PECAM-1 inhibits LDL-induced signaling in platelets

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Submitted
Downregulation of LDL signaling

**Summary**

At physiological concentrations, Low Density Lipoprotein (LDL) increases the sensitivity of platelets to aggregation- and secretion inducing agents without acting as an independent activator of platelet functions. LDL sensitizes platelets by inducing a transient activation of p38\(^{MAPK}\), a Ser/Thr kinase which is activated by the simultaneous phosphorylation of Thr\(^{180}\) and Tyr\(^{182}\) and which is an upstream regulator of cytosolic phospholipase A\(_2\) (cPLA\(_2\)). A similar, transient phosphorylation of p38\(^{MAPK}\) is induced by a peptide mimicking amino acids 3359-3369 in apoB100 called the B-site. Here we report that the transient nature of the p38\(^{MAPK}\) activation is caused by PECAM-1, a receptor with an immunoreceptor tyrosine-based inhibitory motif. PECAM-1 activation by cross-linking induces tyrosine phosphorylation of PECAM-1 and a fall in phosphorylated p38\(^{MAPK}\) and cPLA\(_2\). Interestingly, also LDL and B-site peptide induce tyrosine phosphorylation of PECAM-1 and studies with immunoprecipitates indicate the involvement of c-Src. Inhibition of Ser/Thr-phosphatases PP1/PP2A (okadaic acid) makes the transient p38\(^{MAPK}\) activation by LDL and B-site peptide persistent. Inhibition of Tyr-phosphatases (vanadate) increases Tyr-phosphorylated PECAM-1 and blocks the activation of p38\(^{MAPK}\).

Together these findings suggest that following a first phase in which LDL, through its B-site, phosphorylates and thereby activates p38\(^{MAPK}\), a second phase is initiated in which LDL activates PECAM-1 and induces dephosphorylation of p38\(^{MAPK}\) via activation of the Ser/Thr phosphatases PP1/PP2A.
Introduction

The contact between platelets and Low Density Lipoprotein (LDL) particles is known to enhance their responsiveness to aggregation- and secretion-inducing agents. Part of this sensitization is mediated via activation of p38MAPK and cytosolic phospholipase A2 (cPLA2), which together with a second stimulus leads to more liberation of arachidonic acid and formation of thromboxane A2. A second mechanism involved in sensitization is the activation of p125 Focal Adhesion Kinase (FAK) with still poorly characterized effects on the formation of focal adhesions and cytoskeletal rearrangements. Activation of p38MAPK by LDL is rapid (within 10 seconds at 1 g/L LDL) and occurs at LDL concentrations within the physiological range (0.6 – 1.0 g/L LDL), suggesting that it may affect platelets in the circulation during diet-induced changes in lipoprotein profile. P38MAPK is a member of the family of proline directed serine/threonine kinases, which is activated by the simultaneous phosphorylation of Thr180 and Tyr182.

LDL-induced p38MAPK activation is insensitive to many inhibitors of signal transduction in platelets including the Ca2+ chelator BAPTA, suggesting that it is an early step in the activation cascade initiated by LDL. An exception is an increase in cAMP, which is a potent inhibitor of p38MAPK activation.

The receptor through which LDL initiates p38MAPK and FAK activation in platelets has not yet been characterized but is probably not identical to the classical apoB/E receptor that mediates LDL uptake in fibroblast and smooth muscle cells as an antibody directed against the ligand binding domain of the classical LDL-receptor did not alter the binding of LDL to platelets. Furthermore, LDL binding to platelets existed in platelets from patients with familial hypercholesterolemia, who lack the apoB/E receptor.

We have recently identified an activating domain in LDL that induces p38MAPK activation in platelets. The major apolipoprotein of LDL is apoB100, a 4563 amino acid long protein that is wrapped around the lipid particle and possesses a recognition site for the apoB/E receptor in the so called B-site. A B-site peptide mimetic consisting of 11 amino-acids with a strong positive charge induced a rapid phosphorylation of p38MAPK reaching a peak value after 30 seconds and returning to pre-stimulation values 5 to 10 minutes later. The extent of p38MAPK activation was in the range found with 1 g/L LDL and also the sensitivity to inhibitors of signaling steps resembled that of LDL. This is an important observation since variations between donors, a long isolation procedure and specifically its susceptibility to oxidative modification have led to conflicting interpretations with respect to the platelet activating properties of LDL.

