L D-glucose, pH 7.35). Red blood cells were washed twice with 150 mmol/L NaCl containing 5 mmol/L D-glucose (2000 × g, 5 min), and finally cells were pelleted (2000 × g, 15 min). Platelets were mixed with red cells to obtain reconstituted blood with a hematocrit of 40% and a platelet count of 1 x 10$^{11}$ platelets/L.

For the isolation of murine platelets, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/mL), ketamine (40 mg/L) and atropine (0.05 mg/mL), and blood was subsequently collected into 0.1 volume 130 mmol/L trisodium citrate and 0.1 volume of ACD by heart puncture. PRP was obtained by centrifugation (87 x g, 15 min, 20°C). The remainder of the blood was diluted with Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na$_2$HPO$_4$, 1 mmol/L MgSO$_4$, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5) and 0.1 volume of ACD and centrifuged again (87 x g, 15 min, 20°C). PRP samples were pooled, and platelets were isolated by centrifugation (350 x g, 15 min, 20°C) in the presence of 0.1 volume of ACD buffer and resuspended in Hepes-Tyrode buffer (pH 6.5). PGI$_2$ was added to a final concentration of 10 ng/mL, and the washing procedure was repeated once. The
platelet pellet was resuspended in Hepes-Tyrode buffer (pH 7.2). Platelet count was adjusted to $1 \times 10^{11}$ platelets/L.

Platelets were incubated with 1.0 g/L LDL, native or oxidized to different extents for 1 min at 37°C unless stated otherwise.

**P38 MAPK Assay**

The phosphorylation of p38 MAPK was measured as described elsewhere. In short, platelets were incubated at 37°C with nLDL or oxLDL as indicated. Samples of 100 µL were fixed with 1% formaldehyde (15 min, 4°C), centrifuged (5600 x g, 30 sec, 20°C) and resuspended in 60 µL Laemmli sample buffer. Proteins were analyzed by SDS-PAGE and Western blotting. One part of a sample was applied to SDS-PAGE to identify dual phosphorylated p38 MAPK phosphorylation using a phospho-specific anti-p38 MAPK polyclonal antibody (Thr-180/Tyr-182; 1:2000, 16 hrs, 4°C). Another part was applied to SDS-PAGE and total p38 MAPK was detected with an antibody against p38 MAPK as a control for equal lane loading (1:2000, 16 hrs, 4°C). Both antibodies are raised against residues 171-186 of human p38 MAPK. After washing, the membranes were incubated with horseradish peroxidase labeled anti-rabbit IgG (1:5000, 1 hr, 4°C). Protein bands were visualized using the enhanced chemiluminescence reaction. For semi-quantitative determination, the density of the bands was analyzed using ImageQuant software (Molecular Dynamics). The data express the semi-quantification of dual-phosphorylated p38 MAPK from the blots after correction for the phosphorylation at time point 0 min.

**ApoER2’ Tyrosine Phosphorylation**

Tyrosine phosphorylation of ApoER2’ was determined as described. In short, human platelets were incubated at 37°C with nLDL or partially oxidized LDL as indicated and mixed (1:10 v/v) with ice-cold lysis buffer consisting of 10% (v/v) Nonidet P-40, 5% (w/v) octylglucoside, 50 mmol/L EDTA, 1% (w/v) SDS supplemented with 5 mmol/L NaVO₃ and 10% (v/v) protease inhibitor mixture. ApoER2’ was
precipitated using a goat polyclonal antibody directed against apoER2 (1 µg/mL) and protein G-Sepharose for 3 hrs at 4°C. Precipitates were washed 3 times with lysis buffer (containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaVO$_3$ and 1 µg/mL leupeptin) and taken up in non-reducing Laemmli sample buffer. Samples were analyzed by SDS-PAGE and Western blotting. Tyrosine phosphorylation of apoER2$'$ was visualized by incubation with 4G10, an antibody directed against phosphorylated tyrosine residues (0.5 µg/mL, 16 hrs, 4°C), followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 hr, 4°C), and the enhanced chemiluminescence reaction. As a control for equal lane loading, the blots were stripped and incubated with a monoclonal anti-α-actinin antibody (1:5000 (v/v), 16 hrs, 4°C), since specific apoER2-detecting antibodies are unavailable. This was followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 hr, 4°C). The data express the semi-quantification of tyrosine phosphorylation of apoER2$'$ relative to the density of the bands representing α-actinin after correction for the density at time point 0 min.

Perfusion studies

Fibrinogen was immobilized onto Thermanox® coverslips by a coating procedure (100 µg/mL, 1 hr, 20°C). Coverslips were blocked (1 hr, 20°C) with 1% human albumin in phosphate-buffered saline (PBS). Perfusions were carried out with reconstituted blood in a three single-passage parallel-plate perfusion chambers as described. The blood was preincubated with vehicle or the indicated inhibitors, stimulated with partially oxidized LDL (0.2 g/L) in the presence of CaCl$_2$ (3 mmol/L) for 5 min at 37°C, and perfused over the fibrinogen-coated coverslips for 5 min at a shear rate of 300 s$^{-1}$. After perfusion, slides were washed with Hepes buffer (10 mmol/L Hepes, 150 mmol/L NaCl, pH 7.35) and fixed in 0.5% glutaraldehyde in PBS. Slides were dehydrated in methanol and stained with May-Grünwald and Giemsa. Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software [Dutch Vision Systems (DVS), Breda, the Netherlands]. Platelet attachment to surface-bound fibrinogen was expressed as surface coverage (expressed as percentage).
**Statistical Analysis**

Results are expressed as means±S.E.M. and analyzed with the Student's t test for unpaired observations. Differences were considered significant at \( p < 0.05 \).

**References**


**Figure Legends**

**Fig. I. Oxidation of nLDL introduces apoER2’-independent platelet signaling.**

(A) Platelets were incubated with 1.0 g/L nLDL or partially oxidized LDL for 1 min, stimulated with a second dose of nLDL or partially oxidized LDL (1 min) and p38MAPK phosphorylation was determined. (B-D) Platelets were incubated with (B,C) GST-RAP at the indicated concentrations (10 min) or (D) chondroitinase ABC (ABC; 1.0 U/mL, 10 min), stimulated with 1.0 g/L nLDL or partially oxidized LDL (B,D: 1 min; C: 0.5 min) and (B,D) p38MAPK phosphorylation or (C) tyrosine phosphorylation of apoER2’ was determined. Data were expressed as percentage of the density of incubations with nLDL (A,B,D) or oxLDL (B). Means±S.E.M., n=3.
Figure I

A

B

C

D

Platelet receptors for oxLDL