Low-density lipoprotein activates the small GTPases Rap1 and Ral in human platelets

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Physiological concentrations of low-density lipoprotein (LDL) sensitize blood platelets to α-thrombin- and collagen-induced secretion, and after prolonged contact trigger secretion independent of other agonists. Here we report that LDL activates the small GTPases Rap1 and Ral but not Ras, as assessed by specific precipitation of the GTP-bound enzymes. In unstimulated suspensions, the inhibitor SB203580 blocks Rap1 activation by 60–70%, suggesting activation via p38 mitogen-activated protein kinase and a second, unidentified route. Inhibitors of cyclooxygenase (indomethacin) and the thromboxane A₂ (TxA₂) receptor (SQ30741) induce complete inhibition, indicating that Rap1 activation is the result of TxA₂ formation. Stirring reveals a second, TxA₂-independent Rap1 activation, which correlates quantitatively with a slow induction of dense granule secretion.

Both pathways are unaffected by inhibitors of ligand binding to integrin αIIbβ₃. The results suggest that Rap1 and Ral, but not Ras, may take part in signalling routes initiated by LDL that initially enhance the sensitivity of platelets to other agonists and later trigger LDL-dependent secretion.

Key words: GTP, lipid oxidation, lipoproteins, thrombocyte, thromboxane, thromboxane.

INTRODUCTION

Physiological concentrations of low-density lipoprotein (LDL) increase the sensitivity of human platelets to agonist stimulation [1–4], but during 0–30 min contact with platelets LDL is not an independent inducer of shape change [4,5] and aggregation [3,4,6]. There is little insight into the mechanisms which respond to LDL. LDL receptors on platelets differ from the classical LDL receptors on hepatocytes, lymphocytes and fibroblasts [7]. Binding depends critically on intact lysine residues in LDL, suggesting that the protein moiety bears the signal-inducing elements [8]. Among the signalling steps activated by LDL are phosphoinositide turnover [3,5], Ca²⁺ mobilization [3,9], protein kinase C [5,10] and αIIbβ₃-mediated outside-in signalling [10]. Concurrently, thromboxane A₂ (TxA₂) is formed, reflecting activation of cytosolic phospholipase A₂ (cPLA₂) [2,5,11]. Inhibition of this pathway reveals both TxA₂-dependent and TxA₂-independent sensitization by LDL [2]. During prolonged contact (2–4 h), LDL becomes an independent secretion-inducing agonist triggering release of dense granule contents independent of TxA₂ formation [11].

Human platelets contain several low-molecular-mass GTPases of the Ras family which are activated during stimulation [12–14]. These GTPases cycle between an active GTP-bound form and an inactive GDP-bound state, via reactions controlled by exchange factors and GTPase-activating proteins. Rap1 is abundantly expressed in platelets and accounts for 0.1% of total platelet protein. It is approx. 50% homologous to Ras, with the strongest similarity within the core effector domain. Rap1 is expressed as two isoforms, Rap1A and Rap1B, which are 95% identical and differ predominantly in the C-terminal part. Human platelets contain predominantly the Rap1B isoform. In platelets stimulated by α-thrombin, collagen and platelet activating factor, Rap1 is activated within seconds, which critically depends on an increase in cytosolic Ca²⁺ [14]. Platelet inhibition by prostacyclin inactivates Rap1. Thus activation and inactivation of Rap1 closely follow the activation state of the platelet, suggesting that Rap1 contributes to the signalling mechanisms that control platelet functions.

The Ras-like GTPase Ral also exists as two isoforms, RalA and RalB [15]. It is strongly expressed in platelets, testes and brain [16–18]. In platelets, Rap1 and Ral are activated through similar pathways [12]. The small GTPase Ras is also activated in platelets, but this activation requires high concentrations of α-thrombin and TxA₂ analogue [12,13].

In the present study we investigated whether Ras, Rap1 and Ral were affected by LDL which would make them possible intermediates in the pathways that increase the sensitivity of platelets for secretion-inducing agonists, and after prolonged contact, make LDL an independent secretagogue. The active GTP-bound state of these GTPases was identified using activation-specific probes, based on the differential affinity of the GDP- and GTP-bound forms of the GTPases for their respective downstream effector molecules.

