Lysophosphatidic acid-independent platelet activation by low-density lipoprotein

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Abstract Mildly oxidized low-density lipoprotein activates platelets through lysophosphatidic acid (LPA). Hence, the platelet-activating properties attributed to native low-density lipoprotein (nLDL) might be caused by LPA contamination. We show that nLDL enhances thrombin receptor-activating peptide (TRAP)-induced fibrinogen binding to $\alpha_{IIb}\beta_3$. The LPA receptor blocker *N*-palmitoyl-L-serine-phosphoric acid did not affect nLDL-enhanced fibrinogen binding induced by TRAP, but reduced TRAP-induced binding. cAMP and inhibitors of protein kinase C and Ca^{2+} rises completely blocked ligand binding by TRAP and nLDL/TRAP. Inhibitors of $p38^{MAPK}$ and ADP secretion interfered only partially. Blockade of Rho-kinase increased ligand binding 2–3-fold. We conclude that nLDL enhances TRAP-induced fibrinogen binding independent of LPA. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Lysophosphatidic acid; Low-density lipoprotein; *N*-Palmitoyl-L-serine-phosphoric acid; $\alpha_{IIb}\beta_3$

1. Introduction

Increased levels of native low-density lipoprotein (nLDL) are related to an increased risk for atherosclerosis. Platelets are key elements in the initiation of arterial thrombosis. At physiological concentrations (0.6–0.9 g protein/l), nLDL sensitizes platelets to natural agonists such as α-thrombin, collagen and ADP [1–4]. The sensitization is slow (30 min or more), however, and nLDL alone fails to trigger shape change, integrin $\alpha_{IIb}\beta_3$ activation, or secretion of granule contents. Sensitization of platelets occurs through phosphorylation of p38 mitogen-activated protein kinase ($p38^{MAPK}$) within 10 s with 1.0 g/l nLDL and within 10 min with 0.1 g/l nLDL and results in cytosolic phospholipase A_2 ($cPLA_2$)-activated arachidonic acid release, a precursor of thromboxane (TxA_2) formation [5]. At higher concentrations (3 g/l) and longer incubation times (>4 h), nLDL becomes an independent initiator of platelet activation triggering aggregation and secretion [6]. Thus, depending on concentration and time, nLDL synergizes with other activators or is an independent platelet agonist.

At the early stages of atherosclerotic development, endothelial cells become activated and bind monocytes and T-lymphocytes, which subsequently migrate through the endothelium into the subendothelium. nLDL accumulation has been found in these areas [7] and minimally modified LDL (mm-LDL) has been shown to cause an increase of monocyte binding to the endothelium [8]. nLDL also increases intracellular Ca^{2+} levels in endothelial cells. The Ca^{2+} rises induce VCAM-1 expression and subsequent binding of monocytes to the endothelium [9].

Particularly modified forms of nLDL are atherogenic, and the potency of nLDL as a platelet activator increases upon oxidative modification, leading to faster responses at lower concentrations. Oxidized LDL (ox-LDL) enhances aggregation and secretion induced by α-thrombin and ADP [10,11] or induces spontaneous aggregation, adhesion and serotonin release [12–14].

The effects of oxidatively modified LDL have been attributed to lysophosphatidic acid (LPA), which is generated during oxidation of nLDL [15]. Human platelets possess a G-protein-coupled receptor for LPA, which is a member of the endothelial differentiation genes (Edg) receptor family [16]. The LPA receptor responds to low concentrations of LPA (EC_{50} of 18 nM) with platelet shape change [15,17]. At higher concentrations ($EC_{50} \sim 1.6$ μM), LPA increases cytosolic Ca^{2+} levels and activates Src family tyrosine kinases and the tyrosine kinase Syk, both pathways being independent of platelet shape change [17].

During nLDL isolation, oxidation is prevented by ethylenediaminetetraacetic acid (EDTA). However, a minimal degree of oxidation might be inherent to nLDL isolation procedures, raising the possibility that nLDL lacks any platelet-activating properties.

The present study addresses the question whether platelets respond to nLDL, independent of LPA-mediated cell signaling.

