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 unstirred 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 A_2 (TxA_2) receptor (SQ30741) induce complete inhibition, indicating that

Rap1 activation is the result of TxA_2 formation. Stirring reveals a second, TxA_2 -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 $\alpha_{IIb}\beta_3$. 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.

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], Ca^{2+} mobilization [3,9], protein kinase C [5,10] and $\alpha_{IIb}\beta_3$ -mediated outside-in signalling [10]. Concurrently, thromboxane A_2 (TxA_2) is formed, reflecting activation of cytosolic phospholipase A_2 (cPLA₂) [2,5,11]. Inhibition of this pathway reveals both TxA_2 -dependent and TxA_2 -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 TxA_2 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 Ca^{2+} [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, Ral and Rap1 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 TxA_2 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 A_2 ; ERK2, extracellular-signal-regulated protein kinase 2; LDL, low-density lipoprotein; LPA, 1-oleoyl-L- α -lysophosphatidic acid; MAPK, mitogen-activated protein kinase; TxA_2 , thromboxane A_2 .

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MATERIALS AND METHODS

Materials

Radiolabelled [5,6,8,9,11,12,14,15-³H]arachidonic acid (specific radioactivity 5.5–8.5 TBq/mmol) and 5-hydroxy [side chain-2-¹⁴C]tryptamine creatinine sulphate ([¹⁴C] 5-hydroxytryptamine; specific activity 1.85 GBq/mmol) were from Amersham International. Silicagel 60 was from Merck, Darmstadt, Germany. RalA 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 (p38^{MAPK}) 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-L- α -lysophosphatidic acid (LPA) were from Sigma. The inhibitor of the LPA receptor, *N*-palmitoyl-L-serine-phosphoric acid was from Biomol, Plymouth Meeting, PA, U.S.A. Iloprost was from Behring, Berlin, Germany; bisindolylmaleimide I was from Boehringer Mannheim; demineralized BSA was from Organon Teknika, Eppelheim, Germany; and bis-(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxy-methyl ester) (BAPTA/AM) was from Calbiochem (La Jolla, CA, U.S.A.). Fibrinogen-derived peptide GRGDS was kindly provided by Dr H. M. Verheij, department of Biochemistry, University of Utrecht. SQ30741 was from Bristol-Myers-Squibb (Maarssen, The Netherlands), and SB203580 was from Alexis Corporation (San Diego, CA, U.S.A.). The cDNA encoding Rap1B was a gift of Dr Veronique Pizon (Paris, France), and 2,2'-azobis(2-amidinopropane) dihydrochloride was a kind gift of Dr Wolfgang Sattler, Karl-Franzens University, Graz, Austria.

LDL isolation, lysine modification and oxidation

Fresh non-frozen plasma from four healthy subjects each containing less than 100 mg lipoprotein(a)/l was pooled and LDL (density range 1.019–1.063 kg/l) was isolated [10,19]. To prevent lipid modification and bacterial contamination, 0.25 mM PMSF, 0.2 mM thimerosal, 2 mM NaN₃ and 4 mM EDTA (final concentrations) were present during the first centrifugation. A control purification was carried out in the absence of thimerosal, which, if not fully removed, might contribute to the activating properties of LDL. Subsequent centrifugations were carried out in the absence of additives except for NaN₃ and EDTA; a final dialysis was done without these inhibitors. Further details have been described in [10,11]. The concentrations of LDL were expressed as g of apo B100 protein/l.

For modification of apo B100 lysines [20], LDL (20 g of apo B100/l) in 0.15 M NaCl, 0.01 % EDTA, was diluted with 0.3 M sodium borate buffer, pH 8.0 (2:1, v/v). Potassium cyanate (20 mg/mg of apo B100) was added and the mixture was incubated for 2 h at 35 °C. Excess reagent was removed by two dialysis steps against 0.15 M NaCl, 0.01 % EDTA (pH 7.0), for 12 h at 4 °C. LDL was oxidized with CuCl₂ [21] or 2,2'-azobis(2-amidinopropane) dihydrochloride [22]. Mildly oxidized LDL was prepared by incubation of 20 g/l of LDL with 100 μ M CuCl₂ or 10 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (2 h, 37 °C); oxidized LDL was prepared by incubation of the same amount of LDL with 750 μ M CuCl₂ or 25 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (16 h, 37 °C). Data for thiobarbituric acid-reactive substances for mildly oxidized LDL were 1.62 $\pm$ 0.25, for oxidized LDL 5.28 $\pm$ 3.34, and for native LDL 0.07 $\pm$ 0.04 nmol/mg of apo B100 respectively.

