Modification of Protein Moiety of Human Low Density Lipoprotein by Hypochlorite Generates Strong Platelet Agonist

Ivo Volf, Edith Bielek, Thomas Moeslinger, Franz Koller, Elisabeth Koller

Abstract—Conflicting reports exist about the effects of mildly or extensively oxidized low density lipoproteins (LDLs) on the reactivity of human platelets. This platelet response is mainly caused by modification of the protein and lipid moiety, giving rise to very differently modified species with hardly predictable properties. The aim of this study was to prepare oxidized LDL with modifications essentially restricted to the protein moiety and to determine the eventual platelet responses. We treated LDL at 0°C for 10 minutes with a 60- to 1000-fold molar excess of sodium hypochlorite in borate buffer in the presence of the radical scavenger butylated hydroxytoluene. Under these conditions, neither fragmentation of apolipoprotein B-100 nor formation of LDL aggregates was observed, and lipid oxidation products did not exceed the amount present in untreated LDLs. The degree of modification and the respective effects on platelet function were highly reproducible. Hypochlorite-modified LDLs act as strong platelet agonists, inducing morphological changes, dense granule release, and irreversible platelet aggregation. The evoked platelet effects are completely suppressed by inhibitors of the phosphoinositide cycle but not by EDTA or acetylsalicylic acid. Most likely, these effects are transmitted via high-affinity binding to a single class of sites, which does not recognize native or acetylated LDL. Obviously, modified lysines, and the secondary lipid modifications derived from them, are not essential for this interaction. We conclude that bioactive oxidized lipids are not directly involved in the stimulation of platelets by hypochlorite-modified LDLs. (Arterioscler Thromb Vasc Biol. 2000;20:2011-2018.)

Key Words: atherosclerosis ■ oxidized LDL ■ apoB-100 ■ human platelets ■ platelet aggregation

Platelet–vessel wall interaction (adhesion) and platelet–platelet interaction (aggregation) play a central role in vascular occlusion but are also involved in the earlier stages of development of atherosclerotic plaques. Therefore, conditions leading to altered platelet function are accompanied with an enhanced risk of atherosclerosis and thrombosis. Among others, severe disorders of plasma lipids lead to enhanced platelet reactivity. In particular, it has been known for >25 years that platelets from hyperlipidemic patients are hyperreactive. Furthermore, LDLs from patients with homozygous familial hypercholesterolemia show enhanced susceptibility to oxidative modification, thus generating a form that is substantially more atherogenic than unmodified LDLs. Native LDLs (nLDLs) reportedly stimulate human platelets, but minimally modified LDLs rather than nLDLs may be responsible for these effects. LDLs oxidized in vitro by various agents (oxLDLs) showed even more pronounced platelet activation. Because activated platelets may themselves contribute to oxidative modification of LDLs, the platelet-stimulatory effect of plasma lipoproteins is potentially a key event in atherogenesis.

Free radicals as well as nonradical oxidants are involved in the oxidative modification of LDL in vivo, so oxLDL certainly does not represent a well-defined species. Accordingly, it proves difficult to assess the atherogenicity contributed by individual modifications. Eventually, all oxidants will cause modification of the lipid moiety (lipid peroxidation, oxidation and loss of cholesterol esters, hydrolysis of phospholipids, and consumption of LDL-bound antioxidants) and the apoB moiety (oxidation and derivatization of amino acids, fragmentation, and cross-linking). The extent and order of these reactions strongly depend on the nature of the oxidizing agent and on the presence of antioxidants within the lipoprotein particle and in its environment. The stimulation of platelets by oxLDLs may be due to the uptake of bioactive oxidized lipids and/or to changes in the lipid domain of platelet membranes. Additionally, the interaction of oxidized areas of apoB with the platelet surface may give rise to signal transduction. Which of these 2 principal possibilities contributes to the effects of oxLDL on platelet function and how much is contributed remain controversial.
We describe the effects of LDLs modified with hypochlorite on washed platelets. Hypochlorite-modified proteins were found in atherosclerotic plaques, so the oxidation of LDLs by this agent is likely to occur in vivo. Unlike free radical oxidants, hypochlorite is known to preferentially modify the modified apoB-100 moeity of LDLs, and we applied reaction conditions under which only minimal amounts of lipid peroxides were detectable. The reactivity of hypochlorite-modified lipoproteins is highly reproducible, whereas the effects of LDLs oxidized by free radicals are much less predictable.

