Binding of Low Density Lipoprotein to Platelet Apolipoprotein E Receptor 2′ Results in Phosphorylation of p38MAPK *

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Binding of low density lipoprotein (LDL) to platelets enhances platelet responsiveness to various aggregation-inducing agents. However, the identity of the platelet surface receptor for LDL is unknown. We have previously reported that binding of the LDL component apolipoprotein B100 to platelets induces rapid phosphorylation of p38 mitogen-activated protein kinase (p38MAPK). Here, we show that LDL-dependent activation of this kinase is inhibited by receptor-associated protein (RAP), an inhibitor of members of the LDL receptor family. Confocal microscopy revealed a high degree of co-localization of LDL and a splice variant of the LDL receptor family member apolipoprotein E receptor-2 (apoER2) at the platelet surface, suggesting that apoER2′ may contribute to LDL-induced platelet signaling. Indeed, LDL was unable to induce p38MAPK activation in platelets of apoER2-deficient mice. Furthermore, LDL bound efficiently to soluble apoER2′, and the transient LDL-induced activation of p38MAPK was mimicked by an anti-apoER2 antibody. Association of LDL to platelets resulted in tyrosine phosphorylation of apoER2′, a process that was inhibited in the presence of PP1, an inhibitor of Src-like tyrosine kinases. Moreover, phosphorylated but not native apoER2′ co-precipitated with the Src-family member Fgr. This suggests that exposure of platelets to LDL induces association of apoER2′ to Fgr, a kinase that is able to activate p38MAPK. In conclusion, our data indicate that apoER2′ contributes to LDL-dependent sensitization of platelets.

Platelets and low density lipoproteins (LDL)¹ are key elements in the development of atherothrombotic complications.

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¹ The abbreviations used are: LDL, low density lipoprotein; apoB100, apolipoprotein B100; apoER2, apolipoprotein E receptor-2; BSA, bovine serum albumin; FAK, p125 focal adhesion kinase; GFP, gel-filtered platelets; GST, glutathione S-transferase; LDL-R, LDL receptor; LRP1*

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The interplay between both elements is apparent from the notion that LDL particles markedly enhance the responsiveness of platelets to various aggregation-inducing agents (1–4). These agonists mediate the release of growth factors, vasoactive substances, and chemotactic agents that are known to stimulate atherosclerotic plaque formation. Sensitization of platelets by LDL involves the major LDL constituent apolipoprotein B100 (apoB100) (5), a 4563 amino acid protein that is wrapped around the LDL particle (6). LDL particles are recognized by the classical hepatic LDL receptor (LDL-R) through the apoB100 moiety, and in particular through a region within the apoB100 protein that is enriched in positively charged amino acids, the so-called B-site (7). Like LDL, a synthetic peptide mimicking this B-site associates to the platelet surface (5). Moreover, this peptide interferes with binding of LDL to platelets (5), suggesting that both elements share similar binding sites. This possibility is supported by the observation that binding of either LDL or the B-site peptide to the platelet results in a near immediate activation of the intracellular enzyme p38 mitogen-activated protein kinase (p38MAPK) (5, 8). Activation of this Ser/Thr kinase is associated with downstream phosphorylation and activation of cytosolic phospholipase A₂, which leads to the formation of thromboxane A₂ (9, 10). Finally, enhanced platelet function occurs via exposure of the integrin α₁β₃ and fibrinogen binding (11).

The mechanism by which LDL particles signal to p38MAPK is yet unclear, but it seems conceivable that it involves a receptor-dependent pathway. The finding that the signaling pathway is initiated by the apoB100 component of LDL may point to the involvement of an LDL-binding receptor. However, platelets are known to lack the classical LDL-R as well as LDL receptor-related protein-1 (LRP1) (12, 13). Recently, a splice variant of apolipoprotein E receptor-2 (apoER2) has been identified in platelets and megakaryocytic cell lines (14). ApoER2, which is also known as LDL receptor-related protein-8, is a member of the LDL receptor family, and is mainly expressed in brain, testes, and vascular cells but not in the liver (15, 16). Its structure is most closely related to the LDL-R and VLDL-R (17). However, transcriptional analysis has revealed that multiple alternative splicing variants of apoER2 exist (18, 19), one

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of which, apoER2, is present in platelets (14). Platelet apoER2 mRNA encodes a 130-kDa protein, which consists of a single ligand-binding domain that comprises four complement type A repeats (compared with seven repeats in full-length apoER2), an epidermal growth factor-like homology region, an O-linked sugar domain, and a single transmembrane domain that connects the extracellular region to the cytoplasmic tail.