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 $\times$ 10¹¹ platelets/l. 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 RalGDS bound to GSH-agarose beads (Sigma). GTP-bound Ral was precipitated with GST-tagged Ral-binding domain of RLIP76 [12]. Activated Ras was precipitated from platelets lysed in Ral buffer with GST-tagged Ras-binding domain of Raf1 [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 *Sma*I and *Not*I. 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 p38^{MAPK}

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 p38^{MAPK} were measured [24] using phosphoplus p38^{MAPK} (Thr¹⁸⁰/Tyr¹⁸²) or anti-p38^{MAPK} antibody for detection. For quantitative determinations, the density of the bands was analysed using ImageQuant software [24].

Analysis of [³H]arachidonate release

Platelet-rich plasma was labelled with 0.01 μ M [³H]arachidonic acid for 60 min at 37 °C and incubated in the presence of 30 μ M 5,8,11,14 eicosatetraynoic acid to prevent metabolism of released arachidonate [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]-arachidonate compared to total radioactivity.

Measurement of dense granule secretion

Platelet-rich plasma was incubated with 1 μ M [14 C]5-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 [14 C]5-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 Simplate bleeding time (> 30 min; normal values < 8 min). Patient 1 suffered from a thrombocytopenia (72000 platelets/ μ l), and showed a faint signal for α_{IIb} and β_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 β_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 % $\alpha_{IIb}\beta_3$ -positive respectively.

Presentation of data

Data are expressed as means $\pm$ 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 α -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 α -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 α -thrombin in platelets from five (LDL) or three (α -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 α -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 α -thrombin (0.1 units/ml, 1 min; $n = 3$), indicating that LDL is a slower and slightly weaker activator than α -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 N -palmitoyl-L-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

Figure 1 Time course of LDL-induced Rap1 activation

(A) Platelets were incubated with α -thrombin (α -Thr, 0.1 units/ml) and LDL (1.2 g/l) for the indicated time periods without stirring. After lysis in Ral buffer, GTP-bound Rap1 was precipitated with GST-RBD, coupled to GSH-agarose beads, and identified by Western blotting using a polyclonal antibody against Rap1. The upper band on the immunoblot is caused by specific binding to the GSH-agarose beads. Data are representative of 5 similar experiments. (B) LDL- (●) and α -thrombin- (□) induced activation of Rap1. For each experiment, the band intensity at 10 min LDL-stimulation was set at 100% (○). $n \geq 3$; means $\pm$ S.D.; $P < 0.05$ for all LDL-incubations ≥ 2 min, and for all α -thrombin incubations ≥ 30 s compared to control. Data indicated by (Δ) reflect Rap1 activation by LDL prepared in the absence of thimerosal ($n = 1$).

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 $\alpha_{IIb}\beta_3$ (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 $\alpha_{IIb}\beta_3$ 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 α -chain and inhibits fibrinogen binding [27] as well as the effect of LDL

Figure 2 Dose-response relation for LDL-induced Rap1 activation

(A) Platelets were incubated with LDL at the indicated concentrations for 10 min without stirring (●). GTP-Rap1 was identified and band intensities were measured and expressed as percentage of the intensity at 2 g/l (○). $n = 3$; means $\pm$ S.D.; * $P < 0.05$ compared to control. (B) Control incubation for possible involvement of LPA in the LDL-induced activation of Rap1. Unstirred platelet suspensions were incubated without (Rest) and with 1.2 g/l LDL for 1 min and 30 min, and for 30 min with LPA in the indicated concentrations. Incubations with the inhibitor of the LPA-receptor *N*-palmitoyl-L-serine-phosphoric acid (5 min preincubation; [26]) are indicated by NP. Similar results were obtained in stirred suspensions (results not shown). Note that the blot is overexposed to reveal a possible weak activation by LPA.

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 TxA_2 formation

Earlier work showed that activation of Rap1 by α -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, illustrating a similar dependence on intracellular Ca^{2+} (Table 1). Since LDL induces TxA_2 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 TxA_2 . 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^{MAPK}). The role of this kinase in LDL-induced Rap1 activation was studied using the p38^{MAPK} inhibitor SB203580 [28,29]. SB203580 binds covalently, or within close proximity, to the ATP-binding site of p38^{MAPK} [30,31]. Preincubation of platelets with 10 μ M inhibitor decreased the ability of LDL to activate Rap1 by approx. 70%. In contrast, α -

Figure 3 Effect of LDL-modification and platelet $\alpha_{IIb}\beta_3$ -deficiency on Rap1 activation

