PLATELET ACTIVATION BY OXIDIZED LOW DENSITY LIPOPROTEIN IS MEDIATED BY CD36 AND SCAVENGER RECEPTOR A

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Methods

Mice

C57Bl/6 mice were obtained from Charles River (Maastricht, the Netherlands). CD36−/− mice were kindly provided by Dr. M. Febbraio (Department of Medicine, Weill Medical College of Cornell University, New York, USA),1 and Mex-4 SR-A−/− mice by Dr. T. Kodama (Department of Molecular Biology and Medicine, University of Tokyo, Tokyo, Japan).2 CD36 x SR-A double knockout mice were generated by cross-breeding of the CD36−/− animals with the Mex-4 SR-A−/− mice. 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 (RM3, Special Diet Services, Witham, UK). All experimental protocols were approved by the local ethics committees for animal experiments.

Materials

We obtained bovine serum albumin (acid free; BSA) from ICN Biomedicals (Aurora, Ohio, USA), chondroitinase ABC from Seikagaku America (Falmouth, MA, USA), Renaissance chemiluminescence Western blot reagent from PerkinElmer Life Sciences (Boston, MA, USA), fucoidan, protease inhibitor cocktail and sodium orthovanadate (NaVO₃) from Sigma (St.Louis, MO, USA), fibrinogen from Kordia (Leiden, the Netherlands), prostacyclin (PGI₂) from Cayman Chemical (Ann Arbor, MI, USA), and protein G-Sepharose from Amersham (Uppsala, Sweden). All other chemicals used were of analytical grade.

Antibodies and Proteins

We obtained FA6.152, a monoclonal antibody directed against the oxLDL binding domain of human CD36 (amino acids 155-183)3 from Immunotech (Marseille, France), polyclonal antibodies against dual phosphorylated p38MAPK (phosphoplus p38MAPK) and p38MAPK, and horseradish peroxidase-labeled anti-rabbit IgG from New England Biolabs (Beverly, USA), the goat polyclonal antibody directed against the ectodomain of ApoER2 (D-18) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the anti-
phosphotyrosine monoclonal antibody 4G10 from Upstate Biotechnology (Bucks, UK), monoclonal anti-
α-actinin from Sigma (St.Louis, MO, USA), and peroxidase-linked goat anti-mouse antibody from
DAKO (Glostrup, Denmark). Receptor-associated protein (RAP) fused to glutathione S-transferase
(GST)\(^4\) was prepared as described previously.\(^5\) The ADP receptor P2Y\(_{12}\) antagonist, the ATP analogue
N\(^6\)-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a
kind gift from Astra Zeneca (Loughborough, UK).

**Lipoprotein Isolation and Modification**

Fresh, non-frozen plasma from 3 donors, each containing less than 150 mg lipoprotein(a)/L, was pooled
and nLDL (1.019 - 1.063 kg/L) was isolated by sequential flotation.\(^6\) The concentration of nLDL was
determined on the Behring Nephelometer 100 (Dade Behring, Marburg, Germany) and expressed as g
apoB100/L. The quality of these preparations has been described.\(^6\) Prior to each experiment, nLDL was
dialyzed overnight against 10\(^4\) volumes of 150 mmol/L NaCl. nLDL was oxidized to different extents by
dialysis of 5 g/L EDTA-free nLDL in 5 µmol/L FeSO\(_4\)\(\cdot\)H\(_2\)O in phosphate-buffered saline (PBS) and 150
mmol/L NaCl containing 1 mmol/L NaN\(_3\) (pH 7.2, 24-72 hrs, 20\(^°\)C).\(^7\) After modification, the preparations
were dialyzed against 10\(^3\) volumes of buffer containing 150 mmol/L NaCl, 1 mmol/L NaN\(_3\) and 1 mmol/L
EDTA. Prior to each experiment, oxLDL was dialyzed overnight against 10\(^4\) volumes of 150 mmol/L
NaCl. The degree of lipid modification was inferred from the formation of conjugated dienes at 234 nm
and expressed as % oxidation.\(^8\)