Several studies have shown the ability of apoER2 to bind and internalize apoE-containing lipid vesicles (14, 15). However, the contribution of apoER2 to the general lipid metabolism is probably limited, since mice genetically deficient for apoER2 do not suffer from increased plasma lipoprotein levels (20). Because also the endocytosis rate of apoER2 is almost 20-fold lower compared with LRP1 (21), it seems conceivable that apoER2 serves an alternative physiological function as well. Indeed, there is firm support for a role of apoER2 in cellular signaling processes. For example, apoER2 has been reported to contribute to the neuronal signaling pathway that governs the layering of the developing cortex (20). In this process, apoER2 functions as a receptor for the signaling molecule reelin, and transmits the reelin signal to the intracellular adaptor protein disabled-1, which associates to the cytoplasmic tail of apoER2 (20, 22, 23, 24). This tail contains an xSNPXy sequence (where ψ is a hydrophobic amino acid residue), which may function as a potential binding site for phosphotyrosine binding domains of signaling molecules, including disabled-1. In addition, the cytoplasmic region of apoER2 contains three proline-rich areas that correspond to the consensus sequence (PXXP) for Src homology-3 recognition (14). All of these motifs that potentially link apoER2 to various signaling pathways are also present in the platelet variant of this receptor (14).

In the present study, we investigated whether apoER2 has the potential to transmit the LDL signal to intracellular signaling components, with particular reference to p38MAPK. We found that LDL bound efficiently to soluble apoER2 and that an anti-apoER2 antibody was similar to LDL particles in activating p38MAPK. Furthermore, the absence of apoER2 in platelets was associated with a lack of LDL-dependent p38MAPK activation, while inhibition of ligand binding to apoER2 by the 39 kDa receptor-associated protein (RAP) interfered with LDL-dependent phosphorylation of p38MAPK. Finally, incubation of platelets with LDL particles resulted in a rapid tyrosine phosphorylation of p38MAPK. Finally, incubation of platelets with LDL particles resulted in a rapid tyrosine phosphorylation of p38MAPK. This process was mediated by Src-like kinases and allowed the association with the signaling molecule Fgr. In view of our data, we propose that apoER2 serves a critical role in the LDL-initiated signaling pathway that governs platelets with increased sensitivity toward its aggregation-inducing agents.

**EXPERIMENTAL PROCEDURES**

**Mice—**Wild-type C57Bl/6 mice and mice genetically deficient for the LDL receptor (LDL-R (−/−), CD36 (CD36 (−/−)), scavenger receptor BI (SR-BI (−/−)), and scavenger receptor A (SR-A (−/−)) were used in this study. C57Bl/6 mice were obtained from Charles River (Maastricht, The Netherlands). Mice genetically deficient for apoER2 have been described previously (20). Homozygous LDL-R (−/−) mice, originally generated by Ishibashi et al. (25), were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred at the Garleaus Laboratories (Leiden, The Netherlands). CD36 (−/−) mice were kindly provided by Dr. M. Febbraio (Department of Medicine, Weill Medical College of Cornell University, New York) (26). SR-BI (−/−) mice were kindly provided by Dr. M. Krieger (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA) (27). apoER2-expressing mice were obtained by Dr. T. Kodama (Department of Molecular Biology and Medicine, University of Tokyo, Tokyo, Japan) (28). All mice were backcrossed at least 4 generations to the C57Bl/6 background. Mice had unlimited access to water and regular chow diet, containing 4.3% (w/w) fat with no added cholesterol (RM, Special Diet Services, Witham, UK). All experimental protocols were approved by the local ethics committees for animal experiments.

**Materials—**Nonfat dry milk was obtained from Nutricia (Zoetermeer, the Netherlands). Bovine serum albumin (BSA), a monoclonal antibody against α-actinin, protease inhibitor mixture, phenol-diazose-conjugated anti-goat IgG, and sodium vanadate (NaVO₃) were obtained from Sigma. Protamex (PGL) was from Cayman Chemical (Ann Arbor, MI). Protein G-Sepharose was obtained from Amersham Biosciences. PP1 was from Alexis Biochemicals (San Diego, CA). Renaissance chemiluminescence Western blot reagent was from PerkinElmer Life Sciences. Polyclonal antibodies against p38MAPK and dual phosphorylated p38MAPK (phosphoryl p38MAPK), and horseradish peroxidase-labeled anti-rabbit IgG were from New England Biolabs (Beverly, MA). An antibody against the ligand binding domain of apoER2 (anti-apoER2 antibody 186) has been described previously (24). A polyclonal antibody directed against c-Fgr, a polyclonal antibody directed against Src kinases, and a goat polyclonal antibody directed against the ectodomain of apoER2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LDL-R monoclonal antibody was obtained from Oncogene research Products (Boston, MA). Anti-CD36 antibody 4G10 was from Upstate Biotechnology (Bucks, UK). The anti-human apoB100 monoclonal antibody 2D8 used for confocal microscopy was obtained from the University of Ottawa Heart Institute (Ontario, Canada), and has been described previously (29). The anti-apoB100 antibody used in the solid phase assay was from BioPacific (Emeryville, CA). Fibrinogen (human) was purchased from HyClone Laboratories, Logan, UT. The peptide RL-TRKRGLKLA (M, 1311) designated B-site peptide, represents the reelin domain within 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 peptides was >99% as determined by HPLC, and the molecular weights were verified by matrix-assisted laser desorption mass spectrometry by the manufacturer. Preparation of recombinant murine soluble apoER2 (s-apoER2) fused to mannose-binding protein was performed as described (30).