The aim of the present study was to assess the specific contribution of the modified apoprotein moeity of the effects of oxLDLs on platelet function.

Methods

NaOCl was from Aldrich; its concentration was determined spectrophotometrically before use ($\epsilon_{400}=350 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

Isolation and Modification of LDLs

Lipoprotein concentrations are always given as microgram protein per milliliter. All manipulations were performed at 4°C. LDLs (density 1.019 to 1.063 g/mL) were isolated from fresh normal human acid-citrate-dextrose plasma by sequential flotation, filtered (0.45 μm), and immediately used for further modification. Only LDL preparations with thioribarbituric acid–reactive substances (TBArS) <0.7 nmol/mg protein were used. KBr was removed by Sephadex G-25 chromatography, and butylated hydroxytoluene (BHT) was added to a final concentration of 15 μmol/L.

Hypochlorite modification of LDL was based on the method of Arnhold et al. with the following essential modifications: nLDLs were transferred to 0.1 mol/L sodium borate buffer (pH 7.3) and 0.1 mmol/L EDTA, and 15 μmol/L BHT was added. At 0°C, 10-μL portions of 0.15 mol/L NaOCl were added to give the final concentrations and ratios of NaOCl/apoB-100 as indicated. After 10 minutes, unreacted NaOCl was removed by Sephadex G-25 (0.1 mol/L borate buffer, 0.1 mmol/L EDTA, and 50 mmol/L NaCl, pH 7.3). Hypochlorite-modified LDLs (hypoxLDLs) were always kept on ice and used within 1 week. They were purified by gel filtration on Sepharose-CL 4B immediately before use to remove any aggregates of modified LDL.

Reductive methylation was carried out according to the method of Shepherd and Packard. Under these conditions, 60% to 65% of the free amino groups were methylated. Methylated LDLs (metLDLs) were used as such or further modified by hypochlorite (hypmetLDLs), as described above. Acetylation was performed by the method of Basu et al. Sixty-five percent to 75% of the free amino groups were modified under these conditions. Acetylated LDLs (acLDLs) were used as such or further modified by hypochlorite (hypacLDLs), as described above.

The electrophoretic mobility of native and modified LDL under nondenaturing conditions was assessed by agarose electrophoresis. SDS-PAGE (3% acrylamide) of nLDL and modified LDL was performed to detect any degradation or cross-linking of the apoB moeity.

Amino groups were estimated with 2,4,6-trinitrobenzenesulfonic acid. TBArS were determined according to Mihara and Uchiyama. Formation of malondialdehyde and 4-hydroxynonenal was followed by fluorescence measurement at excitation/emission of 400/470 nm and 360/410 to 430 nm, respectively.

Iodination of Proteins

Radiiodination was performed by the Iodo-Beads method as described in detail in the online publication (which can be accessed at http://atvb.ahajournals.org).

Platelet Procedures

Platelets were isolated from freshly drawn blood, as described previously, out of a pool of 34 healthy donors. In binding studies, adenosine (2 mmol/L) and theophylline (1 mmol/L) were added to prevent platelet aggregation. Nonspecific binding was determined as the amount of platelet-associated radioactivity in the presence of 500 μg/mL of the unlabeled LDL species. Competition for binding was determined by the addition of a 7- to 30-fold excess of the unlabeled competitor species to incubations with either ~5 or ~20 μg/mL of the radiolabeled ligand.

Further details are published online at http://atvb.ahajournals.org.

Electron Microscopy

Aggregation was performed in a final volume of 1 mL with different concentrations of agonists or hypoxLDL, chosen to yield 50% or 100% light transmission, respectively. When the desired extent of aggregation was reached, the reaction was stopped by the addition of an equal volume of 0.2% aqueous glutardialdehyde (GDA). Samples were further treated as described elsewhere in detail.