**Isolation of Platelets**

Human platelets were isolated as previously described.\(^9\) In short, blood was collected from healthy
volunteers (with informed consent) into 0.1 volume of 130 mmol/L trisodium citrate. The donors claimed
not to have taken any medication 10 days prior to blood collection. Platelet-rich plasma (PRP) was
prepared by centrifugation (156 x g, 15 min, 20\(^°\)C). Then, 0.1 volume of ACD (2.5% trisodium citrate,
1.5% citric acid and 2% D-glucose) was added to lower the pH to 6.5. Platelets were further purified by centrifugation (330 x g, 15 min, 20°C). The platelet pellet was resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5). Prostacyclin (PGI₂) was added to a final concentration of 10 ng/mL and the washing procedure was repeated once. The platelet pellet was resuspended in Hepes-Tyrode buffer, pH 7.2. Platelet count was adjusted to 2.0 x 10¹¹ platelets/L, unless stated otherwise.

For perfusion studies, reconstituted blood was prepared as described. In short, PRP was acidified by addition of 0.1 volume of ACD, and the platelets were spun down. The platelet pellet was resuspended in Hepes-Tyrode buffer, pH 6.5 and PGI₂ (10 ng/mL) was added to prevent platelet activation during the subsequent washing step. Platelets were spun down and resuspended in a small volume of Hepes-Tyrode buffer and diluted in human albumin solution (HAS; 4% human albumin, 4 mmol/L KCl, 124 mmol/L NaCl, 20 mmol/L NaHCO₃, 2 mmol/L Na₂SO₄, 1.5 mmol/L MgCl₂, 5 mmol/L D-glucose, pH 7.35). Red blood cells were washed twice with 150 mmol/L NaCl containing 5 mmol/L D-glucose (2000 × g, 5 min), and finally cells were pelleted (2000 × g, 15 min). Platelets were mixed with red cells to obtain reconstituted blood with a hematocrit of 40% and a platelet count of 1 x 10¹¹ platelets/L.

For the isolation of murine platelets, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/mL), ketamine (40 mg/L) and atropine (0.05 mg/mL), and blood was subsequently collected into 0.1 volume 130 mmol/L trisodium citrate and 0.1 volume of ACD by heart puncture. PRP was obtained by centrifugation (87 x g, 15 min, 20°C). The remainder of the blood was diluted with Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5) and 0.1 volume of ACD and centrifuged again (87 x g, 15 min, 20°C). PRP samples were pooled, and platelets were isolated by centrifugation (350 x g, 15 min, 20°C) in the presence of 0.1 volume of ACD buffer and resuspended in Hepes-Tyrode buffer (pH 6.5). PGI₂ was added to a final concentration of 10 ng/mL, and the washing procedure was repeated once. The
platelet pellet was resuspended in Hepes-Tyrode buffer (pH 7.2). Platelet count was adjusted to $1 \times 10^{11}$ platelets/L.

Platelets were incubated with 1.0 g/L LDL, native or oxidized to different extents for 1 min at 37°C unless stated otherwise.

**P38\(^{\text{MAPK}}\) Assay**

The phosphorylation of p38\(^{\text{MAPK}}\) was measured as described elsewhere.\(^{11}\) In short, platelets were incubated at 37°C with nLDL or oxLDL as indicated. Samples of 100 µL were fixed with 1% formaldehyde (15 min, 4°C), centrifuged (5600 x g, 30 sec, 20°C) and resuspended in 60 µL Laemmli sample buffer. Proteins were analyzed by SDS-PAGE and Western blotting. One part of a sample was applied to SDS-PAGE to identify dual phosphorylated p38\(^{\text{MAPK}}\) phosphorylation using a phospho-specific anti-p38\(^{\text{MAPK}}\) polyclonal antibody (Thr-180/Tyr-182; 1:2000, 16 hrs, 4°C). Another part was applied to SDS-PAGE and total p38\(^{\text{MAPK}}\) was detected with an antibody against p38\(^{\text{MAPK}}\) as a control for equal lane loading (1:2000, 16 hrs, 4°C). Both antibodies are raised against residues 171-186 of human p38\(^{\text{MAPK}}\). After washing, the membranes were incubated with horseradish peroxidase labeled anti-rabbit IgG (1:5000, 1 hr, 4°C). Protein bands were visualized using the enhanced chemiluminescence reaction. For semi-quantitative determination, the density of the bands was analyzed using ImageQuant software (Molecular Dynamics). The data express the semi-quantification of dual-phosphorylated p38\(^{\text{MAPK}}\) from the blots after correction for the phosphorylation at time point 0 min.