Abbreviations used: BAPTA/AM, bis-(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid tetrakis(acetoxymethyl ester); cPLA₂, cytosolic phospholipase A₂; ERK2, extracellular-signal-regulated protein kinase 2; LDL, low-density lipoprotein; LPA, 1-oleoyl-α-lysophosphatidic acid; MAPK, mitogen-activated protein kinase; TxA₂, thromboxane A₂.

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Materials and Methods

Materials

Radio-labelled [5,6,8,9,11,12,14,15-³H]arachidonic acid (specific radioactivity 5.5–8.5 TBq/mmol) and 5-hydroxy [side chain-2,³¹C]tryptamine creatinine sulphate (³¹C⁴H₅O₂N₃S·H₂O) 5-hydroxytryptamine; specific activity 1.85 GBq/mmol) were from Amersham International. Silicagel 60 was from Merck, Darmstadt, Germany. RasA antibody was from Transduction Laboratories, Lexington, KY, U.S.A.; antibody 4–4B–3C against cPLA₂ and antibody C14 against extracellular-signal-regulated protein kinase 2 (ERK2), were from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; and total- and dual-phosphorylated mitogen-activated protein kinase (p38MAPK) antibodies were from New England Biolabs, Beverly, MA, U.S.A. The antibodies against Rap1, Ras and Rap1B were prepared in our own laboratory. 5,8,11,14-Eicosatetraynoic acid, human α-thrombin, indomethacin and 1-oleoyl-2-acetyl-sn-glycerol (lipoarsphenamin) (LPA) were from Sigma. The inhibitor of the LPA receptor, 2,6-diaminopimelic acid was from Bioworld (.Alterra, Dublin, Ireland); antibody 4-4B-3C against cPLA₂ and antibody C14. Phosphorylated and total p38 MAPK proteins were transferred to PVDF membranes by electroblotting. cPLA₂ was detected using the mouse monoclonal antibody C14. Phosphorylated and total p38MAPK were measured [24] using phosphoplas p38MAPK (Thr¹⁸⁷/Tyr¹⁸⁷) or anti-p38MAPK antibody for detection. For quantitative determinations, the density of the bands was analysed using ImageQuant software [24]. Platelet isolation

Freshly drawn venous blood from healthy volunteers was collected with informed consent into 0.1 vol. of 130 mM trisodium citrate. Platelet-rich plasma was prepared by centrifugation (200 g for 15 min at 22 °C). Gel-filtered platelets were isolated by gel filtration through Sepharose 2B equilibrated in Ca²⁺-free Tyrode’s buffer and adjusted to 2 × 10⁹ platelets/ml. Platelet suspensions were incubated with LDL or α-thrombin (control) at 37 °C without or with stirring (900 rev./min), as indicated.

Measurement of active GTPases and expression of recombinant GST–Rap1B

GTP-bound Rap1 was precipitated from gel-filtered platelets lysed in Ral buffer [12] with the GST-tagged Rap–binding domain of Rab25GDS bound to GSH–agarose beads (Sigma). GTP-bound Ras was precipitated with GST–tagged Ras-binding domain of RLI25 [12]. Activated Ras was precipitated from platelets lysed in Ral buffer with GST–tagged Ras-binding domain of RalF [23]. The GTPases were identified by Western blotting with the appropriate antibodies [14]. To account for donor variation, different concentrations of recombinant GST–Rap1B were applied to PAGE and analysed densitometrically using ImageQuant software. A linear correlation was obtained between the amount of GST–Rap1B and densitometric intensities up to 7500 arbitrary units (results not shown); subsequent analysis was within this range. The cDNA encoding Rap1B was inserted into the pGEX-4T3 vector by restriction with SmaI and NotI. GST–Rap1B was expressed in Escherichia coli AD202 after induction with isopropyl β-D-thiogalactoside. After lysis of the bacteria, GST–Rap1B was bound to GSH–agarose beads and washed extensively. Protein concentrations were estimated by SDS/PAGE and Coomassie staining, with BSA as a standard.