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Abbreviations: nLDL, native low-density lipoprotein; MAPK, mitogen-activated protein kinase; $cPLA_2$, cytosolic phospholipase A_2; TxA_2, thromboxane A_2; mm-LDL, minimally modified LDL; ox-LDL, oxidized LDL; LPA, lysophosphatidic acid; Edg, endothelial differentiation gene; PEP, phosphoenolpyruvate; PK, pyruvate kinase; L-NASPA, *N*-palmitoyl-L-serine-phosphoric acid; TRAP, thrombin receptor-activating peptide; mox-LDL, mildly oxidized LDL; PKC, protein kinase C

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2. Materials and methods

2.1. Materials

Prostacyclin (PGI_2) was from Cayman Chemical, Ann Arbor, MI, USA. Indomethacin was from Sigma, St. Louis, MO, USA. Iloprost was from Schehring AG, Berlin, Germany. Bisindolylmaleimide I (GF109203X), phosphoenolpyruvate (PEP) and pyruvate kinase (PK) were all obtained from Boehringer Mannheim, Mannheim, Germany. Bis-(*o*-amino-phenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) was from Calbiochem Corporation, La Jolla, CA, USA. SB203580 was obtained from Alexis, San Diego, CA, USA. *N*-palmitoyl-L-serine-phosphoric acid (L-NASPA) was from Biomol, Plymouth, PA, USA. Rho-kinase inhibitor Y-27632 was kindly provided by Yoshitomi Pharmaceuticals, 3-7-25 Koyata, Iruma-Shi Saitama, Japan. The thrombin receptor-activating peptide SFLLRN (TRAP) was synthesized with a semi-automatic peptide synthesizer (Labortec AG SP650, Switzerland) according to van Scharrenburg et al. [18]. Fibrinogen was from Chromogenix, Mölndal, Sweden, and made fibrin- and fibronectin-free by passage through a gelatin-Sepharose 4B column [19]. Sepharose 4B was from Pharmacia Biotech, Uppsala, Sweden. FITC-conjugated anti-human fibrinogen was from DAKO, Glostrup, Denmark. All other chemicals used were of analytical grade.

2.2. nLDL isolation

Fresh, non-frozen plasma from three donors, each containing less than 200 mg lipoprotein(a)/l, was pooled and nLDL (1.019–1.063 kg/l) was isolated by sequential flotation in a Beckman L-80 ultracentrifuge [20]. To prevent bacterial contamination and lipid modification, 2 mM NaN_3 and 4 mM EDTA were present during each run (175 000 × *g*, 20 h, 10°C). KBr was used for density adjustment. nLDL was dialyzed against 10^3 volumes of buffer containing 150 mM NaCl, 1 mM EDTA and 1 mM NaN_3, and subsequently filtered through a 0.45 μm filter. Prior to each experiment, nLDL was dialyzed overnight against 10^4 volumes of 150 mM NaCl.

Levels of apoB100 were measured using the Behring Nephelometer 100. Possible oxidative modification was determined by monitoring the formation of conjugated dienes at 234 nm of 50 mg apoB100/l with 5 μM $CuCl_2 \cdot 2H_2O$. The final nLDL preparation showed an absorption at 234 nm of 0.26 ± 0.08. This was in the range of values reported by Weidtmann [3] for nLDL isolated in the presence of thimerosal (0.28 ± 0.04). All concentrations of nLDL are expressed as g apoB100/l.

2.3. Isolation of human platelets

Human platelets were isolated from freshly drawn venous blood, collected from healthy volunteers (with informed consent) into 0.1 vol of 3.8% (wt/vol) trisodium citrate. The donors claimed not to have taken any medication 10 days prior to blood collection. Platelet-rich plasma was prepared by centrifugation (156 × *g*, 15 min, 20°C). Then, 0.1 vol of ACD (2.5% trisodium citrate, 1.5% citric acid, 2% D-glucose) was added to lower the pH of the plasma to 6.5 and thus prevent platelet activation during further isolation. Platelets were further purified by centrifugation at 330 × *g* for 15 min at 20°C. The platelet pellet was resuspended in HEPES–Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES, pH 6.5) containing 5 mM D-glucose. 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 containing 5 mM D-glucose to a final concentration of 0.4×10^{11} platelets/l. Prior to the experiments, the platelets were left at room temperature for at least 30 min to ensure a resting state.