**Lipoprotein Isolation—**Lipoproteins were isolated as described before (11). In short, fresh, non-frozen plasma from 3 healthy subjects each containing less than 100 mg of lipoprotein(a)/liter was pooled, and LDL (density range 1.019–1.063 g/ml) was isolated by sequential flotation in a Beckman L-80 ultracentrifuge (33). Centrifugations (175,000 g, 20 h, 30 °C) were carried out in the presence of NaC225, and the quality of these preparations has been described (11). Lipoproteins were stored at 4 °C under nitrogen for not longer than 14 days and before each experiment dialyzed overnight against 10⁶ volumes 150 mmol/liter NaCl. apoB100 and lipoprotein(a) concentrations were measured using theBehring Nephelometer 100. The concentration of LDL was expressed as grams of apoB100 protein/liter.

**Isolation of Platelet Lysates—**Platelet lysates were prepared from volunteers who were collected with informed consent into 0.1 volume 130 mmol/liter trisodium citrate. The donors claimed not to have taken any medication during 2 weeks prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation (200 × g, 15 min, 22 °C). Gel-filtered platelets (GFPs) were isolated by gel filtration through Sepharose 2B equilibrated in Ca²⁺-free Tyrode's solution (137 mmol/liter NaCl, 2.88 mmol/liter KCl, 0.42 mmol/liter NaHCO3, 1.7 mmol/liter MgCl2, and 11.9 mmol/liter NaHCO3, pH 7.25) containing 0.2% BSA and 5 mmol/liter glucose. GFPs were adjusted to a final count of 2 × 10¹¹ platelets/liter.

For the isolation of murine platelets, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/ml), ketamine (40 mg/ml), and atropine (0.05 mg/ml), and blood was subsequently collected into 0.1 volume of 130 mmol/liter trisodium citrate and 0.1 volume of ACD buffer (2.5 g of trisodium citrate, 1.5 g of citric acid, and 2 g of Na-glucose in 100 ml of distilled water) by heart puncture. PRP was obtained by centrifugation (87 × g, 15 min, 20 °C). The remainder of the blood was diluted with Hapes-Tyrode buffer (145 mmol/liter NaCl, 5 mmol/liter KCl, 0.5 mmol/liter NaHPO4, 1 mmol/liter MgSO4, 10 mmol/liter Hepes, 5 mmol/liter NaHCO3, 0.5 mmol/liter Na2-ACD buffer and centrifuged again (87 × g, 15 min, 20 °C). PRP samples were pooled, and platelets were isolated from PRP by centrifugation (350 × g, 15 min, 20 °C) in the presence of 0.1 volume of ACD buffer and resuspended in Hapes-Tyrode buffer (pH 8.5). PG12 was added to a final concentration of 10 ng/ml, and the washing procedure was repeated once. The platelet pellet was resuspended in Hapes-Tyrode buffer (pH 7.2). Platelet count was adjusted to 1 × 10¹¹ platelets/liter.
ApoER2 Mediates LDL Platelet Binding

p38MAPK Assay—Human GFPs or washed murine platelets were incubated at 37 °C with LDL, anti-apoER2 antibody 186 or anti-LDL-R antibody as indicated. After incubation, 100-μl aliquots were mixed with 1:16 dilution of cold wash buffer consisting of 10% (v/v) nonfat P-40, 5% (v/v) octylglucoside, 50 mmol/liter EDTA, 1% (w/v) SDS supplemented with 5 mmol/liter NaVO₃ and 10% (v/v) protease inhibitor mixture and subsequently taken up in Laemmli sample buffer. Samples were heated prior to SDS-polyacrylamide gel electrophoresis (12%). Proteins were electrophoretically transferred (1 h, 100 volts) to a nitrocellulose membrane using a mini-protein system (Bio-Rad). The blots were blocked in 5% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) (1 h, 4 °C) and incubated with the phosphoprotein (p38MAPK) (recognizing p38MAPK phosphorylated at Thr¹⁸⁰ and Tyr¹⁸²) or p38MAPK antibody, which recognizes both phosphorylated and non-phosphorylated isoforms (1:2000 (v/v) in 1% (w/v) nonfat dry milk, 0.1% (v/v) Tween (in PBS, 16 h, 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 (v/v), 1 h, 4 °C) and p38MAPK was visualized using the enhanced chemiluminescence reaction. 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).