Results

Modification of LDLs with hypochlorite was performed in borate buffer at different final concentrations of NaOCl, including the range that may occur in vivo in the course of a phagocytic burst. The concentration of nLDL was varied at any given concentration of oxidant, leading to final molar ratios of NaOCl/apoB ranging between ~60 and ~1000 (based on $M_w$ of apoB-100). Data from representative experiments are summarized in Table 1. The relative electrophoretic mobility (REM) gives an estimate of the
obtained degree of modification. Electronegativity increased with increasing final concentration of oxidant and with increasing NaOCl/LDL ratios as well. Unlike the unpredictable pattern of products obtained when the oxidation of LDL is catalyzed by transition metal ions, the modification of LDL by hypochlorite turned out to be highly reproducible. The observed REM of identically treated lipoproteins from individual donors generally ranged within a margin of 15%, most likely reflecting differences in the antioxidant load of the individual donors.

The morphology of the lipoproteins was characterized by native electrophoresis and SDS-PAGE. Figure 2A demonstrates the correlation between the aggregation power and the relative mobility in native electrophoresis, which is proportional to the extent of LDL modification by hypochlorite. Therefore, different samples of hypoxLDL are indicated by their respective relative Rf values (REM) in this paper.

The relationship between the conditions of the modification reaction and the resulting properties of hypoxLDL is documented in more detail in Figure 2B. For any given constant concentration of lipoprotein, the platelet aggregation potential increased with increasing concentrations of NaOCl. However, when prepared at identical concentrations of NaOCl, the platelet aggregation potential of the resultant modified LDL decreased with increasing concentrations of nLDL. Thus, in vitro the reaction of rather low concentrations of oxidant with low amounts of LDL gives rise to a modified LDL species that is able to induce platelet aggregation.

The hypoxLDL-induced changes of light transmittance resemble those induced by known platelet agonists, reflecting platelet activation followed by aggregation. Nevertheless, similar traces could be produced by agglutination or cell lysis without activation of the platelets. To assess the nature of the evoked platelet effects, we tested for typical indicators of platelet activation, including morphological changes, release from cellular compartments, and the expression of fibrinogen binding sites.

The morphological effects of hypoxLDL and of different agonists were compared by electron microscopy. Figure 3 shows a representative series of electron micrographs, includ-
thrombin and collagen. Aggregation, and they show more aggressive power than shown). Taken together, hypoxLDLs appear to induce true higher amounts of the examined agonists, ie, conditions following order: ADP, thrombin, collagen, and hypoxLDL. Detailed results are shown in Figure II (published online at http://atvb.ahajournals.org). Under conditions leading to full aggregation (100% increase in light transmission), the hypoxLDL-induced release was more pronounced than that evoked by thrombin and collagen.

We tested the effects of different inhibitors on platelet aggregation induced by hypoxLDL to find out which known intracellular second messengers might be involved. The results are summarized in Table II (published online at http://atvb.ahajournals.org). Pretreatment of platelets with 10 mmol/L EDTA for 30 minutes did not completely prevent their aggregation after the addition of hypoxLDL but virtually suppressed any aggregation response to ADP or thrombin. We conclude that an intact platelet integrin αIIbβ3 (platelet glycoprotein IIb/IIIa complex) is not required for activation induced by hypoxLDL. Because acetylsalicylic acid had no effect, pathways involving cyclooxygenase(s) are not essential in hypoxLDL-induced platelet activation. Taken together, the effects of inhibitors reflect a complex pattern of activation pathways common with other agonists: elevation of cyclic nucleotides as well as inhibition of phospholipase A2 and phospholipase C completely suppressed platelet aggregation in a dose-dependent manner, whereas inhibition of protein kinase C caused partial inhibition of platelet responses.

In the absence of any further agonist, fibrinogen-receptor sites were expressed on the addition of hypoxLDLs (REM 1.87), which could induce aggregation, whereas nonaggregating hypoxLDLs (REM 1.34) as well as nLDLs did not show this effect. Data are given in Table III (available online at http://atvb.ahajournals.org).