2.4. Binding of fibrinogen

A volume of 0.3 ml platelets was treated with the cyclooxygenase inhibitor indomethacin (30 μM, 30 min, 37°C) to prevent secondary effects from the TxA_2-dependent signaling pathway. The platelets were then incubated with various inhibitors followed by pretreatment with nLDL (1.0 g/l, 30 min, 37°C) or an equal volume of 150 mM NaCl and subsequently stimulated with TRAP (15 μM, 5 min) in the presence of fibrinogen (1 μM, 2 min) at room temperature. Platelets were fixed in phosphate-buffered saline (PBS) containing 1% formaldehyde after a 64-min incubation period with FITC-conjugated anti-human fibrinogen (1:30 dilution of 1.4 g/l). Fibrinogen binding to $\alpha_{IIb}\beta_3$ was analyzed by flow cytometry (FACScalibur, Becton Dickinson).

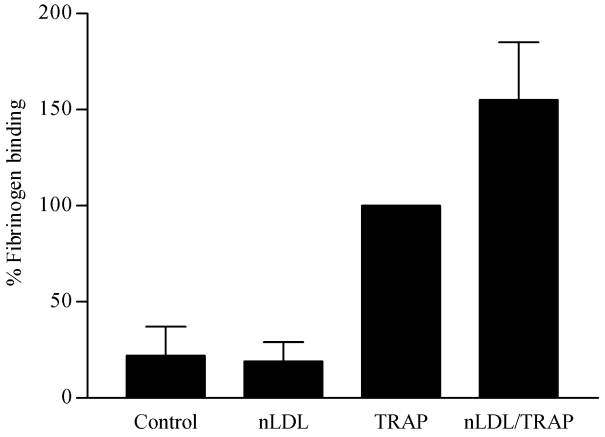

Fig. 1. nLDL-enhanced fibrinogen binding induced by TRAP. Indomethacin-treated platelets (0.4×10^{11}/l) were preincubated with vehicle (150 mM NaCl) or with 1.0 g/l nLDL for 30 min at 37°C. Subsequently, the platelets were stimulated without (150 mM NaCl) or with 15 μM TRAP (5 min) in the presence of 1 μM fibrinogen (2 min) at room temperature. Platelets were fixed in PBS/1% formaldehyde 64 min after the addition of FITC-conjugated anti-human fibrinogen (1.4 g/l; dilution 1:30). Fibrinogen binding was analyzed by flow cytometry. Data are means ± S.D., $n = 5$–10.

2.5. Statistical analysis

Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test as post test. Results are expressed as means ± S.D. from 5–10 experiments without inhibitors, and from three experiments with inhibitors.

3. Results

Indomethacin-treated platelets were stimulated with nLDL, TRAP or a combination of TRAP and nLDL, and fibrinogen binding to $\alpha_{IIb}\beta_3$ was determined by flow cytometry. nLDL alone failed to induce fibrinogen binding, but a 30-min preincubation of platelets with nLDL enhanced the TRAP-induced fibrinogen binding (100%) with 55 ± 30% (Fig. 1). Since recent findings indicate that mildly oxidized LDL (mox-LDL) induces platelet activation via LPA [15], nLDL-enhanced fibrinogen binding induced by TRAP was measured in the presence of L-NASPA, a blocker of the LPA receptor. At 1, 10, 20, and 30 μM L-NASPA, TRAP-induced fibrinogen binding decreased to 67 ± 26%, 32 ± 11%, 47 ± 23%, and 54 ± 38%, respectively ($P < 0.001$, Fig. 2). Surprisingly, following pretreatment with nLDL, fibrinogen binding remained undisturbed at 178 ± 27%, 133 ± 5%, 132 ± 8%, and 143 ± 34% at the respective L-NASPA concentrations ($P = 0.19$). Thus, the LPA receptor functions in TRAP-induced signaling, whereas the enhancement of TRAP signaling by nLDL appears independent of LPA or its receptor.