Since platelet sensitization by LDL might be one of the factors that contribute to the development of thrombo-atherosclerotic disease, its downregulation is equally important as it prevents persistent platelet sensitization. At present, there is little insight in this mechanism.
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The presence of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is of specific interest since its cytoplasmic tail contains a so called immunoreceptor tyrosine-based inhibitory motif (ITIM), which is a characteristic of receptors that mediate inhibitory signals. The ITIM family comprises several members such as PECAM-1, FcgammaRIIb, signal regulating protein, CD22 and killer inhibitory receptor of which only PECAM-1 is present on human platelets. PECAM-1 is a 130 kDa transmembrane glycoprotein of the immuno-globulin superfamily of cell adhesion molecules. Its 574 amino acid extracellular domain is organized into six Ig-like homology domains. There is a single transmembrane domain and a 118 amino acid cytoplasmic tail. The ITIM motif is characterized by the consensus sequence L/I/V/S-x-Y-x-x-L/V. Expression of PECAM-1 is restricted to hematopoietic and vascular cell types such as platelets, monocytes, neutrophils and endothelial cells. The functions of PECAM-1 are diverse and include roles in angiogenesis, vasculogenesis, integrin regulation and transendothelial migration of leukocytes. Activation of PECAM-1 is accompanied by phosphorylation of the cytoplasmic part of the molecule. The cytoplasmic tail of PECAM-1 contains 12 serine, 4 threonine and 5 tyrosine residues. Which of these residues are phosphorylated depends on the type of agonist. Both resting and TRAP-stimulated platelets show predominantly serine phosphorylation of PECAM-1 provided that the cells do not aggregate. When suspensions are stirred and aggregates are formed PECAM-1 becomes tyrosine phosphorylated. Contact of platelets with collagen leads to strong PECAM-1 phosphorylation on tyrosine residues. A second means to activate PECAM-1 in platelets is by cross-linking with the specific antibody PECAM-1.3, which results in tyrosine phosphorylation of the cytoplasmic tail. Upon receptor activation, the cytoplasmic ITIM-motif recruits and activates Src homology 2 (SH2) domain containing protein-tyrosine phosphatases such as SHP-1 and SHP-2. Both SHP-1 and SHP-2 are essential components in PECAM-1 mediated generation of inhibitory signals and their recruitment and activation depends on phosphorylation of tyrosine residues on the PECAM-1 cytoplasmic tail. Earlier studies have shown that p38MAPK activation by the B-site in LDL is maximal after 1-2 minutes and followed by a gradual decline to pre-activation values. In the present study we investigated whether PECAM-1 took part in the downregulation of LDL-induced p38MAPK activation. Furthermore, we determined whether the downregulation of p38MAPK activation was mediated by the action of phosphatases.

Materials and Methods

Antibodies and Reagents
The monoclonal antibody PECAM 1.3 and the polyclonal antibody SEW, both directed against PECAM-1, were kindly provided by Prof. P. J. Newman (The Blood Center of Southeastern Wisconsin, Milwaukee). Anti-mouse F(ab')2 fragments were from Southern biotechnology Associates, Inc (Birmingham, USA).
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The goat polyclonal anti-PECAM-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody IV.3 was purified from hybridoma cell culture medium. Antiphosphotyrosine mAb 4G10 was from Upstate Biotechnology (Bucks, UK). The antibody directed against serine-P was kindly provided by Dr. B.M.T. Burgering (Laboratory for Physiological Chemistry, University Medical Center Utrecht, The Netherlands). MoAb directed against cPLA₂ (4-4B-3C) and a polyclonal antibody directed against c-Src were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against p38MAPK and dual phosphorylated p38 MAPK (phosphoplus p38MAPK) and horseradish peroxidase labeled anti-rabbit IgG were from New England Biolabs (Beverly, USA). Renaissance chemiluminescence Western-blot reagent was from NEN-Dupont, Boston, MA, USA. Non-fat dry milk was obtained from Nutricia (Zoetermeer, the Netherlands). PP1 was obtained from Alexis Biochemicals (San Diego, CA, USA). Okadaic acid was obtained from Calbiochem (San Diego, CA, USA); vanadate from Sigma (St. Louis, MO, USA). Human α-thrombin was purchased from Kordia Life Science (Leiden, The Netherlands).