The modification of LDLs by hypochlorite is largely confined to the protein moiety of the particle; therefore, specific binding of the modified apoB to the platelet surface is likely to be the basis for the activation evoked by hypoxLDLs. Because nLDLs and Cu2+-oxidized LDLs show saturable binding to sites on the platelet surface (although nonidentical),27 we also tested whether hypoxLDLs bind to either of these binding sites. To restrict oxidation of the lipid moiety to the minimum level, iodination was performed in the presence of BHT. This procedure slightly reduced the specific radioactivity of the labeled lipoprotein but kept TBA RS in the final product <1.5 nmol/mg protein. Binding isotherms were performed with 3 preparations of 125I-hypoxLDL modified to different extents (REM 1.29 to 1.94), and the results are summarized in Table II. In each case, saturable binding was observed with only a minor (ε15% of the total binding) contribution of nonspecific binding (ie, binding that could not be displaced by an excess of the respective unlabeled hypox-LDLs). The apparent binding strength significantly increases with the increasing extent of modification. The species that could not induce platelet aggregation (REM 1.29) apparently bind to a single class of sites, roughly comparable to the respective behavior of nLDLs. nLDLs effectively suppressed the binding of this hypoxLDL species, and we conclude that both lipoprotein species bind to the same receptor sites. The 2 more extensively modified preparations could induce plate-
Figure 3. Electron micrographs of unstimulated washed human platelets (A) and platelets treated with different agonists (B through E). They are representative of the results obtained with 5 platelet preparations from individual donors. On average, 20 fields on 4 grids corresponding to a total area of 3 mm² were examined. Agonist doses were adjusted to yield 50% light transmission. A, Control platelets without added agonist. B, Platelets +15 μmol/L ADP. C, Platelets +0.02 U/mL thrombin. D, Platelets +3 μg/mL collagen. E, Platelets +15 μg/mL hypoxLDL (REM 1.81). Bar=1 μm.
let aggregation. The species inducing strong platelet aggregation (REM 1.94) bound to a single class of high-affinity sites. This binding affinity is in perfect accordance with the concentration range, which is effective in platelet aggregation (Figure III, published online at http://atvb.ahajournals.org). Binding of this sample was not affected even by a large excess of unlabeled nLDL (500 µg/mL), so both species apparently bind to independent sites. Table 2 also includes the competition of this binding by reductively methylated LDL. Binding of 125I-hypoxLDL could be displaced by an excess of hypmetLDL nearly as efficiently as by unlabeled hypoxLDL, whereas methylated LDL, which was not further modified with hypochlorite (metLDL), proved as ineffective as nLDL. Maleylated human serum albumin (malHSA) strongly suppressed the binding of hypoxLDL, whereas acLDL had no effect at all (data not shown).

The less extensively modified sample (REM 1.76) revealed intermediate behavior with respect to binding strength and competition for binding by nLDL. We conclude that the regions confining interaction with the nLDL-specific binding sites are partially retained, together with modified stretches responsible for binding to a surface protein specifically recognizing hypoxLDL.

**Discussion**

Unlike radical-induced oxidation of LDL, controlled modification with hypochlorite gives rise to a lipoprotein species with highly reproducible effects on blood platelets. Hypox-LDLs with markedly increased electronegativity (REM ≥1.5) can induce irreversible aggregation of resting platelets in suspension as well as in platelet-rich plasma. HypoxLDLs induce functional morphological changes and extensive release from dense granules. Key enzymes of the arachidonate cycle obviously are not required for platelet activation by hypoxLDLs, whereas the phosphoinositide cycle appears to be essential for signal transduction from the platelet surface into the cell.

