Supplemental Material:

FLow-Induced PRotrusions (FLIPRs): a platelet-derived platform for retrieval of microparticles by monocytes and neutrophils

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Detailed Materials and Methods

Materials

Human serum albumin (HSA) was purchased from MP Biomedicals (ImmunoO fraction V; Amsterdam, the Netherlands). Fibrinogen was obtained from Enzyme Research Laboratories (Swansea, UK) and von Willebrand Factor (VWF) from Biotest AG (Dreieich, Germany). GFOGER and CRP-XL were produced as described before(1,2). RGD-W was purchased from the NKI (Amsterdam, the Netherlands). Calpeptin, 2-aminoethoxydiphenylborate (2-APB) and bongkrekic acid were obtained from Enzo Life Sciences (Antwerpen, Belgium), and MDL 28170, thapsigargin and FK-506 from Sigma (Zwijndrecht, the Netherlands). Cyclosporin A, GSK 429286, ZCL-278 and NSC 23766 were obtained from Tocris Biosciences (Abingdon, UK). Cyclophilin A inhibitor was purchased from EMD Millipore (Billerica, USA) and 1-oleoyl-2-acetyl-glycerol (OAG) from Santa Cruz (Heidelberg, Germany).

The following antibodies were used for immunofluorescent staining: anti-GPIb (HIP-1) (eBiosciences, Vienna, Austria); anti-P-selectin, anti-fibrinogen, and anti-GPIb (SZ2) (Santa Cruz); anti-PF4 and anti-βTG (R&D systems); annexin A5-FITC (Biovision, MountainView, CA, USA); phalloidin-FITC (Invitrogen, Bleiswijk, the Netherlands); and anti-α-tubulin (eBiosciences). Secondary antibodies labeled with Alexa fluor 488, Alexa fluor 555, and Alexa fluor 680 were purchased from Invitrogen. Lactadherin-FITC (Haematologic Technologies, Essex Junction, VT, USA) and FITC-, PE- or APC-labeled monoclonal antibodies against GPIb, P-selectin, CD14, CD66b, CD11b and CD62L (BD Biosciences, Franklin Lakes, NJ, USA) were used for FACS analysis.

Blood collection

Blood was collected from healthy volunteers who had not been on anticoagulant or antiplatelet medication for at least 10 days before blood withdrawal. The study was approved by the University Medical Center Utrecht (UMCU) Ethics Committee. Blood was anticoagulated with 10% sodium citrate (3.2% w.v.). For annexin A5 adhesion stainings, blood was anticoagulated using 100 U/mL hirudin. Platelet rich plasma (PRP) and washed platelets were prepared as described before(3). Platelet count was determined using CellDyn1800 (Abbott, Hoofddorp, the Netherlands) and set to $350x10^9$/L.

Real-time perfusion studies

Glass coverslips were cleaned with chromic-sulfuric acid (Sigma), rinsed with dH_2O and placed in a polydimethylsiloxane (PDMS) perfusion chamber with a channel height of 75 μm and width of 2 mm. The channels were coated with fibrinogen (100 μg/mL), VWF (10 μg/mL), fibronectin (100 μg/mL) or GFOGER+CRP-XL (100 μg/mL and 200 μg/mL, respectively) for 1.5 hours at room temperature and blocked with 1% HSA overnight at 4˚C. PRP and washed platelets were perfused using a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a shear rate of 300 sec^{-1}. Washed platelets that were perfused over fibrinogen were activated using collagen related peptide (CRP, 500 ng/mL) after 15 minutes of perfusion. FLIPR formation was visualized with an inverted microscope (Zeiss observer Z.1, Carl Zeiss, Sliedrecht, the Netherlands). Movies and snapshots were recorded with differential interference contrast (DIC) microscopy, using a 40x/1.25 oil or 100x/1.25 oil EC-plan Neofluar objective (Carl Zeiss). For reflection interference contrast microscopy (RICM) analysis, a 63x/1.25 oil EC-plan Neofluor antifluor objective (Carl Zeiss) was used in combination with an HBO lamp. All images were analyzed with AxioVision software (Release 4.6, Carl Zeiss) and ImageJ software (Release 1.41, National Institutes of Health, Bethesda, MD, USA).