To evaluate the signaling steps involved in the effect of nLDL on TRAP-induced fibrinogen binding, studies were repeated with metabolic inhibitors known to interfere with signal transduction in platelets. The cAMP-raising agent iloprost, the protein kinase C (PKC) inhibitor bisindolylmaleimide and the Ca^{2+} chelator BAPTA-AM all abolished fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by TRAP alone or in combination with nLDL (Table 1). Thus, low cAMP, a rise in Ca^{2+} and activation of PKC are prerequisites for TRAP-induced $\alpha_{IIb}\beta_3$ activation with and without nLDL. Only par-

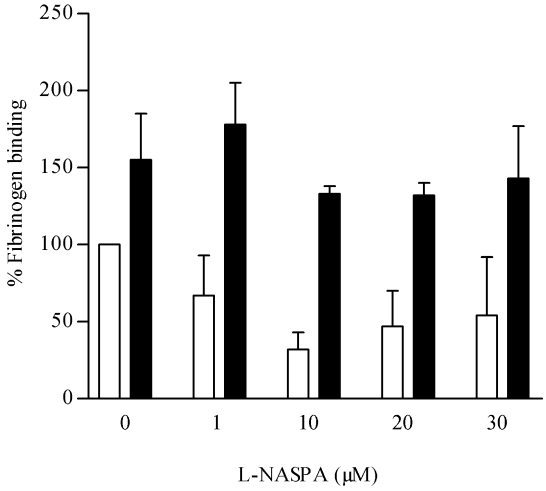

Fig. 2. nLDL activates platelets independent of LPA/LPA receptor-mediated signaling. Indomethacin-treated platelets were incubated with vehicle (DMSO) or the LPA receptor blocker L-NASPA (1, 10, 20 or 30 μM) for 5 min at 37°C. Prior to stimulation with TRAP in the presence of fibrinogen at room temperature, platelets were pretreated with 1.0 g/l nLDL (closed bars) or 150 mM NaCl (open bars) at 37°C. Fibrinogen binding was determined as described in the legend of Fig. 1. Data are means ± S.D., $n = 5–10$, for the inhibitor $n = 3$.

tial inhibition was found with SB203580, an inhibitor of $p38^{MAPK}$.

Mox-LDL induces platelet shape change via Rho/Rho-kinase activation [21]. To study the involvement of Rho-kinase in the LPA-induced signaling pathway to $\alpha_{IIb}\beta_3$ activation, the platelets were preincubated with Y-27632, a Rho-kinase inhibitor. This treatment increased both TRAP-induced fibrinogen binding and nLDL/TRAP-induced binding (Table 1).

Finally, studies were repeated with PEP (0.28 mM) and PK (3 U/ml) to interfere with released ADP. This treatment reduced fibrinogen binding induced by TRAP alone or with nLDL almost 3-fold (Table 1). Thus, a major part of the effect of nLDL originates from stimulation of TRAP-induced dense granule secretion.

4. Discussion

Recent data show that oxidation of nLDL is accompanied by LPA formation. LPA is the activating ligand for platelet shape change induced by mox-LDL [15]. The present study shows that a potent inhibitor of the LPA receptor strongly interferes with the TRAP-induced ligand binding to $\alpha_{IIb}\beta_3$ but has no significant effect when platelets have been pretreated with nLDL. Thus, nLDL activates platelets independent of LPA-mediated signaling. This nLDL-dependent pathway is sensitive to iloprost and inhibited by bisindolylmaleimide and BAPTA-AM, indicating that it shares common steps in signal transduction by TRAP and other platelet activators. nLDL-induced activation is sensitive to an ADP removing mixture, indicating that a major part of the activation originates from TRAP-induced ADP secretion. Hackeng et al. [22] already showed that 1.0 g/l LDL enhances the collagen-induced release of serotonin from about 20% to 40% of maximal secretion. Similar results were obtained with stirred suspensions stimulated with TRAP.