Phosphorylation of cPLA₂, ERK2 and p38MAPK

cPLA₂ phosphorylation [24] and ERK2 phosphorylation were measured by mobility shifts on SDS/PAGE, which accompanies phosphorylation of these proteins. Platelets were incubated with LDL, and samples were withdrawn and centrifuged (30 s, 9000 g). Pellets were suspended in Laemmli sample buffer, sheared through a needle, heated (5 min, 100 °C) and stored at −20 °C until analysis [24]. The running buffer for electrophoresis of cPLA₂, was pH 8.3, and for ERK2, pH 8.8. After separation, proteins were transferred to PVDF membranes by electroblotting. cPLA₂ was detected using the mouse monoclonal antibody 4–4B–3C, and ERK2 using the rabbit polyclonal antibody C14. Phosphorylated and total p38MAPK were measured [24] using phosphorplas p38MAPK (Thr¹⁸⁷/Tyr¹⁸⁷) or anti-p38MAPK antibody for detection. For quantitative determinations, the density of the bands was analysed using ImageQuant software [24].

Analysis of [³H]arachidonic acid release

Platelet-rich plasma was labelled with 0.01 μM [³H]arachidonic acid for 60 min at 37 °C and incubated in the presence of 30 μM 5,8,11,14-eicosatetraynoic acid to prevent metabolism of released arachidonic acid [24]. Lipids were extracted according to Bligh and Dyer [25]. For separation of phospholipids, phosphatidic acid and free arachidonic acid, the mixture was chromatographed on Silica gel 60 at 22 °C. Spots were visualized with phosphomolybdic acid and the radioactivity measured. Release of arachidonic acid was expressed as percentage free [³H]arachidonic acid compared to total radioactivity.
Measurement of dense granule secretion

Platelet-rich plasma was incubated with 1 µM \(^{3}H\)S-hydroxytryptamine for 30 min at 37 °C followed by gel filtration. At different times, samples were collected in formaldehyde, centrifuged, and the supernatant was analysed for \(^{3}H\)S-hydroxytryptamine. Data were expressed as percentage of maximal secretion, as defined in [11].

Patients

Three unrelated patients with Glanzmann’s thrombo-asthenia (designated Patient 1, 2 and 3) were studied and have been described in [10]. The patients had a prolonged Simplex bleeding time (> 30 min; normal values < 8 min). Patient 1 suffered from a thrombocytopenia (72,000 platelets/µl), and showed a faint signal for \(\alpha_{IIa}\) and \(\beta_{3}\) after two-dimensional electrophoresis of platelet-membrane lysates with subsequent silverstaining, as well as in lysates of \(^{125}\)I-labelled platelets analysed by autoradiography. On FACS, the mean fluorescence of \(\beta_{3}\)-expressing cells was 0.3 % of that observed with normal platelets. Platelet fibrinogen was normal. Platelets from Patient 2 and Patient 3 were 0.7 and 0.2 % \(\alpha_{IIa}\beta_{3}\)-positive respectively.

Presentation of data

Data are expressed as means ± S.D. for the given number of observations \(n\), and were analysed with the Student’s \(t\) test for unpaired observations. Differences were considered significant at \(P < 0.05\).

RESULTS

LDL activates the small GTPase Rap1 in human platelets

Unstirred platelet suspensions were incubated with LDL (1.2 g/l) or \(\alpha\)-thrombin (0.1 units/ml) and GTP-bound Rap1 was precipitated with GST–RalGDS Rap-binding domain. Activation of Rap1 was observed 1–3 min after incubation with LDL (Figures 1A and 2B). This is relatively slow compared with \(\alpha\)-thrombin, which activated Rap1 after approx. 5 s, and induced full activation within 30 s [14]. Figure 1(B) shows the time course of Rap1 activation by LDL and \(\alpha\)-thrombin in platelets from five (LDL) or three (\(\alpha\)-thrombin) different subjects. Activation of Rap1 by LDL started after 1–5 min (depending on the donor), was maximal at 5 min, and remained high for 1 h. A similar activation was observed with LDL, prepared in the absence of thimerosal (Figures 1A and 2B). This is relatively slow compared with \(\alpha\)-thrombin, which activated Rap1 after approx. 5 s, and induced full activation within 30 s [14]. Figure 1(B) shows the time course of Rap1 activation by LDL and \(\alpha\)-thrombin in platelets from five (LDL) or three (\(\alpha\)-thrombin) different subjects. Activation of Rap1 by LDL started after 1–5 min (depending on the donor), was maximal at 5 min, and remained high for 1 h. A similar activation was observed with LDL, prepared in the absence of thimerosal.