Immunofluorescent staining of adhered platelets and FLIPRs

After perfusion, adhered platelets and FLIPRs were fixed under flow using 2% paraformaldehyde (PFA). Samples were blocked using 10% normal goat serum for 30 minutes. Immunofluorescent staining was performed using antibodies against GPIb, P-selectin, PF4, β-TG, fibrinogen, α-tubulin and CD14. Secondary antibodies containing either AF488 or AF555 were used

to visualize the stainings. Stains were analyzed using a Zeiss LSM 510 meta confocal microscope (Carl Zeiss).

Determination of GPIb distribution across membranes

Images of GPIb-stained platelets were taken using confocal microscopy. ImageJ was used to determine the intensity of the staining, with the histogram option being directed at selected platelets with and without a FLIPR. The intensity of the normal platelets was set at 100% and used to calculate the intensity of the platelets with a FLIPR in the same microscopic picture.

Real-time calcium flux microscopy

Calcium flux was visualized in washed platelets (suspended in HEPES-Tyrode buffer, pH 7.3) that had been incubated with 1 μM Oregon green BAPTA-1, AM (Invitrogen) at 37˚C for 30 minutes. Platelets were centrifuged again in the presence of 10ng/mL PGI_2 and resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM $CaCl_2$. Perfusion was performed and calcium flux was visualized and analyzed using AxioVision software (Release 4.6, Carl Zeiss).

FACS analysis and westernblot on microparticles

Washed platelets were perfused in a calcium rich buffer over fibrinogen. FLIPR formation was created by perfusion of 500 ng/mL CRP after 15 minutes (=perfusion with FLIPRs). The control perfusion was not activated with CRP (=perfusion no FLIPRs). Flowthroughs were collected and centrifuged twice at 1500xg for 15 minutes in the presence of PGI_2 and 20 mM EDTA to prevent platelet activation during centrifugation in the presence of CRP remnants. For FACS analysis, the supernatant containing microparticles was then incubated with antibodies against GPIb and lactadherin for 20 minutes and fixed using 0.2% formaldehyde in 0.9% NaCl. All samples were analyzed using the FACSCanto from BD Biosciences. Microparticles were gated based on forward- and side-scatter properties, and the number of $GPIb^+$/$lactadherin^+$ events was counted and compared to that of the isotype controls. For westernblots, the supernatant was centrifuged at 20,000xg for 1 hour, and the microparticle-containing pellet resuspended in sample buffer in the presence of dithiothreitol (DTT). Proteins were separated by SDS-PAGE and stained with anti-GPIb (SZ-2).

Electron microscopy of FLIPRs and microparticles

Platelets were perfused over fibrinogen-coated formvar grids, and fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer. Platelets and FLIPRs were counterstained with uranyl acetate and analyzed in a JEOL 1200CX electron microscope at 80 kV (JEOL, Nieuw-Vennep, The Netherlands). Microparticle-containing samples were collected after perfusion (as described in the FACS and western blot section) at the outlet of the perfusion chamber and isolated using differential centrifugation(4). Isolated vesicle fractions were examined in the electron microscope after uranyl staining and embedding(4).

Monocyte and neutrophil perfusions

For monocyte isolation, citrated whole blood was diluted with HEPES-Tyrode buffer (pH 7.3) and a Ficoll separation was performed. The pellet was resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM $CaCl_2$. Monocytes were isolated using $CD14^+$ magnetic beads (Miltenyi Biotec, Leiden, the Netherlands). Neutrophils were isolated from the supernatant obtained after static incubation of blood (from which platelets were already isolated) with a 6% Dextran/0.9% NaCl solution. The supernatant was spun down and the pellet was shocked with ice-cold water. 0.6M KCl was added, followed by HBS and cells were again spun down. Neutrophils were isolated using a Ficoll separation and resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM $CaCl_2$. The purity of both monocytes and neutrophils was established using the CellDyn1800, which measures the cell type based on light scattering, as well as with FACS analysis for CD14 and CD66b. Both types of analysis indicated a purity of >98%.