The peptide RLTRKRGLKLA (Mw = 1311 Da), designated B-site peptide, represents the apoB receptor-binding domain (Arg 3359- Ala 3369) of apoB100. The peptide was synthesized by standard solid-phase peptide synthesis and purified by C18 reverse-phase chromatography (HPLC, Genosphere biotechnologies, Paris, France). The purity of the peptide was >99% as determined by HPLC and the molecular weight was verified by matrix-assisted laser desorption mass spectrometry by the manufacturer.

Lipoprotein Isolation

Lipoproteins were isolated as described previously [27]. In short, fresh, non-frozen plasma from 4 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 by sequential flotation in a Beckman L-70 ultracentrifuge. Centrifugations (20 hr, 17,5000 g, 10°C) were carried out in the presence of NaN₃ and EDTA. The LDL preparations contained only minimal amounts TBARS (0.20 ± 0.07 nmol/mg), lipid peroxides (6.7 ± 1.9 nmol/mg) and contaminating plasma proteins (below or within reported values for native LDL). Lp(a) concentrations, determined with the use of a specific antibody (Apotech, Organon Technika, Rockville, U.S.A), were below 14 mg/L. Lipoproteins were stored at 4°C under nitrogen for not longer than 14 days and before each experiment dialyzed overnight against 10⁴ volumes 150 mmol/L NaCl. ApoB100 and lipoprotein(a) concentrations were measured using the Behring Nephelometer 100. The concentration of LDL was expressed as g apoB100 protein/L.

Platelet Isolation

Freshly drawn venous blood from healthy volunteers was collected with informed consent into 1:10 v/v 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication during two weeks prior to blood collection. Platelet-rich plasma was prepared by centrifugation (200 g, 15 minutes, 20°C). Gel-filtered platelets (GFP) were isolated by gel filtration through Sepharose 2B equilibrated in Ca²⁺-free Tyrode’s solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 1.7 mmol/L MgCl₂, and 11.9 mmol/L NaHCO₃, pH 7.25) containing 0.2 % BSA and 5 mmol/L glucose. GFP were adjusted to a final count of 2 × 10¹¹ platelets/L and incubated with LDL and other agonists with and without stirring (900 rev/min) at 37°C as indicated in “Results”.

Measurement of p38MAPK and cPLA₂

GFP were incubated at 37°C with LDL, B-site peptide or thrombin as indicated. After incubation, 100 mL aliquots were mixed (1:10 v/v) with cold lysis buffer (RIPA buffer containing 10% protease inhibitor cocktail, 5 mM NaVO₃) and subsequently taken up in Laemmli sample buffer. Samples were heated prior to SDS-polyacrylamide gel electrophoresis (12%). Proteins were electrophoretically transferred (1 hr, 100 volts) to a nitrocellulose membrane using a mini-protean system (Biorad, Richmond, CA, USA). The blots were blocked in 5% non-fat dry milk, 0.1% Tween 20 in phosphate buffered saline (1 hr, 4°C) and incubated with the phosphopubs p38MAPK (Thr180/Tyr182) or p38MAPK antibody (1:2000 in 1% non-fat dry milk, 0.1% Tween in PBS, 16 hr, 4°C). Both antibodies are raised against residues 171-186 of human p38MAPK. After washing, the membranes were incubated with horseradish peroxidase labeled anti-rabbit (1:2000, 1 hr, 4°C) and p38MAPK was visualized using the enhanced chemiluminescence reaction.
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For semi-quantitative determination of the amount of dual phosphorylated or total p38MAPK, the density of the bands was analyzed using ImageQuant software (Molecular Dynamics). For the measurement of cPLA₂ phosphorylation, samples were withdrawn and collected in Laemmili sample buffer. Measurement of cPLA₂ was based on the mobility shift on SDS-PAGE that accompanies phosphorylation of the protein. The running buffer for electrophoresis of cPLA₂ was pH 8.3. cPLA₂ was detected using the moAb 4-4B-3C. Immune complexes were detected by enhanced chemiluminescence.