Formation of LDL aggregates on oxidation can be virtually excluded, so platelet aggregation is obviously induced by interaction with monodispersed hypoxLDLs. We report high-affinity binding of hypochlorite-modified LDLs. Dose/response diagrams of the specific binding and the induced platelet aggregation almost perfectly parallel each other, so we conclude that this binding is the limiting step in platelet activation induced by hypoxLDLs. Most likely, this interaction gives rise to transmembrane activation signals. However, we cannot completely rule out the possibility that some modified and thus reactive site of platelet-bound hypoxLDLs might oxidize some component of the platelet plasma membrane on contact. This presumptive oxidation, if taking place at all, apparently is not transmitted by free radicals or free reactive oxygen species, because the addition of oxidant scavengers like catalase or BHT to the platelet-lipoprotein reaction mixtures had no effect (data not shown).

We conclude that 2 independent classes of binding sites exist on the platelet plasma membrane. nLDLs and slightly modified LDLs compete for one type of membrane protein, presumably identified as the platelet integrin (platelet glycoprotein Ibα/IIa complex). More extensively modified LDLs are no longer recognized by this receptor but tightly bind to a different protein on the platelet surface. Intermediate species can interact with both binding sites, leading to a total binding capacity larger than in the 2 former cases. Experiments to identify the sites to which LDLs bind when sufficiently modified with hypochlorite are under way. Interaction with these sites may be due to a major rearrangement of apoB on the treatment of LDLs with hypochlorite, which exposes parts of the protein otherwise inaccessible, as has been reported for several globular proteins. Alternatively, some protein in the platelet membrane could recognize (clusters of) modified side chains. This possibility is strengthened by the observation that some monoclonal antibodies, which do not cross-react with other types of modified LDLs, also recognize epitopes on other hypochlorite-modified proteins. Furthermore, treatment with hypochlorite can also transform other proteins into platelet-aggregating agents. This includes delipidated HSA and fibrinogen, oxidized with NaOCl/protein ratios between 500 and 1000, whereas several other proteins are ineffective (authors’ unpublished data, 2000). MalHSA, on the other hand, does not activate platelets but strongly inhibits platelet aggregation induced by either thrombin, hypoxLDL, or hypochlorite-treated HSA. Because neither acLDLs nor hypochlorite-modified proteins in general act as platelet agonists, we further conclude that pronounced electronegativity alone is not sufficient to acquire this capability.

The ligand specificity of the platelet receptor recognizing hypoxLDL reflects some relatedness with the macrophage scavenger receptor system. Currently, a broad spectrum of partly unrelated oxLDL receptors has been identified. Most of them, however, are able to bind both oxLDL and acLDL, whereas human platelets are unable to bind acLDL. So far, only 2 strictly oxLDL-specific receptors have been identified, namely, CD32 (the Fcγ receptor IIb/IIa) and CD36; both are present on human platelets. There is strong evidence that Cu2+-oxLDL binding to human platelets is mediated by CD36, which therefore represents a likely candidate for hypoxLDL binding.

One major question concerns the nature of the modification responsible for the reported platelet effects. It is generally
accepted that hypochlorite preferentially oxidizes apoB with little or no lipid peroxidation, and that α-tocopherol is not depleted, and that LDL cholesterol is not oxidized. Extensive lipid peroxidation requires prolonged treatment of LDLs or liposomes with NaOCl at 37°C. Very recently, it was confirmed by Hazell et al that most hypochlorite is rapidly consumed by nonradical reactions with apoB; the formation of radicals from chloramines with the subsequent oxidation of lipids is a secondary effect. Large excess of hypochlorite should favor lipid peroxidation, because amino acid side chains other than amino groups would be modified, which would more easily give rise to radicals. The reaction conditions stated in the present study (0°C, rather short incubations, and the addition of the radical scavenger BHT) appear sufficient to largely restrict lipid oxidation. BHT reportedly even completely inhibits TBarS formation in VLDL and phosphatidylcholine vesicles treated with hypochlorite for up to 3 hours. On the other hand, some oxidative modification of the lipid moiety of LDL still may occur. Treatment of phospholipids with hypochlorite could lead to the formation of products not detectable by the methods applied in the present study, eg, the accumulation of small amounts of lysolechithin, chloramines, and other oxidative products derived from the polar part of phospholipids. Additionally, small amounts of oxidized lipids may be formed only transiently during the oxidation reaction. Secondary reactions of these primary products may themselves initiate modifications of the apoprotein, and covalent adducts of oxidized lipids with suitable sites at the polypeptide chain may occur. Finally, even trace amounts of lipid peroxides, isoprostanates, and platelet-activating factor-like substances may be important. However, some potentially formed oxidized lipids reportedly inhibit platelet function (eg, oxysterols, lyssolethithin, and 4-hydroxynonenal); no effects of oxLDL are mediated not only by various bioactive lipids but also by the protein moiety.