Solid Phase Binding Assay—Microwell plates were incubated with Tris-buffered saline (TBS) 2 mmol/liter CaCl₂ containing 0.1 g/ml s-apoER2 (16 h, 4 °C). After blocking (1 h, 22 °C) in blocking solution (2% BSA in TBS, 2 mmol/liter CaCl₂, 0.05% (v/v) Tween) (in vitro phosphorylation of p70S6K with 10 μg/ml polyclonal antibody as indicated. After incubation, 100-μl aliquots were mixed with 1:16 dilution of cold wash buffer consisting of 10% (v/v) nonfat P-40, 5% (v/v) octylglucoside, 50 mmol/liter EDTA, 1% (w/v) SDS supplemented with 5 mmol/liter NaVO₃ and 10% (v/v) protease inhibitor mixture and subsequently taken up in Laemmli sample buffer. Samples were heated prior to SDS-polyacrylamide gel electrophoresis (12%). Proteins were electrophoretically transferred (1 h, 100 volts) to a nitrocellulose membrane using a mini-protein system (Bio-Rad). The blots were blocked in 5% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) (1 h, 4 °C) and incubated with the phosphoprotein (p38MAPK) (recognizing p38MAPK phosphorylated at Thr¹⁸⁰ and Tyr¹⁸²) or p38MAPK antibody, which recognizes both phosphorylated and non-phosphorylated isoforms (1:2000 (v/v) in 1% (w/v) nonfat dry milk, 0.1% (v/v) Tween (in PBS, 16 h, 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 (v/v), 1 h, 4 °C) and p38MAPK was visualized using the enhanced chemiluminescence reaction. 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).

Surface Plasmon Resonance Analysis—Surface plasmon resonance (SPR) experiments were performed using a BiaCore 3000 system. A biotinylated peptide corresponding to the apoB100 (residues 171–186 of human p38MAPK) was immobilized at a density of 239 fmol/mm². A control channel was prepared by immobilization of a biotinylated irrelevant monoclonal antibody. Binding of s-apoER2 was corrected for binding to this control channel (less than 2%). SPR analysis was performed in 150 mmol/liter NaCl, 2.5 mmol/liter CaCl₂, 0.05% (v/v) Tween-20, 25 mmol/liter HEPES (pH 7.4) at 25 °C with a flow rate of 5 μl/min. Regeneration of the sensor chip surface was performed by incubating with 10 mmol/liter taurodeoxycholic acid, 100 mmol/liter Tris (pH 9.0) for 2 min. Data were analyzed as described previously (34).

ApoER2 Tyrosine Phosphorylation—Human GFPs were incubated at 37 °C with LDL or B-site peptide as indicated, mixed with ice-cold wash buffer consisting of 10% (v/v) BSA and 0.1% (v/v) glycine (pH 7.4), and incubated with the monoclonal anti-apoB100 2D8 (5) and the goat polyclonal anti-apoER2 antibody. Afterward, the coverslips were incubated with a tetramethylrhodamine B isothiocyanate (TRITC)-labeled anti-goat antibody, diluted 1:40,000 in blocking solution. All incubations were carried out at room temperature for 1 h. For the color reaction, a solution of 0.1 mol/liter sodium acetate, pH 6.0 containing 0.1 mg/ml of 3,3′,5,5′-tetramethylbenzidine and 10 mmol/liter H₂O₂ was used. The reaction was stopped after 5 min by the addition of 0.3 mol/liter H₂SO₄ and bound secondary antibody was photometrically quantified at 450 nm.

RESULTS

LDL-dependent Activation of p38MAPK in the Absence of LDL-R, CD36, SR-BI, or SR-A—LDL-particles are recognized by the classical hepatic LDL-R through the apoB100 moiety (7). The scavenger receptors SR-BI and CD36 also bind LDL, in contrast to SR-A that only binds LDL after modification (35). To investigate the contribution of these receptors to LDL-dependent platelet sensitization, platelets were obtained from wild-type C57Bl/6 mice and mice that were genetically deficient for one of the following receptors: LDL-R, CD36, SR-BI, or SR-A. These platelets were incubated with freshly purified LDL (1 g/liter) for 5 min, and activation of p38MAPK was subsequently assessed by comparing the binding of antibodies directed against dual-phosphorylated p38MAPK to the binding of antibodies recognizing total p38MAPK. In the absence of LDL, low levels of phosphorylated p38MAPK relative to the total amount of the kinase were present in platelets from wild-type as well as the various knockout mice (Fig. 1). As expected, a substantial increase in phosphorylation was observed upon incubation of wild-type platelets with LDL. Moreover, an increase in phosphorylation of p38MAPK was also observed when platelets obtained from the various knockout mice were incubated with LDL (Fig. 1). Thus, at least in mice, LDL-mediated p38MAPK activation is a process that may occur independently of LDL-R, CD36, SR-BI, and SR-A—ligand binding to members of the LDL receptor family is blocked in the presence of RAP. To study whether LDL-induced platelet signaling involves a RAP-sensitive receptor, LDL-dependent phosphorylation of p38MAPK in human platelets was determined in the absence and presence of GST-RAP (0.15–0.45 μmol/liter). As shown in Fig. 2A, similar amounts of p38MAPK were present in all samples, and the kinase was efficiently phosphorylated upon incubation of the human platelets with LDL (1 g/liter for 1 min). The presence of GST-RAP, however, was associated with a dose-dependent decrease in p38MAPK phosphorylation, with over 70% inhibition at 0.3 μmol/liter GST-RAP. As shown in Fig. 2B, GST alone (0.45 μmol/liter) failed to affect LDL-induced p38MAPK phosphorylation (Fig. 2B, inset).
indicates that LDL-dependent activation of \( p38^{\text{MAPK}} \) is mediated by a RAP-sensitive receptor, only one of which has been reported to be present in platelets: apoER2. As such, apoER2 is a candidate receptor for LDL particles at the platelet surface. To test the contribution of this receptor, \( p38^{\text{MAPK}} \) activation was monitored using platelets obtained from apoER2-deficient mice. As shown in Fig. 2C, LDL was unable to induce the phosphorylation of \( p38^{\text{MAPK}} \) in the absence of apoER2. Thus, these data show that apoER2 is an essential link in the process of LDL-dependent \( p38^{\text{MAPK}} \) activation in platelets.