PECAM-1 tyrosine phosphorylation
Platelets were incubated with LDL, B-site peptide or thrombin as indicated (37 °C). After incubation, 500 µl aliquots were mixed with cold lysis buffer (1:10 v/v) as described above and precipitated with goat polyclonal anti-PECAM-1 (1 µg) and protein G-Sepharose for 3 hr (4°C). After washing with lysis buffer, samples were taken up in Laemmlli sample buffer. Samples were heated (5 min, 100°C) prior to the western blotting procedure. The blots were blocked in 5% BSA, 0.05% Tween 20 in triss-buffered saline (TBS) (1 hr, 4°C) and incubated with monoclonal 4G10 antibody (1:2000 in 1% BSA, 0.05% Tween in TBS, 16 hr, 4°C) or polyclonal SEW antibody (1:2000 in 1% BSA, 0.05% Tween in TBS, 16 hr, 4°C). After washing, the membranes were incubated with peroxidase linked anti-mouse (1:5000, 1 hr, 4°C) or anti-rabbit IgG (1:10000, 1 hr, 4°C) and phosphorylation was visualized using the enhanced chemiluminescence reaction.

Statistics
Data are expressed as means ± SD with number of observations n.

Results

PECAM-1 inhibits LDL-induced phosphorylation of p38MAPK
As reported previously, B-site peptide (100 µmol/L) induced a rapid phosphorylation of p38MAPK reaching a peak value after 30 seconds and which returned to pre-stimulation values after 5 - 10 minutes (Figure 1). Thus, following a first phosphorylation step, phosphatases were activated that brought p38MAPK back to the range found in resting platelets. The same rapid phosphorylation of p38MAPK was induced by LDL (1 g/L), reaching a maximum within 1 minute and decreasing to basal levels after 20 minutes or more. This dephosphorylation of p38MAPK is distinctly faster than observed with LDL preparations isolated in the presence of the anti-bacterial agent thimerosal, which was common use in earlier studies.

![Figure 1. P38MAPK activation by LDL and B-site peptide](image)

Platelets were incubated with LDL (1 g/L, 37°C, closed symbol) or B-site peptide (100 µmol/L, 37°C, open symbol). Dual phosphorylated p38MAPK was measured by SDS-PAGE and Western blotting using a phosphospecific anti-p38MAPK polyclonal antibody. The blots were semi-quantified and the data were expressed as percentage of the p38MAPK phosphorylation after 1 min (100%, open symbol). Data are expressed as means ± SD, n = 4.
Thus, following an initial phase in which p38MAPK was phosphorylated, both LDL and a peptide mimetic of the B-site of apoB100 induced dephosphorylation of p38MAPK, bringing the enzyme back to the state found in resting platelets.

To investigate whether PECAM-1 played a role in the dephosphorylation of p38MAPK, the receptor was activated with antibody PECAM-1.3 that is specific for the ectodomain of PECAM-1. Incubation of platelets with this antibody (1 µg/mL) for 10 min resulted in tyrosine phosphorylation of PECAM-1, illustrating that the receptor was activated (Figure 2A). No further activation of PECAM-1 was found by cross-linking PECAM-1.3 antibody with anti-mouse Fab-fragments.
When platelets were first treated with the PECAM-1.3 antibody for 10 minutes and thereafter stimulated with LDL (1 g/L) for 1 minute, phosphorylation p38\(_{\text{MAPK}}\) was reduced to 70% compared with platelets that were not treated with PECAM-1.3 antibody (Figure 2B).

A further cross-linking of PECAM-1 resulted in a reduction in phosphorylation of p38\(_{\text{MAPK}}\) to 40%. A similar inhibition by PECAM was observed at the level of cPLA\(_2\), which is a downstream target of p38\(_{\text{MAPK}}\) in LDL-induced signaling 6.

To assess a possible involvement of the FcgammaRIIa-receptor mediated pathway in the anti-PECAM-1.3 induced activation of PECAM-1 and concomitant de-phosphorylation of p38\(_{\text{MAPK}}\), studies were repeated in the presence of antibody IV.3, an inhibitor of FcgammaRIIa (data not shown). The inhibition by PECAM-1 activation was unchanged. This observation, together with literature data that show that p38\(_{\text{MAPK}}\) is activated by FcgammaRIIa activation rather than inhibited, illustrate that the inhibition by PECAM of LDL-induced p38\(_{\text{MAPK}}\) phosphorylation is independent of FcgammaRIIa.