The number of intact primary amino groups declines with increasing electrophoretic mobility of the modified LDLs. However, although this increase may serve as a suitable parameter to predict the interaction of hypoxLDLs with platelets, oxidized lysines apparently are not directly involved. Modification of the majority of solvent-exposed lysine residues of LDLs (by reductive methylation or by acetylation) before hypochlorite modification hardly had any effect on their platelet aggregation power (Figure I and Table I, published online at http://atvb.ahajournals.org), and hypoxLDL was indistinguishable from hypoxoLDL in binding experiments (Table 2). Obviously, neither intact nor modified lysine residues are essential in hypoxoLDL-platelet interaction, whereas clusters of charge-neutralized (acyetylated but not dimethylated) lysine residues are responsible for the binding of oXLDL to macrophage scavenger receptors. Consequently, any secondary reactions involving chloramines are not likely to be essential for the conversion of hypoxoLDL into a platelet agonist. Thus, hypoxoLDL could serve as an in vitro model of protein-specific atherogenic lipoprotein transformations. HypoxoLDL leads to complete platelet aggregation at doses far below those necessary with Cu+-oxidized LDL to achieve comparable effects. Furthermore, it can induce platelet aggregation even in platelet-rich plasma, although higher doses are required. This demonstrates that plasma components, including other lipoproteins and antioxidants, cannot completely counteract the specific action of hypoxoLDL and that platelet stimulation by this lipoprotein species may also occur in vivo.

Acknowledgment

Part of this work was supported by grant No. 6021 of the Jubiläumsfonds der Österreichischen Nationalbank.

References


Modification of Protein Moiety of Human Low Density Lipoprotein by Hypochlorite Generates Strong Platelet Agonist
Ivo Volf, Edith Bielek, Thomas Moeslinger, Franz Koller and Elisabeth Koller

doi: 10.1161/01.ATV.20.8.2011
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 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/20/8/2011

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2000/07/25/20.8.2011.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/
Methods

Radioiodination
A single N-chloro-benzenesulfonamide conjugated polystyrene bead (Iodo-Beads, Pierce, Rockford, IL, USA) was added to 0.1 to 0.2 mL of a solution of native or modified LDL (0.5-1.5 mg/mL) and 250 µCi Na$^{125}$I in 0.1 mol/L borate buffer, 15 µmol/L BHT, pH 7.0 on ice. After 15 min the Iodo-Bead was removed and washed with 0.1 mL of the respective unlabeled LDL species, and the supernatants were pooled. After addition of 20 µl 2 mol/L aqueous KI, unreacted Na$^{125}$I was removed by gel filtration with Sephadex G-25.

Isolation of human platelets
Throughout the isolation PGI$_2$ was added to a final concentration of 25 µg/L. After the final washing step the platelets were resuspended in Tyrode buffer with Ca$^{2+}$ (137 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO$_3$, 1.0 mmol/L MgCl$_2$, 2 mmol/L CaCl$_2$, 0.42 mmol/L NaH$_2$PO$_4$, 5.5 mmol/L d-glucose, 3.5 g/L human serum albumin, pH 7.35), without PGI$_2$. The platelet suspension was kept 30 min at room temperature to recover from PGI$_2$ inhibition.

Aggregation studies
Platelet aggregation was determined in a single (LumiAggregometer) or four-channel (490-4D) aggregometer (Chronolog Corp., Havertown, PA, USA). Platelet suspensions (adjusted to 2x10$^5$ cells/µL in a final volume of 0.5 mL) were supplemented with all additives and were stirred for 3 min in siliconized glass cuvettes at 1,100 rpm in the aggregometer before adding the aggregating agents. Controls with 5 to 30 µmol/L ADP were performed and the experiments were discontinued after the first decline of reactivity was observed.