**ApoER2 and LDL Co-localization on the Platelet Surface**—As apoER2 is indispensable for LDL-dependent phosphorylation of \( p38^{\text{MAPK}} \), we further examined whether LDL particles indeed are able to interact with this receptor. Therefore, we first analyzed the location of apoER2 and of platelet-bound LDL employing confocal immunofluorescence microscopy analysis. Platelets were incubated with LDL (1 g/liter) in the presence or absence of GST-RAP (0.3 \( \mu \text{mol/liter} \)). By using antibodies directed against apoER2 and the LDL constituent apoB100, it was found that both proteins were selectively present at the platelet surface (Fig. 3). Merging of both images suggested a high degree of co-localization of apoB100 and apoER2, indicating that LDL may bind to or in close proximity of apoER2. Furthermore, in the presence of GST-RAP only minor amounts of apoB100 were detectable at the platelet surface, demonstrating that GST-RAP interferes with LDL binding to the platelet surface.

**Interaction between s-ApoER2' and LDL**—Since LDL and ApoER2' appear to co-localize at the platelet surface, direct binding studies were performed to address the interaction between both components. First, various concentrations of LDL (0–10 \( \mu \text{g/ml} \)) were incubated with immobilized murine ApoER2' (1 \( \mu \text{g/well} \)), and bound LDL was detected employing an anti-apoB100 directed antibody. A dose-dependent and saturable binding isotherm was observed, with half-maximal binding at 0.28 \( \mu \text{g/ml} \) LDL (Fig. 4A). Little, if any, binding was observed in the presence of EDTA. In a complementary approach, the interaction between this receptor and the LDL constituent apoB100 was examined by SPR analysis. To this end, various concentrations of murine s-apoER2' (50–300 nmol/liter) were perfused over a biotinylated peptide corresponding to the B-site of apoB100 immobilized on a streptavidin sensorchip (239 fmol/mm²). Association of soluble apoER2'
to the peptide was found to be dose-dependent as the highest response was observed for the highest concentration (Fig. 4B), whereas binding to the control channel was less than 2% compared with the peptide-coated channel (not shown). Analysis of the sensorgrams revealed that the association and dissociation curves were best fitted to a model describing the interaction with a single class of binding sites. The apparent dissociation and association rate constants were calculated to be $1.21 \pm 0.12 \times 10^{-3}$ s$^{-1}$ and $2.96 \pm 0.33 \times 10^{4}$ M$^{-1}$ s$^{-1}$, resulting in an apparent affinity constant of 41 nmol/liter. Hence, the soluble form of apoER2 constitutes a binding site for LDL, and more specifically, the ligand-binding region of the LDL constituent apoB100.

**An Anti-ApoER2 Antibody and LDL Are Similar in Activation of p38MAPK**—To further assess the contribution of apoER2 to the activation of p38MAPK, human platelets were incubated with anti-apoER2 antibody 186 (1:1000 (v/v) (24)). This antibody has previously been reported to induce dimerization of ApoER2, and does not cross-react with other members of the LDL receptor family (24). As a control, p38MAPK phosphorylation was determined similarly after incubation of platelets with LDL (1 g/liter) or in the presence of an anti-LDL-R antibody at the indicated concentrations. As expected, the anti-LDL-R antibody was unable to induce p38MAPK activation (Fig. 5C), whereas the presence of LDL was associated with a rapid increase in the amount of phosphorylated p38MAPK with a maximum at 1 min (Fig. 5A). This rapid increase was followed by a gradual decline to baseline levels of phosphorylated p38MAPK in the following 10 min. A similar time-dependent pattern was detected when platelets were incubated with anti-apoER2 antibody 186, with maximal phosphorylation of p38MAPK observed after 1 min (Fig. 5B). This suggests that ligand binding to and/or dimerization of apoER2 is associated with a rapid activation of p38MAPK. 