**LDL activates PECAM-1**

The observation that PECAM inhibited the LDL-induced phosphorylation of p38\(_{\text{MAPK}}\) raised the question whether LDL itself was capable of activating PECAM-1 thereby downregulating the initial activation of p38\(_{\text{MAPK}}\) activating pathways. Platelets were incubated with LDL and the phosphorylation of PECAM-1 was visualized on western blots after immunoprecipitation of the receptor (Figure 3A). As a control, platelets were stimulated with thrombin (0.5 U/mL, 2 minutes, 37°C) with and without stirring. Stirring resulted in an enhanced tyrosine phosphorylation of PECAM-1 whereas serine phosphorylation was equal under both conditions such in agreement with earlier observations 23. Western blots with an appropriate antibody revealed that LDL induced tyrosine phosphorylation of PECAM-1 but was incapable of inducing phosphorylation of serine residues. The LDL (1 g/L) -induced tyrosine phosphorylation of PECAM-1 was transient, showing a maximum after 1 minute stimulation and downregulation after prolonged incubation (Figure 3B). Also the B-site peptide induced tyrosine phosphorylation of PECAM-1 which is in line with the concept that the B-site mediates the activation of PECAM-1 by LDL. After immunoprecipitation of PECAM-1 an additional band at approximately 125 kDa was co-precipitated (Figure 3B). Reprobing of the blots with the polyclonal anti-PECAM-1 (SEW) revealed that the upper band represents PECAM-1.

The inhibitor of Src kinases, PP1 (10 \(\mu\)mol/L), abrogated tyrosine phosphorylation of PECAM-1 suggesting a role for Src kinases (Figure 3C). Indeed, upon LDL stimulation, a 60 kDa band co-precipitated with PECAM-1 upon LDL stimulation. After reprobing with a specific antibody this band could be identified as c-Src, indicating that a complex was formed between c-Src and PECAM-1 upon stimulation by LDL (Figure 3D).
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Downregulation of LDL-signaling by PECAM-1 depends on serine/threonine phosphatases

PECAM-1 is known to initiate inhibitory signaling pathways by recruitment of the tyrosine phosphatases SHP-1 and SHP-2. As p38MAPK is activated upon phosphorylation of both threonine and tyrosine residues, the involvement of serine/threonine phosphatases as well as tyrosine phosphatases was investigated.

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Figure 3. LDL induces tyrosine phosphorylation of PECAM-1