(A) Platelets were stimulated with lysine-modified LDL or native LDL (1.2 g/l) for 10 min without stirring. (B) Platelets were incubated with 1.2 g/l LDL, mildly oxidized (mox) or oxidized (ox) LDL for 10 min. Shown here are the results of incubations with $CuCl_2$ -oxidized LDL. Experiments with 2,2'-azobis(2-amidinopropane) dihydrochloride-oxidized material gave similar results. (C) Platelets from a control and a Glanzmann patient deficient in integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb/IIIa) were incubated with vehicle or LDL (1.2 g/l). The data are from Patient 3 and are representative of the results from the two other patients

thrombin-induced Rap1-activation was not affected (results not shown). At 10 μ 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 μ M, which inhibits p38^{MAPK} by approx. 90% [28,29], and a similar inhibition was found compared with 10 μ M (results not shown). Thus LDL activates cPLA₂ via p38^{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^{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^{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 μ M, 1 min), BAPTA/AM (30 μ M, 30 min), indomethacin (30 μ M, 30 min), SQ30741 (400 nM, 15 min) or SB203580 (10 μ M, 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 $\pm$ S.D., $n \geq 4$. * $P < 0.05$ for the incubation with LDL.

Inhibitor	Rap1 activation (% of control)
None	100
GRGDS	92 $\pm$ 36
BAPTA/AM	8 $\pm$ 8*
Indomethacin	10 $\pm$ 9*
SQ30741	10 $\pm$ 10*
SB203580	32 $\pm$ 15*

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 p38^{MAPK} 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 $\pm$ 0.3%). Data are means $\pm$ S.D., $n = 5$. * $P < 0.05$.

Stimulator	% Arachidonic acid release	% Of control
LDL	5.30 $\pm$ 1.77	100
LDL + SB203580	1.75 $\pm$ 1.09*	33 $\pm$ 21
α -Thrombin	8.00 $\pm$ 1.32	100
α -Thrombin + SB203580	8.23 $\pm$ 1.84	103 $\pm$ 23

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 p38^{MAPK} 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 p38^{MAPK} was expressed as the percentage of LDL-induced phosphorylation after 10 min (1.2 g/l LDL, 37 °C, 100%). Analysis of p38^{MAPK} blots with an antibody recognizing both phosphorylated and unphosphorylated protein showed that protein concentration was similar in all samples (results not shown). $n \geq 3$; means $\pm$ S.D.

induced cPLA₂-phosphorylation [28]. Unstimulated platelets contained little free arachidonate, but incubation with LDL (1.2 g/l for 10 min) released approx. 5% of the incorporated [³H]arachidonic acid (Table 2). SB203580 inhibited this release by more than 60%. α -Thrombin-induced arachidonate release was insensitive to SB203580, in accordance with a previous report [34]. Hence these data indicate that in unstirred suspensions LDL signals to cPLA₂ via p38^{MAPK} and a second,

Figure 5 TxA₂-independent Rap1 activation by LDL

(A) Platelet suspensions without (○) and with (▲, ▽) pretreatment with indomethacin (30 μ M, 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 $\pm$ S.D.) were 0 (○, 3 $\pm$ 1%), 0.5 (▲, 24 $\pm$ 6%), 1.0 (▽, 33 $\pm$ 9%), 1.5 (◆, 41 $\pm$ 8%), and 2.0 (●, set at 100%). Data are representative for 2 similar experiments with equivalent results.

Figure 6 LDL activates the small GTPase Ral but not Ras or ERK2

(A) Platelets were incubated with α -thrombin (α -Thr, 1 unit/ml) and LDL (1.2 g/l) for the indicated time periods without stirring and lysed in Ral buffer. Activated Ras was precipitated with GST-tagged Raf1-Ras binding domain (GST-Raf1RBD) coupled to GSH-agarose beads, and identified by Western blotting using an antibody against Ras (the lower band on the immunoblot is caused by aspecific binding to the GSH-agarose beads). (B) Platelets were incubated with α -thrombin (α -Thr, 0.1 unit/ml) or LDL (1.2 g/l, 37 °C) for the indicated time periods without stirring, lysed in Ral buffer, and activated Ral was precipitated with GST-Raf1-binding domain of RLIP76 coupled to GSH-agarose beads, and identified by Western blotting using an antibody against RalA. (C) Platelets were incubated with α -thrombin (α -Thr, 1 unit/ml) and LDL (1.2 g/l, 37 °C) as indicated. Samples were centrifuged and pellets were dissolved in Laemmli sample buffer. ERK2 phosphorylation was measured as a mobility shift by Western blotting with a polyclonal antibody against ERK2.