Activation of Rap1 by \(\alpha\)-thrombin was faster, showing a maximal response after 30 s. Figure 2(A) illustrates the dose-response relationship of the LDL-induced Rap1 activation after 10 min. Rap1 was activated at 0.7 g/l or more, reaching a maximum at approx. 1.2 g/l LDL. The maximal Rap1 activation by LDL (1.2 g/l, 10 min) was 69 ± 4 % of the activation induced by \(\alpha\)-thrombin (0.1 units/ml, 1 min; \(n\) = 3), indicating that LDL is a slower and slightly weaker activator than \(\alpha\)-thrombin. LDL preparations might become slightly oxidized during prolonged storage, resulting in formation of LPA [26]. To investigate whether LPA contributed to the LDL-induced activation of Rap1, control experiments were carried out with LDL in the presence of \(\mathrm{N}\)-palmitoyl-\(\ell\)-serine-phosphoric acid, an inhibitor of the LPA-receptor, and with LPA in the absence of LDL. As shown in Figure 2(B), this inhibitor did not change the effect of LDL, and LPA was unable to activate Rap1.

To get a better insight into the nature of the activating properties of LDL, studies were focused on the protein moiety (mainly apo B100) and the lipid components. Figure 3(A) shows that modification of lysine residues in apo B100, which abolishes specific LDL-platelet binding [8], blocked Rap1 activation. This indicates that the binding of the apo B100 moiety to the platelet is a crucial step in LDL signalling to Rap1. As LDL may activate cells via oxidation [26], the effect of mildly oxidized and oxidized LDL on Rap1 activation was studied. Figure 3(B) shows that with an increasing degree of CuCl\(_{2}\)-induced oxidation, the ability of LDL to activate Rap1 decreases. Similar results were obtained using 2,2’-azobis(2-amidinopropane) dihydrochloride-oxidized LDL (results not shown). Taken together, these results suggest that both protein and lipid moieties play an important role in Rap1 activation by LDL, and that LDL-induced Rap1 activation is not due to oxidative modification of the LDL preparation.

Recently [10], we reported that LDL enhances platelet sensitivity to collagen via a mechanism which involves exposure of ligand-binding sites on integrin \(\alpha_{IIa}\beta_{3}\) (glycoprotein IIb-IIIa). To clarify the role of this complex in Rap1 activation, studies were repeated in platelets from patients with Glanzmann’s thrombo-asthenia which lack integrin \(\alpha_{IIa}\beta_{3}\), and are insensitive to LDL-induced sensitization [10]. The same activation was observed in platelets from healthy subjects (Figure 3C). Also, the presence of GRGDS, a peptide that mimicks part of the fibrinogen \(\alpha\)-chain and inhibits fibrinogen binding [27] as well as the effect of LDL.
on collagen-induced secretion [10], failed to change Rap1 activation by LDL (Table 1). Thus LDL-induced Rap1 activation occurred independently of integrin $\alpha_{IIb}\beta_3$.

**LDL-induced Rap1 activation is mediated by TxA2 formation**

Earlier work showed that activation of Rap1 by $\alpha$-thrombin depends critically on a rise in intracellular Ca$^{2+}$ [14]. LDL-induced Rap1 activation was almost completely abolished by the Ca$^{2+}$-scavenger BAPTA/AM, indicating a similar dependence on intracellular Ca$^{2+}$ (Table 1). Since LDL induces TxA2 formation in platelets [2,5,11], studies were performed in the presence of the cyclooxygenase inhibitor indomethacin and the thromboxane receptor antagonist SQ30741. Both inhibitors blocked Rap1 activation almost completely, suggesting that LDL signals towards Rap1 via formation of TxA2. Thromboxane formation is the result of arachidonate release from membrane phospholipids by cPLA2. Recent reports indicate that platelet cPLA2 is phosphorylated and activated by the p38 mitogen-activated protein kinase (p38$\text{MAPK}$). The role of this kinase in LDL-induced Rap1 activation was studied using the p38$\text{MAPK}$ inhibitor SB203580 [28,29]. SB203580 binds covalently, or within close proximity, to the ATP-binding site of p38$\text{MAPK}$ [30,31]. Preincubation of platelets with 10 $\mu$M inhibitor decreased the ability of LDL to activate Rap1 by approx. 70%. In contrast, $\alpha$-thrombin-induced Rap1-activation was not affected (results not shown). At 10 $\mu$M, SB203580 also inhibits kinases of the stress-activated protein kinase/c-Jun N-terminal kinases family [32]. The experiments were therefore repeated with 1 $\mu$M, which inhibits p38$\text{MAPK}$ by approx. 90% [28,29], and a similar inhibition was found compared with 10 $\mu$M (results not shown). Thus LDL activates cPLA2 via p38$\text{MAPK}$ and a second, unidentified pathway.