Platelets were incubated with LDL (1 g/L, 37°C) or thrombin without stirring (Thrombin -, 0.5 U/mL, 2 min, 37°C) or thrombin under stirred conditions (Thrombin +, 0.5 U/mL, 900 rev. per min., 2 min, 37°C). PECAM-1 was immunoprecipitated from platelet lysates and applied to gel. With the use of antibody 4G10 tyrosine phosphorylation (A, left) and with the use of anti-Ser antibody serine phosphorylation (A, right) of PECAM-1 was visualized. The lower panel shows similar samples in which PECAM-1 was detected with a polyclonal antibody against this receptor as a control for equal lane loading. (B) Platelets were incubated with LDL (1 g/L, 37°C), B-site peptide (100 µmol/L, 37°C) for the indicated time-periods or with thrombin under stirred conditions (Thrombin +, 900 rev. per min., 2 min, 37°C) and the tyrosine phosphorylation of PECAM-1 was analyzed. (C) Platelets were incubated with the Src family tyrosine kinases inhibitor PP1 (10 µmol/L, 15 min) prior to incubation with LDL (1 g/L, 5 min, 37°C) and PECAM-1 tyrosine phosphorylation was detected. (D) Complex formation between PECAM-1 and Src family tyrosine kinases was measured by incubating platelets with LDL (1 g/L, 37°C) for the indicated time-periods or with thrombin (1 U/mL, 2 min, 37°C, 900 rev.p.m.) followed by immunoprecipitation with polyclonalAb against PECAM-1 and western blotting with an antibody against c-Src. The 60 kDa band was identified as c-Src. As a control for equal lane loading blots were reprobed with a monoclonal antibody against total PECAM-1 (lower panel).
To investigate the contribution of serine/threonine phosphatases, platelets were incubated for 0.5 minute with 1 µmol/L okadaic acid, a concentration known to inhibit the serine/threonine phosphatases PP1 and PP2A. This treatment resulted in a strong potentiation of p38\textsuperscript{MAPK} activation by LDL (1 g/L) and B-site peptide (100 µmol/L) and changed the transient activation into a more sustained activation compared with platelets stimulated with LDL in the absence of the inhibitor (Figure 4A). We next investigated whether inhibition of tyrosine phosphatases with vanadate interfered with the phosphorylation states of p38\textsuperscript{MAPK}. Platelets were preincubated for 30 minutes with 100 µmol/L sodium vanadate. This treatment completely abolished the phosphorylation of p38\textsuperscript{MAPK} by LDL and B-site peptide (Figure 4B). Furthermore, the tyrosine phosphorylation of PECAM-1 induced by LDL and B-site peptide was preserved by vanadate treatment and even increased to levels above the range found in the absence of the inhibitor (Figure 4C). Thus, inhibition of tyrosine phosphatases augmented the Tyr-phosphorylation of PECAM-1 induced by LDL and B-site peptide thereby potentiating the dephosphorylation of p38\textsuperscript{MAPK} to such an extent that any rise in phosphorylated p38\textsuperscript{MAPK} was prevented. This finding strengthens the role of PECAM-1 in downregulation of p38\textsuperscript{MAPK}.

Discussion

ApoB100 is the main protein constituent of LDL and consists of 4563 amino acids, wrapped around the lipid particle. The B-site is the domain in apoB100 that binds to the apoB/E receptors on cells that remove plasma cholesterol from the circulation. A peptide that mimics this region between Arg 3359 and Ala 3369 of apoB100 induced a transient activation of platelet p38\textsuperscript{MAPK} showing a maximal phosphorylation after 1 minute stimulation. Also LDL induced a rapid and transient phosphorylation of p38\textsuperscript{MAPK} with approximately similar kinetics as observed with the B-site peptide. It is possible that this downregulation at prolonged incubation times might reflect a protection mechanism that prevents extensive platelet activation in the circulation by LDL.

Here we report that the downregulation of phosphorylated p38\textsuperscript{MAPK} is mediated by PECAM-1. PECAM-1 activation by a specific receptor cross-linking antibody led to Tyr-phosphorylation of PECAM-1 and p38\textsuperscript{MAPK} dephosphorylation. A second cross-linking Fab fragment in addition to PECAM-1 antibody did not induce much more PECAM-1 tyrosine phosphorylation although literature data suggest that Fab fragments are required for optimal PECAM-1 activation. Activation of PECAM-1 with the PECAM-1.3 antibody resulted in a decrease in LDL-induced phosphorylation of p38\textsuperscript{MAPK} and a further activation of PECAM with additional Fab fragments led to a further reduction in the phosphorylation of the enzyme. These observations make PECAM-1 an important inhibitor of LDL-induced p38\textsuperscript{MAPK} phosphorylation.
P38MAPK is activated by dual phosphorylation of p38MAPK of Thr^{180} and Tyr^{182} and the inhibition by PECAM-1 is therefore likely to impair further signaling to downstream effectors. Indeed, activation of PECAM-1 also inhibits the phosphorylation of cPLA₂, which is a key step in the mobilization of arachidonic acid and further formation of thromboxane A₂. This makes PECAM-1 a key factor in the control of LDL-induced platelet sensitization.

There is little insight in the mechanisms by which PECAM-1 is activated under physiological conditions. The activation of PECAM-1 could be the result of agonists present in the circulation that induce a protection mechanism against cardiovascular...
complications. Platelet activation through the collagen receptor glycoprotein VI results in PECAM-1 tyrosine phosphorylation which may function to negatively limit growth of platelet thrombi on collagen surfaces. Also, platelet activation through thrombin receptors leads to PECAM-1 activation. Activated non-aggregated platelets exhibit serine phosphorylation of PECAM-1 in contrast to aggregated platelets which show tyrosine phosphorylated PECAM-1. The present experiments suggest a role for LDL as an agonist for PECAM-1-mediated platelet inhibition. Incubation of platelets with LDL or B-site peptide induced tyrosine phosphorylation of PECAM-1 with an optimum after 1 minute followed by a downregulation after prolonged incubation. Neither LDL nor B-site peptide induced phosphorylation of serines on PECAM-1, such in contrast to PECAM-1 stimulation by thrombin. Our data reveal that also in non-aggregated platelets PECAM-1 can be tyrosine phosphorylated since LDL is not an aggregation-inducing agent.