**LDL-dependent Phosphorylation of Platelet ApoER2**—LDL receptor-related proteins have been implicated in various signaling events, and participation in signaling processes may be associated with intracellular phosphorylation of these receptors. We therefore addressed the possibility that association of LDL particles to the platelet surface results in phosphorylation of apoER2. Human platelets were incubated with LDL (1 g/liter), and at various time intervals apoER2 was withdrawn by immunoprecipitation employing a polyclonal anti-apoER2 antibody. Samples were analyzed for the presence of phosphorylated tyrosine residues within apoER2 using 4G10, an antibody that recognizes tyrosine-phosphorylated proteins. Gel-loading was monitored employing an antibody directed against α-actinin, a protein that co-precipitated in a nonspecific manner. In non-LDL exposed platelets minor amounts of tyrosine-phosphorylated apoER2 could be detected (Fig. 6A). However, incubation of platelets with LDL particles resulted in a marked increase in tyrosine phosphorylation of apoER2, a process that appeared to be maximal after 30 s. Prolonged incubations resulted in a gradual decay of phosphorylated apoER2, although after 20 min its levels were still above basal levels (Fig. 6A). Phosphorylation of apoER2 also proved to be dependent on the concentration of LDL (Fig. 6B). Since the synthetic peptide corresponding to the B-site of the LDL component apoB100 induces p38MAPK activation (5), we further tested whether this peptide induced tyrosine phosphorylation of platelet apoER2. Indeed, incubation of platelets with this peptide was associated with phosphorylation of apoER2 to a similar extent as compared with LDL (Fig. 6C). Previously, it has been suggested that the integrin αIIbβ3 serves as a receptor for LDL on platelets (36, 37). However, tyrosine phosphorylation of apoER2 by LDL remained unaffected in the presence of the integrin αIIbβ3 inhibitors, fibrogin γ-chain-derived dodecapeptide (γ400–411) and GPI-562 (Fig. 6D). Thus, both the synthetic apoB100 B-site peptide and native LDL particles induce reversible phosphorylation of the platelet surface receptor apoER2 in a manner that is independent of integrin αIIbβ3.

**Recruitment of Src Kinases upon LDL Binding to apoER2**—Several tyrosine kinases may mediate LDL-dependent tyrosine phosphorylation of apoER2: src kinase family (8, 38, 39). To determine whether src kinases are involved in the tyrosine phosphorylation of apoER2, platelets were incubated with human recombinant apoER2 (apoER2/Fc) and LDL. In the presence of 10 mmol/liter EDTA, bound LDL was detected with anti-apoB100 and peroxidase-conjugated anti-goat antibodies as described under “Experimental Procedures.” A$_{opt}$ represents optical density at 450 nm. B: various concentrations of soluble murine apoER2 (50–300 nmol/liter) were perfused over a biotinylated peptide corresponding to the B-site of apoB100, which was immobilized onto a streptavidin-sensor chip at a density of 239 fmmol/mm$^2$. Perfusion was allowed for 2 min in 150 mmol/liter NaCl, 2.5 mmol/liter CaCl$_2$, 0.005% (v/v) Tween-20, 25 mmol/liter Hepes (pH 7.4) at 25°C, after which dissociation was initiated by perfusion with buffer alone. Binding was corrected for binding to a control channel (biotinylated irrelevant monoclonal antibody), which was less than 2% of binding to the peptide-coated channel.
phosphorylation of the cytoplasmic tail of platelet ApoER2. It has been reported by others that phosphorylation of LRP1 in fibroblastic cells involves the Src family of tyrosine kinases (38). In addition, we have previously shown that the Src kinase inhibitor PP1 interferes with LDL-dependent phosphorylation of p38MAPK (5). Therefore, PP1 was used to examine the role of the Src kinase family in the phosphorylation of apoER2 by LDL. In the absence of LDL and PP1, low levels of phosphorylated apoER2 were present, whereas a marked increase was observed upon incubation with LDL (Fig. 7A). However, preincubation of platelets with PP1 (1, 5 and 10 μM/liter) reduced LDL-induced apoER2 tyrosine phosphorylation up to 80% (Fig. 7A). This strongly suggests that Src-like tyrosine kinases play a dominant role in the phosphorylation of the apoER2 cytoplasmic tail. To identify the Src-like kinase that contributes to this process, LDL-treated platelets were used for co-immunoprecipitation experiments employing a polyclonal anti-apoER2 antibody. In preliminary experiments, analysis of the precipitates with polyclonal antibodies that recognize multiple members of the Src family, including Fyn, c-Src, Yes, and Fgr, revealed a band migrating with an apparent molecular mass of 55 kDa (data not shown). As the molecular mass of Fgr corresponds to 55 kDa, the precipitates were re-examined employing an antibody specifically directed against Fgr. Minor association of Fgr to apoER2 could be detected in platelets that were not exposed to LDL (Fig. 7B). In contrast, incubation with LDL resulted in a transient association of Fgr to apoER2 with maximal co-precipitation of Fgr at 1–2 min. Thus, association of LDL with the platelet surface leads to association of apoER2 with the Src kinase Fgr.