To compare the kinetics of cPLA2 and Rap1 activation by LDL in more detail, enzyme phosphorylation identified by mobility shift on PAGE was compared with enzyme activation assessed by release of arachidonate [33]. cPLA2 from resting platelets migrated as a single band reflecting the non-phosphorylated state (Figure 4A). LDL (1.2 g/l) induced a mobility shift starting at 1.5 min and leading to 60% phosphorylation after 10 min. cPLA2-phosphorylation was preceded by p38$\text{MAPK}$-activation, which was detectable as phosphorylation of the protein after 10 s incubation with 0.1–1.0 g/l LDL (Figure 4B). The activation of p38$\text{MAPK}$ was responsible for phosphorylation of cPLA2, since SB203580 inhibited the mobility shift completely (results not shown). These findings are in contrast with an earlier report for collagen-
Table 1  Effect of inhibitors on LDL-induced Rap1 activation

Unstirred platelet suspensions were incubated with LDL in the absence and presence of GRGDS (100 μM, 1 min), BAPTA/AM (30 μM, 30 min), indomethacin (30 μM, 30 min), SQ30741 (400 nM, 15 min) or SB203580 (10 μM, 15 min), before addition of LDL (1.2 g/l, 10 min). In each experiment, the band intensity after LDL-incubation without inhibitors was set 100%. Data were corrected for background intensities without LDL, and expressed as means ± S.D., n=4. *P<0.05 for the incubation with LDL.

<table>
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<tr>
<th>Inhibitor</th>
<th>Rap1 activation (% of control)</th>
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<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>GRGDS</td>
<td>92 ± 36</td>
</tr>
<tr>
<td>BAPTA/AM</td>
<td>8 ± 8</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10 ± 9*</td>
</tr>
<tr>
<td>SQ30741</td>
<td>10 ± 10*</td>
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<tr>
<td>SB203580</td>
<td>32 ± 15*</td>
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</table>

Figure 4  LDL induces phosphorylation of cPLA2

(A) Platelets were incubated with LDL (1.2 g/l) for the indicated time without stirring. Samples were then centrifuged and pellets were dissolved in Laemmli sample buffer. cPLA2 was identified by Western blotting with an antibody against cPLA2. (B) Bands from cPLA2 (●) and p38MAPK phosphorylation (□) analyses of LDL-stimulated platelets were scanned and quantified using ImageQuant software. Phosphorylation of cPLA2 was expressed as the intensity of the upper band compared to total cPLA2. Activation of p38MAPK was expressed as the percentage of LDL-induced phosphorylation after 10 min (1.2 g/l LDL, 37 °C, 100%). Analysis of p38MAPK blots with an antibody recognizing both phosphorylated and unphosphorylated protein showed that protein concentration was similar in all samples (results not shown). n=3; means ± S.D.

Table 2  Influence of the p38 MAPK inhibitor SB203580 on arachidonic acid release

Unstirred [3H]arachidonic acid-labelled platelets were stimulated with LDL (1.2 g/l, 10 min, 37 °C) or α-thrombin (1 unit/ml, 1 min, 37 °C) in the absence or presence of the p38MAPK inhibitor SB203580, without stirring. 5,8,11,14-Eicosatetraynoic acid was added to prevent metabolism of released arachidonic acid. The release of arachidonic acid is expressed as percentage of total radioactivity (left column) and also compared between suspensions treated without and with SB203580 (right column). Values were corrected for radioactivity in resting platelets (2.7 ± 0.3%). Data are means ± S.D., n=5. *P<0.05.