Upon incubation of platelets with LDL, a band with a slightly lower molecular weight compared to PECAM-1 co-precipitates with this receptor. To date, the nature of the lower molecular weight band is not known, but a possible candidate is ApoER2, a 130 kDa protein which might function as a platelet LDL receptor.

Okadaic acid is an inhibitor of the serine/threonine phosphatases PP1 and PP2A. When platelets were preincubated with this inhibitor, the transient phosphorylation of p38MAPK by LDL and B-site peptide changed into a persistant activation and reached levels far above the maximal phosphorylation observed in untreated platelets. A recent study on p38MAPK phosphorylation by collagen revealed that PP2A mediates the dephosphorylation and a similar role might be present in platelets stimulated by LDL. P38MAPK is activated by dual phosphorylation on tyrosine and threonine residues by dual specific MAPK-Kinases 3 en 6 (MKK3/6). MKK6 is activated by phosphorylation on Ser151 and Thr155 by an upstream enzyme termed MKK kinase. Thus, the dephosphorylation of p38MAPK by PP1/PP2A might be the result of a direct effect of these phosphatases on p38MAPK but an indirect effect caused at the level of upstream kinases is equally feasible.

Vanadate is an inhibitor of tyrosine phosphatases. When platelets were preincubated with this inhibitor, the transient Tyr-phosphorylation of PECAM-1 was increased and more sustained enabling the receptor to transmit a stronger inhibitory signal into the cell. Apparently, vanadate did not increase the phosphorylated state of p38MAPK although the enzyme is phosphorylated on threonine and tyrosine residues. Instead, vanadate treatment completely abolished the increase of phosphorylated p38MAPK by LDL and B-site peptide. This illustrates the potent inhibitory action of PECAM-1 which through its strong activation of PP1/PP2A abolished the accumulation of phosphorylated p38MAPK. The results found in vanadate-treated platelets support the concept that PECAM-1 is a negative regulator of LDL-induced signaling in platelets. At the same time this property makes it difficult to study whether tyrosine phosphatases took part in the dephosphorylation of p38MAPK.
Signal transduction by PECAM-1 depends on the ITIM motif which becomes tyrosine phosphorylated. Since PECAM-1 does not appear to be autophosphorylated, a kinase is thought to phosphorylate the cytoplasmic tyrosine residue. A candidate responsible for the tyrosine phosphorylation is a member of the Src family tyrosine kinase. Immunoprecipitation studies after incubation of platelets with LDL showed co-precipitation of c-Src with PECAM-1. P38MAPK activation was not affected by the okadaic acid treatment until platelets had been stimulated with LDL for 1 minute or more. This is in accordance with the time-dependent activation of PECAM-1 by LDL and recruitment of c-Src. This suggests that c-Src recruitment to the cytoplasmic tail of PECAM-1 induces ITIM-tyrosine phosphorylation and subsequent recruitment of PP2A.

In conclusion, the results reported in this study are best explained by assuming a model in which LDL affects platelet signaling mechanisms in two phases. During a first, initial phase LDL binds via its B-site in apoB100 to the putative LDL receptor on platelets thereby inducing the dual phosphorylation of p38MAPK and activating the enzyme. This step leads to activation of cPLA2 and formation of thromboxane A2. This mechanism explains why aspirin or related drugs abolish at least in part the sensitization of platelets by LDL. This activation phase is followed by a second phase in which LDL, either directly or bound in a complex with the LDL receptor, activates PECAM-1. This results in recruitment of c-Src and phosphatases PP1/PP2A thereby downregulating the phosphorylation of p38MAPK.

Acknowledgements

JWNA is supported by the Netherlands Thrombosis Foundation.
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References