Washed platelets were perfused for 15 minutes followed by activation with CRP for 5 minutes. Monocytes and neutrophils were then perfused, and rolling and adhesion were studied during real-time perfusion. The flowthrough during these perfusions were captured and stained with CD14 (monocytes), CD66b (neutrophils), GPIb, CD11b or CD62L for 20 minutes and fixed using 0.2%

formaldehyde in 0.9% NaCl. $CD14^+$ or $CD66b^+$ cells were gated and GPIb mean fluorescence intensity was measured within this gate to analyze the presence of platelet (microparticles) on the monocytes or neutrophils. All samples were analyzed using the FACSCanto from BD Biosciences.

***In vivo* FLIPR formation in a mouse carotid injury model**

All procedures for animal experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals as defined by European laws. Mice (8-10 weeks) were anesthetized by intra peritoneal administration of ketamine (100 mg kg^{-1}) and xylazine (20 mg kg^{-1}). The common carotid arteries were exposed and vascular injury was induced by application of 7.5% $FeCl_3$ saturated Whatmann filter paper on top of the carotid artery for 2 minutes. The arteries were fixed with 2.5% glutaraldehyde by transcardiac perfusion. The injured artery was excised and post-fixed in 2.5% glutaraldehyde overnight. The isolated segments were cut open in the middle and dehydrated in increasing concentrations of ethanol, followed by treatment with hexamethyldisilazine. Samples were glued on a coverslip with the lumen oriented upwards. The coverslips were sputter-coated with gold and analyzed at 5 kV using a FEG Sirion SEM (FEI, Hillsboro, USA).

Statistics

A Mann-Whitney U test was performed to compare the difference between 2 conditions. A p-value of less than 0.05 was considered significant. All data analyses were performed with computer software (SPSS-pc Version 15.0, SPSS Inc., Chicago, IL, USA).

Online supplemental data

Supplemental movie I: FLIPR formation after stimulation with CRP. Platelet adhesion and spreading on fibrinogen was observed during the first 10 minutes of perfusion. After perfusion with CRP, adhered platelets start forming FLIPRs in the direction of the flow. Note that the platelet body decreases in size as the FLIPR elongates. Video was recorded at four frames/second using DIC microscopy, and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie II: Overview of FLIPR formation. CRP was added to platelets spread on fibrinogen substrate. Multiple platelets are shown forming long membrane protrusions. The video was recorded at four frames/second using DIC microscopy. Actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie III: FLIPR elongation and platelet disintegration. Real-time video recording of a spread platelet adhered to fibrinogen. Extension of the membrane is followed real time to show the length of the FLIPR. Note that at the end of the recording, the morphology of the spread platelet is changed and the platelet starts to disintegrate. Video was recorded at four frames/second using DIC microscopy, and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie IV: Monocyte rolling over FLIPRs. FLIPRs were created by perfusing washed platelets over fibrinogen, followed by CRP activation and isolated $CD14^+$ monocytes were perfused subsequently. Note the FLIPR membrane that is disappearing due to retrieval of membrane by the rolling monocytes. Video was recorded at four frames per second using DIC microscopy and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie V: Inhibited monocyte rolling by blocking P-selectin. FLIPRs were created by perfusing washed platelets over fibrinogen, followed by CRP activation and isolated $CD14^+$ monocytes were perfused subsequently in the presence of anti-P-selectin. Video was recorded by DIC microscopy at a speed of four frames per second. Data were collected and the video is displayed at actual speed. Flow direction is indicated with an arrow.

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