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>% Arachidonic acid release</th>
<th>% Of control</th>
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<tbody>
<tr>
<td>LDL</td>
<td>5.30 ± 1.77</td>
<td>100</td>
</tr>
<tr>
<td>LDL + SB203580</td>
<td>1.75 ± 1.09*</td>
<td>33 ± 21</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td>8.00 ± 1.32</td>
<td>100</td>
</tr>
<tr>
<td>α-Thrombin + SB203580</td>
<td>8.23 ± 1.84</td>
<td>103 ± 23</td>
</tr>
</tbody>
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Figure 5  TxA2-independent Rap1 activation by LDL

(A) Platelet suspensions without (○) and with (●, ▲) pretreatment with indomethacin (30 μM, 30 min) were stirred (900 rev./min) in the presence of 1.2 g/l LDL (○, ▲) or buffer (●) for 30 min, and at different time intervals GTP–Rap1 was analysed as indicated in Figure 1. (B) Indomethacin-treated platelet suspensions were stirred (900 rev./min) with different concentrations of LDL for 30 min and GTP–Rap1 was analysed. Concurrently, indomethacin-treated, [14C]5-hydroxytryptamine-labelled platelets were stirred (900 rev./min) with different concentrations of LDL for 4 h, and at different time intervals dense granule secretion was measured using [14C]5-hydroxytryptamine as a marker. LDL concentrations (g/l) and corresponding Rap1 activation (% of maximal, n=3; means ± S.D.) were 0 (○, ▲), 0.5 (▲, 24 ± 6%), 1.0 (●, ▲, 33 ± 9%), 1.5 (●, 41 ± 8%), and 2.0 (●, set at 100%). Data are representative for 2 similar experiments with equivalent results.
unidentified route, and that activation of cPLA₂ and formation of TXA₂ account for further Rap1 activation.

**TXA₂-independent Rap1 activation**

The observation that Rap1 activation fully depended on the formation of TXA₂ made it difficult to separate the roles of these molecules in LDL-induced sensitization. Earlier studies showed that the faster α-thrombin-induced secretion in LDL-treated platelets was only partly sensitive to inhibitors of TXA₂ formation or blockade of the TXA₂-receptor [2]. These conditions were therefore further explored in a search for a role for Rap1. When platelets were stirred, LDL induced a second, indomethacin-independent secretion of TXA₂ and TXA₂ formation, and further activation via the TXA₂-receptor. Thus it was not possible to separate a role of Rap1 from the well known activating properties of TXA₂. Earlier work had shown that the secretion induced by α-thrombin in stirred platelet suspensions was faster following preincubation with LDL, and that the effect of LDL could only be inhibited in part by indomethacin or the TXA₂-receptor antagonist, SQ30741 [2]. The present study illustrates that stirring also introduces a TXA₂-dependent Rap1 activation. This second pathway of Rap1 activation is followed by a slow secretion of dense granule contents. Studies with different LDL concentrations reveal that LDL activates the small GTPase Rap1 but not Ras.

**DISCUSSION**

The present study shows that an LDL concentration within the physiological range (0.26–1.23 g apo B100/l) activates the small GTPase Rap1, reaching maximal activation within 5 min. The activation of Rap1 by LDL probably involves both the protein and the lipid moieties of the particle. The role of apo B100 is illustrated by the loss of LDL activity after lysine-modification. The role of lipids is illustrated by the decrease in agonist activity after extensive lipid oxidation. The latter finding contrasts with results showing that mildly oxidized LDL, but not native LDL, initiates platelet shape change via formation of LPA, a product also found in atherosclerotic lesions [26].

In unstirred platelet suspensions, LDL triggered the formation of GTP–Rap1 entirely via activation of p38MAPK, cPLA₂, and TXA₂ formation, and further activation via the TXA₂-receptor. Thus it was not possible to separate a role of Rap1 from the well known activating properties of TXA₂. Earlier work had shown that the secretion induced by α-thrombin in stirred platelet suspensions was faster following preincubation with LDL, and that the effect of LDL could only be inhibited in part by indomethacin or the TXA₂-receptor antagonist, SQ30741 [2]. The present study illustrates that stirring also introduces a TXA₂-independent Rap1 activation. This second pathway of Rap1 activation is followed by a slow secretion of dense granule contents. Studies with different LDL concentrations reveal that formation of GTP–Rap1 and secretion show the same dose-response relationship, suggesting, but not proving, that active Rap1 might be an intermediate in this TXA₂-independent secretion.

A previous study revealed an important role for platelet integrin α₁β₃ in LDL-induced sensitization [10]. The sensitization was absent in normal platelets with blocked ligand-induced outside-in signalling, as well as in α₁β₃-deficient platelets. The present data show that Rap1 activation by LDL...
occurs normally in the absence of integrin signalling, indicating that it is not a downstream event of \(x_{int}\). Thus Rap1 may either function in pathways that control integrin activation or is independent of \(x_{int}\). Control studies show that LDL-induced fibrinogen binding, as detected in FACs studies [10], is completely abolished by indomethacin, and the role of Rap1 in integrin regulation therefore remains uncertain (results not shown).