**ApoER2’ Tyrosine Phosphorylation**

Tyrosine phosphorylation of ApoER2’ was determined as described.\(^{12}\) In short, human platelets were incubated at 37°C with nLDL or partially oxidized LDL as indicated and mixed (1:10 v/v) with ice-cold lysis buffer consisting of 10% (v/v) Nonidet P-40, 5% (w/v) octylglucoside, 50 mmol/L EDTA, 1% (w/v) SDS supplemented with 5 mmol/L NaVO\(_3\) and 10% (v/v) protease inhibitor mixture. ApoER2’ was
precipitated using a goat polyclonal antibody directed against apoER2 (1 µg/mL) and protein G-Sepharose for 3 hrs at 4°C. Precipitates were washed 3 times with lysis buffer (containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaVO₃ and 1 µg/mL leupeptin) and taken up in non-reducing Laemmli sample buffer. Samples were analyzed by SDS-PAGE and Western blotting. Tyrosine phosphorylation of apoER2' was visualized by incubation with 4G10, an antibody directed against phosphorylated tyrosine residues (0.5 µg/mL, 16 hrs, 4°C), followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 hr, 4°C), and the enhanced chemiluminescence reaction. As a control for equal lane loading, the blots were stripped and incubated with a monoclonal anti-α-actinin antibody (1:5000 (v/v), 16 hrs, 4°C), since specific apoER2-detecting antibodies are unavailable. This was followed by incubation with peroxidase-linked anti-mouse IgG (1:5000 (v/v), 1 hr, 4°C). The data express the semi-quantification of tyrosine phosphorylation of apoER2’ relative to the density of the bands representing α-actinin after correction for the density at time point 0 min.

**Perfusion studies**

Fibrinogen was immobilized onto Thermanox® coverslips by a coating procedure (100 µg/mL, 1 hr, 20°C). Coverslips were blocked (1 hr, 20°C) with 1% human albumin in phosphate-buffered saline (PBS). Perfusion studies were carried out with reconstituted blood in a three single-passage parallel-plate perfusion chambers as described.¹³ The blood was preincubated with vehicle or the indicated inhibitors, stimulated with partially oxidized LDL (0.2 g/L) in the presence of CaCl₂ (3 mmol/L) for 5 min at 37°C, and perfused over the fibrinogen-coated coverslips for 5 min at a shear rate of 300 s⁻¹. After perfusion, slides were washed with Hepes buffer (10 mmol/L Hepes, 150 mmol/L NaCl, pH 7.35) and fixed in 0.5% glutaraldehyde in PBS. Slides were dehydrated in methanol and stained with May-Grünwald and Giemsa.¹⁴ Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software [Dutch Vision Systems (DVS), Breda, the Netherlands]. Platelet attachment to surface-bound fibrinogen was expressed as surface coverage (expressed as percentage).
Statistical Analysis

Results are expressed as means±S.E.M. and analyzed with the Student's t test for unpaired observations. Differences were considered significant at $p < 0.05$.

References


Figure Legends

Fig. I. Oxidation of nLDL introduces apoER2’-independent platelet signaling.

(A) Platelets were incubated with 1.0 g/L nLDL or partially oxidized LDL for 1 min, stimulated with a second dose of nLDL or partially oxidized LDL (1 min) and p38MAPK phosphorylation was determined. (B-D) Platelets were incubated with (B,C) GST-RAP at the indicated concentrations (10 min) or (D) chondroitinase ABC (ABC; 1.0 U/mL, 10 min), stimulated with 1.0 g/L nLDL or partially oxidized LDL (B,D: 1 min; C: 0.5 min) and (B,D) p38MAPK phosphorylation or (C) tyrosine phosphorylation of apoER2’ was determined. Data were expressed as percentage of the density of incubations with nLDL (A,B,D) or oxLDL (B). Means±S.E.M., n=3.
Figure I

A

![Bar chart showing p38MAPK-P (%) in different conditions](image)

B

![Graph showing P38MAPK-P (%) vs. GST-RAP (μg/mL)](image)

C

![Graph showing IP ApoER2 expression with different GST-RAP concentrations](image)

D

![Graph showing p38MAPK-P (%) with vehicle and ABC](image)