Binding studies
Binding and competition studies were performed as described by us in detail$^{22}$. Briefly, washed platelets resuspended in Tyrode-Ca$^{2+}$ were incubated with the radiolabeled lipoprotein (0.5 - 100 µg/mL) at room temperature for 20 min in triplicates. Platelet-bound radioactivity was determined after centrifugation through 20% aqueous sucrose (45 s at 16,000 x G). Non-specific binding was determined as the amount of platelet-associated radioactivity in the presence of 500 µg/mL of the unlabeled LDL species.
5-Hydroxytryptamine (5-HT) release

After loading platelet rich plasma (PRP) with $[^{14}C]-5$-HT (0.05 µCi/mL) for 30 min, platelet suspensions were prepared as described above. Aggregation was carried out in a final volume of 1 mL with different concentrations of agonists or hypoxLDL. When the evoked aggregation response was complete, the release was terminated by addition of glutardialdehyde (0.5 % final). After centrifugation the radioactivity of aliquots of the supernatant was counted. Control release without addition of agonists ranged between 3 and 8% of the platelet-associated radioactivity.
<table>
<thead>
<tr>
<th>LDL-species</th>
<th>[NaOCl]/[LDL]*</th>
<th>TBARS (nmol/mg)</th>
<th>free NH₂-groups (%)</th>
<th>REM†</th>
<th>Induced platelet aggregation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[concentration of nLDL (µg/mL)*]</td>
</tr>
<tr>
<td>nLDL</td>
<td>–</td>
<td>0.8</td>
<td>100</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>acLDL</td>
<td>–</td>
<td>1.1</td>
<td>25</td>
<td>2.75</td>
<td>2.46</td>
</tr>
<tr>
<td>hypacLDL</td>
<td>570 833</td>
<td>1.2</td>
<td>24</td>
<td>2.67</td>
<td>2.42</td>
</tr>
<tr>
<td>metLDL</td>
<td>–</td>
<td>0.8</td>
<td>41</td>
<td>0.97</td>
<td>1.08</td>
</tr>
<tr>
<td>hypmetLDL</td>
<td>570 833</td>
<td>1.0</td>
<td>29</td>
<td>1.78</td>
<td>2.24</td>
</tr>
<tr>
<td>hypoxLDL</td>
<td>570 555</td>
<td>1.1</td>
<td>52</td>
<td>1.88</td>
<td>1.77</td>
</tr>
<tr>
<td>hypoxLDL</td>
<td>855 833</td>
<td>1.3</td>
<td>40</td>
<td>2.44</td>
<td>2.32</td>
</tr>
</tbody>
</table>

* concentration of nLDL refers to their apoB-100 contents
† relative electrophoretic mobility
‡ % increase in light transmittance after 6 min; the two series were performed with platelets from different donors
Figure IA: Aggregometer tracings showing the effects of hypoxoLDL (REM 1.84) on PRP. 2x10^5 platelets/µL were suspended in 0.5 mL platelet poor plasma (PPP) [tracings (a) and (b)], or in diluted PPP, i.e. PPP/Tyrode-Ca^{2+} 1:1 (v/v) [tracings (c) to (e)]. Aggregation was evoked by addition of (a) 280 µg/mL hypoxoLDL; (b) 100 µg/mL hypoxoLDL; (c) 180 µg/mL hypoxoLDL; (d) 90 µg/mL hypoxoLDL; (e) 45 µg/mL hypoxoLDL. The aggregation-response does not follow a simple saturation behavior in PRP, as shown in fig. IB. The sigmoidity of this curve was less pronounced when platelets were suspended in diluted plasma. In this case much lower concentrations of hypoxoLDL could induce complete aggregation.
Figure IB: Dose-dependence of hypoxLDL (REM 1.84)-induced platelet aggregation with washed platelets (dotted line); with platelets in diluted PPP (broken line); with platelets in undiluted PPP (full line).
Figure II:

[Graph showing the relationship between release (%) and increase in light transmittance (%).]
Figure II: Release of $^{14}$C-5-hydroxytryptamine (5-HT) from pre-loaded platelets upon treatment with different agonists in relation to the degree of aggregation induced by the respective agonist doses. Experiments were performed with 3 (hypoxLDL) or 2 (all others) platelet preparations from individual donors. Symbols for agonists: (●) ADP; (∆) collagen; (◊) thrombin; (□) hypoxLDL (REM 1.97).