FIG. 5. Anti-apoER2 antibody-induced phosphorylation of p38MAPK. Human platelets were incubated with LDL (1.0 g/liter, A), anti-apoER2 antibody 186 (1:1000; B) or an anti-LDL-R antibody (0.1–10 μg/ml, C) at 37 °C for the indicated time periods. p38MAPK phosphorylation was determined as described in the legend to Fig. 1. The graphs show the semi-quantification of dual-phosphorylated p38MAPK from the blots. Data were expressed as percentage of the p38MAPK phosphorylation after 1 min of incubation (open symbol). Means ± S.E., n = 3.

DISCUSSION

Association between platelets and LDL particles is believed to result in the formation of hyperreactive platelets, enhancing the risk for thrombotic complications and atherosclerotic plaque formation. Several studies have been directed to the identification of LDL-induced signaling pathways that are responsible for the increased responsiveness of platelets to its various agonists (8, 39, 40). Although much insight has been gained in these signaling pathways, the identification of the platelet surface receptor(s) mediating LDL binding has remained inconclusive. Previously, the platelet integrin αIIbβ3 has been suggested to serve as a receptor for LDL, as inferred from ligand blotting and immunofluorescence studies (36, 37). However, specific antibodies directed against integrin αIIbβ3 proved unable to inhibit the binding of LDL to platelets. Furthermore, similar binding characteristics were found with platelets obtained from controls and Glanzmann’s thrombasthenia patients, who lack integrin αIIbβ3 (11). Thus, the involvement of αIIbβ3 in LDL binding at the platelet surface is inconclusive (11, 41), leaving the possibility that other receptors are involved in LDL-induced platelet signaling.

In order to identify the platelet receptor for LDL-particles, we focused on LDL-dependent activation of the Ser/Thr kinase p38MAPK (8). Phosphorylation of this kinase was not affected by the genetic deletion of various receptors (LDL-R, CD36, SR-BI, and SR-A) (Fig. 1). In contrast, phosphorylation of p38MAPK was almost completely inhibited in the presence of GST-RAP, pointing to the involvement of a
member of the LDL receptor family in LDL-dependent activation of p38MAPK (Fig. 2). This possibility is supported by our observations that (i) the absence of apoER2 in platelets of mice genetically deficient for this receptor resulted in a lack of LDL-dependent p38MAPK activation (Fig. 2C); (ii) the LDL-component apoB100 co-localized at the platelet surface with the platelet receptor apoER2' (Fig. 3A); (iii) GST-RAP interfered with binding of LDL to the platelet surface (Fig. 3B); (iv) soluble apoER2' interacted efficiently with LDL and the apoB100-derived B-site peptide (Fig. 4); (v) LDL particles and an anti-apoER2 antibody were similar in mediating p38MAPK activation (Fig. 5); and (vi) apoER2' was rapidly phosphoryl-

**Fig. 6. Tyrosine phosphorylation of apoER2' by LDL.** A, platelets were incubated with LDL (1 g/liter, 37 °C). At the indicated time points, apoER2' was immunoprecipitated from platelet lysates and tyrosine phosphorylation was detected by SDS-PAGE and Western blotting with 4G10, an antibody directed against tyrosine-phosphorylated proteins (upper lane). After stripping, the membranes were reprobed with a monoclonal antibody directed against α-actinin as a control for equal lane loading (lower panel). The graph shows the semi-quantification of tyrosine phosphorylation of apoER2' relative to the density of the bands representing α-actinin. Data were expressed as percentage of the density after 1 min of incubation with LDL (open symbol; means ± S.E., n = 3). B, platelets were incubated with the indicated concentrations of LDL (1 min, 37 °C), and tyrosine phosphorylation of apoER2' was monitored as described above. Data were expressed as percentage of the density after incubation with 1 g/liter LDL (open symbol, means ± S.E., n = 3). C, platelets were incubated with LDL (1 g/liter, 1 min, 37 °C) or B-site peptide (100 μmol/liter, 1 min, 37 °C). Tyrosine phosphorylation of apoER2' was determined as described above. D, platelets were incubated with the fibrinogen γ400–411 peptide (100 μmol/liter, 2 min) or the αIIbβ3 blocker GPI-562 (10 nmol/liter, 1 min) prior to incubation with LDL (1 g/liter, 1 min, 37 °C). ApoER2' tyrosine phosphorylation was detected as described. Data were expressed as percentage of apoER2' tyrosine phosphorylation in the absence of the blockers of ligand binding to integrin αIIbβ3. (Means ± S.E., n = 3).
ated upon the incubation of platelets with LDL particles or with a peptide corresponding to the B-site of the LDL constituent apoB100 (Fig. 6).