Rho-kinase is involved in LPA-signaling pathways leading to shape change [21]. To investigate the role of Rho-kinase in LPA-dependent signaling to $\alpha_{IIb}\beta_3$, the platelets were treated with Y-27632. This Rho-kinase inhibitor caused an increase of the fibrinogen binding induced by TRAP or nLDL/TRAP by 2–3-fold. Activated Rho-kinase induces phosphorylation of myosin light chain leading to interaction of the myosin head with actin to form contractile actin/myosin bundles. Rho-kinase also phosphorylates moesin, which subsequently mediates the interaction of the actin/myosin bundles with the plasma membrane [21]. The actin cytoskeleton appears to play a role in regulating $\alpha_{IIb}\beta_3$ function and interruption of actin filament turnover in platelets by cytochalasin D enables a substantial portion of $\alpha_{IIb}\beta_3$ to bind soluble fibrinogen [23]. Thus, the observed $\alpha_{IIb}\beta_3$ activation after Rho-kinase inhibition might have been due to interruption of the cytoskeletal organization.

nLDL activates $\alpha_{IIb}\beta_3$ by a pathway involving activation of $p38^{MAPK}$ resulting in $cPLA_2$-mediated arachidonic acid release, TxA_2 formation and further signaling via the TxA_2 receptor [5]. In addition, nLDL also induces p125 focal adhesion kinase ($p125^{FAK}$) phosphorylation [24]. Both $p38^{MAPK}$ and $p125^{FAK}$ phosphorylation are early events after LDL–platelet contact [5,24]. So far, the role of $p38^{MAPK}$ was thought to be restricted to the TxA_2-dependent pathway. The present study shows that $p38^{MAPK}$ also functions in nLDL signaling in indomethacin-treated platelets. Incubation with SB203580, which inhibits the activation of $p38^{MAPK}$, only partially reduced fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by TRAP or nLDL/TRAP. Four isoforms of $p38^{MAPK}$ (p38α, p38β, p38γ and p38δ) have been identified, which are 60–70% identical in their amino acid sequence [25]. As SB203580 only inhibits the p38α and the p38β isoforms [25], p38α and/or p38β are involved in the TxA_2-independent

Table 1
Effect of metabolic inhibitors on nLDL-enhanced fibrinogen binding to $\alpha_{IIb}\beta_3$

Inhibitor	TRAP-induced fibrinogen binding (%)	Fibrinogen binding induced by nLDL/TRAP (%)
–	100	155 ± 30
Iloprost	8	7
Bisindolylmaleimide	10	8
BAPTA-AM	4	7
SB203580	51 ± 5	48 ± 8
Y-27632	172 ± 63	366 ± 201
PEP-PK	31 ± 9	56 ± 11

Indomethacin-treated platelets were preincubated with vehicle (150 mM NaCl), iloprost (1 μM, 2 min), bisindolylmaleimide (5 μM, 2 min), BAPTA-AM (30 μM, 30 min), SB203580 (10 μM, 30 min), Y-27632 (10 μM, 30 min) or PEP-PK (0.28 mM PEP, 3 U/ml PK, 2 min) at 37°C prior to pretreatment with 1.0 g/l nLDL or 150 mM NaCl, and stimulation with TRAP in the presence of fibrinogen at room temperature. Fibrinogen binding was determined as described in the legend of Fig. 1. Data are means ± S.D., $n = 5–10$, for the inhibitors $n = 1$ or $n = 3$.

$\alpha_{IIb}\beta_3$ regulation. However, SB203580 does not inhibit the phosphorylation of the p38γ or the p38δ isoform, which therefore might also play a role in this nLDL-signaling pathway. Thus, nLDL induces another important signaling pathway independent of TxA_2 formation, in which $p38^{MAPK}$ seems to be involved. Moreover, this signaling pathway is completely insensitive for treatment with L-NASPA.

In conclusion, we have shown that nLDL, independent of LPA, is able to enhance the activation of a TxA_2-independent signaling pathway in TRAP-stimulated platelets. ADP, released from the dense granules, seems to mediate the activation of the TxA_2-independent signaling pathway in which rises of Ca^{2+}, activation of PKC and $p38^{MAPK}$ and cAMP as an inhibitor are involved. Platelets from familial hypercholesterolemia patients have an increased sensitivity to agonists due to increased levels of nLDL in the circulation [26]. Therefore, studies that deepen our insights in the mechanisms by which nLDL sensitizes platelets, might provide tools for therapeutic intervention and reduction of cardiovascular complications.

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