Both the TxA\(_2\)-dependent and the TxA\(_2\)-independent Rap1 activation are inhibited by the Ca\(^{2+}\) chelator BAPTA/AM, and by agents that raise cAMP. There are several steps in the TxA\(_2\)-dependent route both upstream and downstream of the TxA\(_2\) receptor that involve Ca\(^{2+}\), e.g. cPLA\(_2\) and the initial activation of p38MAPK is extremely sensitive to cAMP [24]. The nature of the TxA\(_2\)-independent Rap1 activation remains uncertain but may involve p125 focal adhesion kinase, which is phosphorylated by LDL with little interference by indomethacin [38].

Platelet cPLA\(_2\) associates with the membrane via its Ca\(^{2+}\)-dependent lipid-binding domain located at the N-terminus in a Ca\(^{2+}\)-dependent manner [39], and is phosphorylated at Ser\(^{968}\) and Ser\(^{727}\) by various agonists [40]. This finding agrees with the strong phosphorylation of cPLA\(_2\) induced by LDL, which in turn depends on activation of p38MAPK. An inhibitor of this kinase (SB203580) strongly decreased cPLA\(_2\)-phosphorylation and arachidonic acid release, as well as Rap1 activation by LDL. Therefore in LDL-induced signal generation, phosphorylation and activation of PLA\(_2\) are coupled processes, leading to a release of approx. 6 \% of total \([^{3}H]\)arachidonic acid in 10 min. In contrast, \(\alpha\)-thrombin-induced (1 unit/ml) arachidonate release is rapid (approx. 8 \% in 1 min) and apparently independent of p38MAPK-mediated phosphorylation. The same insensitivity for p38MAPK inhibition is observed for \(\alpha\)-thrombin-induced Rap1 activation. Recent observations in PC12 cells suggest that activation of Rap1 may result in activation of ERK2 via B-raf [41].

Our results in LDL-stimulated platelets show that Rap1 activation is not accompanied by activation of ERK2. Apart from Rap1, the small GTPase Ral is also activated upon platelet stimulation with LDL. Activated Ral might play a role in cytoskeletal rearrangements; it has recently been shown that Ral can play a role in filopodia formation upstream of the filamin ABP280, a protein responsible for crosslinking of actin filaments [42]. Another putative effector protein of Ral, RLIP76, is a GTase-activating protein for CDC42, a member of the Rho/Rac family. CDC42 is responsible for rearrangement of the actin cytoskeleton leading to focal contact formation and filopodia extension. Ral might also contribute to regulation of phospholipase D. In v-Src transformed cells, phospholipase D activity was precipitated with Ral, an interaction that was disturbed after deletion of an N-terminal region of Ral. Overexpression of Ral potentiated phospholipase D-activity, whereas the expression of a dominant negative mutant of Ral was inhibitory [16]. The association of Ral with another small GTase, Arf, seems necessary to render Ral able to stimulate phospholipase D activity.

In fibroblasts, Ral activation critically depends on Ras [43]. However, Wolthuis et al. [12] recently reported the concurrent activation of Rap1 and Rap1, rather than Ras, in platelets stimulated by different agonists, suggesting that Ral might be a signalling molecule downstream of Rap1 in these cells. This also follows from our results showing that Rap1 and Rap1 are regulated by the same upstream signalling components activated by LDL. Indeed, Rap1 activates Ral in cotransfection experiments with the Ral exchange factor Rlf in Cos7 cells [44]. Therefore Rap1 may have taken over the function of Ras in platelets. In accordance with the absence of Ras activation by LDL, no activation of the downstream element of Ras signalling, ERK2, is observed, indicating that both Ras and ERK do not have a function in LDL-induced platelet sensitization. Ras is activated in platelets stimulated by high concentrations of \(\alpha\)-thrombin and TxA\(_2\) analogue. One would therefore expect a similar activation of Ras in LDL-treated platelets, since this leads to TxA\(_2\) formation and TxA\(_2\)-dependent Rap1 activation. The fact that this does not occur might be due to the relatively weak activating properties of LDL which, in contrast to \(\alpha\)-thrombin, requires 3 min or more before activation of signalling sequences can be identified.

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