The finding that apoER2 serves as a receptor for apoB100-containing LDL particles may seem unexpected in view of the report by Kim et al. (16), who observed only minimal binding of 125I-labeled LDL and VLDL to CHO cells expressing full-length apoER2. In contrast, efficient binding of apoE-rich β-VLDL particles to immobilized purified s-apoER2 was observed. It should be mentioned, however, that in those experiments the lipoprotein concentrations did not exceed 5 mg/liter, which is far below the physiological LDL plasma concentration (normal range: 0.6 to 1 g/liter) that is used in our experiments. Of note, the binding isotherm of LDL particles to immobilized purified s-apoER2 indicates saturation of the receptor at LDL concentrations below 0.01 g/liter (Fig. 4A), while LDL concentrations of 0.1–0.3 g/liter are sufficient to induce p38MAPK activation (8). This suggests that platelet apoER2 would be saturated in normal humans, considering the physiological levels of 0.6–1 g/liter LDL.

Whereas binding of LDL to apoER2 appears to be mediated by the apoB100 moiety, binding of β-VLDL to apoER2 involves the apoE component of these lipoprotein particles. Interestingly, incubation of platelets with full-length apoE or synthetic peptides corresponding to the Arg/Lys-rich sequence of apoE resulted in a decrease in ADP-induced platelet aggregation, a process that could be inhibited by RAP (14). Apparently, apoB100 and apoE seem to have different upstream effects upon binding to apoER2 in that apoB100 governs platelets an increased response toward agonists, whereas apoE has the opposite effect. In this regard, the LDL component apoB100 acts in a similar fashion as dimeric β2-glycoprotein I, which was recently shown to enhance platelet deposition to collagen and thrombus size via interaction with apoER2 (42). Thus, different ligands may initiate different effects through the same receptor. This conclusion becomes even more intriguing given the observation that the effects of apoE, apoB100 and dimeric β2-glycoprotein I can be neutralized by GST-RAP (this study and Refs. 14 and 42). In principle, GST-RAP should be considered as a ligand for apoER2, as it shares its interactive region within the receptor with the other ligands. One possibility that may explain these different responses may be related to ligand-dependent association of apoER2 with other cell surface receptors. For instance, LDL-dependent activation of p38MAPK in platelets is followed by a de-activation step of this kinase that is mediated by platelet endothelial cell adhesion molecule-1 (PECAM-1) (43). The mechanism underlying the molecular cross talk between apoER2 and PECAM-1 remains to be elucidated, but the possibility exists that both receptors meet at the platelet surface in an LDL-dependent manner. It should be noted in this respect that in preliminary co-immunoprecipitation experiments no association between apoER2 and PECAM-1 could be observed upon incubation with LDL (data not shown).

Incubation of platelets with LDL resulted in the tyrosine phosphorylation of apoER2, a process that was inhibited in the presence of the Src kinase inhibitor PP1. This suggests that apoER2 phosphorylation is mainly dependent on kinases of the Src family (Fig. 7). ApoER2 phosphorylation coincided with association to the Src kinase Fgr (Fig. 7). Thus, ligand binding may facilitate the interaction with Fgr, which in turn might phosphorylate the receptor. Alternatively, the receptor may become phosphorylated upon ligand binding by a so far unidentified Src kinase, which subsequently results in association to the signaling molecule Fgr. This kinase has the potential to act as an activator of p38MAPK in neutrophils (44). It seems conceivable that Fgr may serve a similar role in platelets. Fgr also mediates the activation of p125 focal adhesion kinase (FAK) (39). Indeed, incubation of platelets with LDL particles results in a prompt activation of FAK (39). A similar pattern of FAK phosphorylation was observed upon incubation of platelets with anti-apoER2 antibody 186 (data not shown). Apparently, distinct pathways can be initiated upon ligation of apoER2.

In conclusion, our study strongly suggests that ApoER2 may act as a receptor for LDL at the platelet surface. Furthermore, we demonstrate that LDL binding to platelets results in phosphorylation of apoER2, and this step appears to be critical to activate further signaling cascades. Thus, apoER2 serves a so far unrecognized role in the pre-activation of platelets, and may therefore be used as a target for the development of therapeutic strategies aiming to reduce platelet hyperreactivity. Interestingly, a polymorphism within the apoER2 gene has recently been reported to be associated with the development of Alzheimer disease (45). It would be of interest to investigate whether this polymorphism or others predispose to thrombotic complications or display a protective effect.

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Binding of Low Density Lipoprotein to Platelet Apolipoprotein E Receptor 2′ Results in Phosphorylation of p38 MAPK

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