With ADP, no release was observed up to 100% aggregation. In all other cases, agonist doses leading to at least 50% aggregation response caused significant release of serotonin.

Controls in the presence of platelet inhibitors were performed to assess that hypoxLDL did not cause non-specific membrane perturbation or leakage. As indicated, the effects of hypoxLDL could be completely suppressed by addition of either 10 µmol/L staurosporine (■); or by 1 mmol/L adenosine and 1 mmol/L theophylline (▲).
TABLE II: Effect of inhibitors on platelet aggregation induced by ADP, thrombin, and hypoxLDL.

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>ADP</th>
<th>thrombin</th>
<th>hypoxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA, (10 mmol/L, 30 min, 37°C)</td>
<td>0</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>ASA, (1 mmol/L, 30 min, 37°C)</td>
<td>30</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td>Adenosine, (1 mmol/L, 5 min, 20°C)</td>
<td>30</td>
<td>n.d.</td>
<td>15</td>
</tr>
<tr>
<td>Adenosine, (2.5 mmol/L, 5 min, 20°C)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Theophylline, (0.6 mmol/L, 5 min, 20°C)</td>
<td>20</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Theophylline, (1.5 mmol/L, 5 min, 20°C)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitroprusside, 0.18µg/mL final</td>
<td>0</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Nitroprusside, 1.8µg/mL final</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sphingosine, (13 µmol/L, 5 min, 20°C)</td>
<td>30</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Sphingosine, (26 µmol/L, 5 min, 20°C)</td>
<td>30</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Staurosporine, (2 µmol/L, 5 min, 20°C)</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Staurosporine, (3 µmol/L, 5 min, 20°C)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Quinacrine, 20 µmol/L final</td>
<td>20</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>Quinacrine, 100 µmol/L final</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Conditions of pre-treatment of platelets with the respective inhibitor are given in parenthesis; otherwise inhibitors were added immediately before addition of agonist in the concentration indicated.

n.d. not determined
TABLE III: Effects of hypoxLDL with different extent of modification, and of nLDL on expression of platelet fibrinogen sites (concentration of $^{125}I$-fibrinogen 10 µg/mL; corrected for non-specific binding, as determined in the presence of 500 µg/mL unlabeled fibrinogen; mean values ± S.D., 5 experiments performed with platelets from different donors).

<table>
<thead>
<tr>
<th>Agonist added</th>
<th>final concentration of agonist</th>
<th>Specific binding of fibrinogen (ng bound per $10^8$ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>59 ± 13</td>
</tr>
<tr>
<td>ADP</td>
<td>12 µg/mL</td>
<td>306 ± 72</td>
</tr>
<tr>
<td>nLDL</td>
<td>45 µg/mL</td>
<td>65 ± 30</td>
</tr>
<tr>
<td>hypoxLDL (REM 1.34)</td>
<td>32 µg/mL</td>
<td>80 ± 49</td>
</tr>
<tr>
<td>hypoxLDL (REM 1.87)</td>
<td>7 µg/mL</td>
<td>357 ± 71</td>
</tr>
<tr>
<td>hypoxLDL (REM 1.87)</td>
<td>28 µg/mL</td>
<td>489 ± 50</td>
</tr>
</tbody>
</table>
Figure III: Correlation of the concentration dependence of platelet binding of hypoxLDL (REM 1.93) (■) with the respective induced platelet aggregation (REM 1.93) (▲). The dose-dependent degree of aggregation induced by hypoxLDL (REM 2.53) is also included (△).