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-insensitive formation of GTP-Rap1 (Figure 5A). This activation was unchanged by inhibitors of ligand binding to integrin $\alpha_{IIb}\beta_3$ ($95 \pm 7\%$ compared with control), the protein kinase C inhibitor bisindolylmaleimide ($110 \pm 8\%$), or the p38^{MAPK}

inhibitor SB203580 ($84 \pm 12\%$). The Ca²⁺-chelator BAPTA/AM ($16 \pm 4\%$) and iloprost, which raises cAMP ($6 \pm 2\%$; $n = 3$, $P < 0.05$), interfered with the formation of GTP-Rap1.

To investigate whether the TxA₂-independent Rap1 activation played a role in the secretion response, GTP-Rap1 and [¹⁴C]5-hydroxytryptamine secretion were compared in stirred, indomethacin-treated platelets (Figure 5B). Although 0.5 g/l already induced 50% Rap1 activation without affecting secretion, a stepwise further increase to 2 g/l was accompanied by a dose-dependent enhancement of 5-hydroxytryptamine release. This suggests that the formation of GTP-Rap1 might play a role in the slow release of dense granule contents induced during prolonged contact with LDL.

LDL activates the small GTPase Ral but not Ras

Apart from Rap1, platelets contain the small GTPases Ral and Ras [12]. Figure 6(A,B) illustrates that Ral is activated by LDL (1.2 g/l), reaching a maximum between 3 and 10 min. Formation of GTP-Ral showed the same sensitivity to indomethacin, SB203580 and SQ30741 as formation of GTP-Rap1 (results not shown). In contrast, no significant activation of Ras was observed in LDL-treated platelets. A downstream element of Ras signalling is the serine/threonine kinase ERK2 (p42^{MAPK}) [35]. Earlier studies have shown that phosphorylation of ERK2 is a reflection of enzyme activation, and leads to a mobility shift on SDS/PAGE [36]. Figure 6(C) shows that in contrast to α -thrombin (1 unit/ml, 1 min), LDL is unable to activate ERK2 in accordance with the absence of Ras activation.

DISCUSSION

The present study shows that an LDL concentration within the physiological range (0.26–1.23 g apo B100/l) [37] 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 p38^{MAPK}, 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 data illustrate 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 $\alpha_{IIb}\beta_3$ in LDL-induced sensitization [10]. The sensitization was absent in normal platelets with blocked ligand-induced outside-in signalling, as well as in $\alpha_{IIb}\beta_3$ -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 $\alpha_{IIb}\beta_3$. Thus Rap1 may either function in pathways that control integrin activation or is independent of $\alpha_{IIb}\beta_3$. 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 TxA₂-dependent and the TxA₂-independent Rap1 activation are inhibited by the Ca²⁺ chelator BAPTA/AM, and by agents that raise cAMP. There are several steps in the TxA₂-dependent route both upstream and downstream of the TxA₂ receptor that involve Ca²⁺, e.g. cPLA₂ and the initial activation of p38^{MAPK} is extremely sensitive to cAMP [24]. The nature of the TxA₂-independent Rap1 activation remains uncertain but may involve p125 focal adhesion kinase, which is phosphorylated by LDL with little interference by indomethacin [38].

Platelet cPLA₂ associates with the membrane via its Ca²⁺-dependent lipid-binding domain located at the N-terminus in a Ca²⁺-dependent manner [39], and is phosphorylated at Ser⁵⁰⁵ and Ser⁷²⁷ by various agonists [40]. This finding agrees with the strong phosphorylation of cPLA₂ induced by LDL, which in turn depends on activation of p38^{MAPK}. An inhibitor of this kinase (SB203580) strongly decreased cPLA₂-phosphorylation and arachidonic acid release, as well as Rap1 activation by LDL. Therefore in LDL-induced signal generation, phosphorylation and activation of PLA₂ are coupled processes, leading to a release of approx. 6% of total [³H]arachidonic acid in 10 min. In contrast, α -thrombin-induced (1 unit/ml) arachidonate release is rapid (approx. 8% in 1 min) and apparently independent of p38^{MAPK}-mediated phosphorylation. The same insensitivity for p38^{MAPK} inhibition is observed for α -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 GTPase-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 GTPase, 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 Ral 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 Ral 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 α -thrombin and TxA₂ analogue. One would therefore expect a similar activation of Ras in LDL-treated platelets, since this leads to TxA₂ formation and TxA₂-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 α -thrombin, requires 3 min or more before activation of signalling sequences can be identified.

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