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Farnesoid X Receptor and its ligands inhibit the function of platelets

Article

Supplemental Material

Materials and Methods (not in main text)

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Materials and methods

Reagents

Aggregation and cell stimulation assays were performed using collagen-related peptide (CRP-XL), a selective agonist for the platelet collagen receptor glycoprotein (GP) VI, from Professor Richard Farndale (University of Cambridge, Cambridge, United Kingdom), collagen (Horm collagen, Nycomed, Austria) and thrombin (Sigma Aldrich, UK). The FXR ligands GW4064 and 6α-ethyl-chenodeoxycholic acid (6-ECDCA) were purchased from Merck Millipore, UK. Chenodeoxycholic acid (CDCA) was purchased from Sigma, Aldrich, UK

Mice

FXR systemic knockout and littermates were produced as described previously. [1, 2] All protocols involving the use of animals were approved by the University of Reading Local Ethical Review Panel and authorized by a Home Office License (UK).

Human washed platelet preparation, aggregation and dense granule secretion

Washed platelets were prepared from fresh blood obtained from healthy, aspirin-free human volunteers with informed consent. Platelets were prepared and re-suspended in modified Tyrodes-HEPES buffer to a final density of $4x10^8$ cells mL^{-1} for aggregation assays as described previously. [3-5] Aggregation studies were performed at 37°C in an optical platelet aggregometer (Chronolog). Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leukocytes were rarely encountered with total cell contamination level was <1 per 13000 platelets. ATP secretion assays were performed as described previously. [4]

Immunoblotting and cyclic nucleotide assays

SDS-PAGE and immunoblotting were performed using standard protocols as described previously. [4-5] Rabbit anti-human 14-3-3ζ (Santa Cruz Biotechnology, USA) was used to detect 14-3-3ζ to ensure equivalent levels of protein loading in immunoblots. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against Syk and LAT were obtained from Epitomics, USA. Phospho-VASP antibody was obtained from Cell Signaling Technology, UK. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK. Phycoerythrin (PE)-Cy5 mouse antihuman CD62P and PE-Cy5 mouse IgG1 κ-isotype control used to measure P-selectin exposure were from BD Biosciences, UK. Luciferin-luciferase

luminescence substrate was obtained from (Chrono-log, Havertown, USA). PAPA nonoate, cGMP and cAMP ELISA detection and, cyclic nucleotide phosphodiesterase assays were obtained from Enzo lifesciences, UK and performed according to manufacture's protocols. Total cellular cGMP ELISA detection for murine platelets (RPN 226) were obtained from Amersham Biosciences, (Buckinghamshire, UK) and performed according to manufacture's protocols.

Flow cytometric analysis: α-granule secretion and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

Platelets were analysed by flow cytometry using whole blood with increasing concentrations of GW4064 or vehicle (containing DMSO (0.1% (v/v)) prior stimulation with CRP-XL (1 μg mL^{-1}) at room temperature for 20 minutes. Platelets were labelled with FITC-labelled rabbit anti-human fibrinogen and PE/Cy5 anti-human-CD62P (P-selectin) as described previously. [4-5] The cells were then fixed in 0.2% (v/v) formyl saline and analysed by counting 5000 events within the gated population using flow cytometry. Flow cytometric acquisition was performed using Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). Data were analysed by calculating median fluorescence intensity (MFI).

Measurement of $[Ca^{2+}]_i$ by spectrofluorimetry

Intracellular calcium mobilisation in platelets was measured in PRP pre-loaded with the fluorescent dye FURA-2AM as described previously and it the presence of EGTA (1mM) to prevent calcium influx. [4] Briefly, PRP were incubated with FXR agonist or vehicle (containing DMSO 0.1% (v/v) for 5 min and then stimulated with CRP-XL (1 μg mL^{-1}) in a luminescence spectrophotometer (LS-50B; Perkin Elmer, Beaconsfield, UK). The ratio of emission values (excitation: 340/380 nm) was calculated and converted to calcium concentration using FLWinLab software (Perkin Elmer).

Clot retraction

Human PRP (200 μl) was mixed with 5μl of red blood cells and vehicle or GW4064, and the final volume raised to 1mL with modified Tyrodes-HEPES buffer as described. [4] Fibrin clot formation was initiated by adding thrombin (1U/mL). Clot retraction around a glass capillary added prior to clot formation was observed over a period of 2 hours at room temperature. Clot weight was measured as a marker of clot retraction.

Platelet spreading

Washed mouse platelets were prepared at a density of $2x10^7$ cells/ml and 200µl of suspension allowed to spread on fibrinogen (100µg/ml) coated cover-glass for 45 minutes. Unbound platelets were washed away using modified Tyrodes-HEPES buffer and adhered cells fixed using 2% formaldehyde in modified Tyrodes-HEPES buffer. Images were obtained using a Nikon A1-R Confocal microscope using 100X objective. The number of platelets in different stages of spreading such as initial adhesion, formation of filapodia, formation of lamellapodia and fully spread were analysed using ImageJ (NIH, USA).

Thrombus formation *in vitro*

Whole citrated blood labelled with the lipophilic dye 3,3'-dihexyloxacarbocyanine iodide ($DIOC_6$) was pre-incubated with vehicle (containing DMSO (0.1% (v/v)) or GW4064 and perfused over a collagen coated (coated using 400µg/mL) Vena8 BioChips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm2 as reported previously. [4-5] Z-stack images of thrombi were obtained using a Nikon eclipse (TE2000-U) microscope or by confocal analysis using an A1R microscope (Nikon Instruments, UK). Fluorescence intensity was calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

Analysis of Thrombosis

Thrombus formation in mice and data analysis were performed as described previously.[3-5] Briefly, C57BL/6 mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg) and atropine (0.25 mg/kg). Anaesthesia was maintained with 5 mg/kg pentobarbital as required and the mouse circulation was accessed via a cannulus placed in the jugular vein, and the platelets labelled with Alexa-488-conjugated anti-mouse-GPIbβ antibody. The cremaster muscle was exteriorized, connective tissue removed, and an incision was made to allow the muscle to be affixed as a single sheet over a glass slide. Injury to cremaster arterioles was induced with a Micropoint Ablation Laser Unit (Andor technology plc, Belfast, UK). Thrombi were observed using an upright Olympus BX microscope. Images were captured prior to and after the injury by a Hamamatsu charged-coupled device digital camera C9300 in 640 x 480 format (Hamamatsu Photonics UK Ltd, Welwyn garden City, UK) and analysed using slidebook5 software (Intelligent Imaging Innovations, Denver, CO).

Analysis of transgenic mouse platelets

Blood was obtained from FXR+/+ and FXR-/- mouse platelets via cardiac puncture after termination. Blood (1 mL) was drawn into a syringe containing acidic citrate dextrose as

anticoagulant. Flow cytometry analysis was performed using whole blood with increasing concentrations of GW4064 prior stimulation with CRP-XL (1 μg mL^{-1}) at room temperature for 20 minutes with FITC-labelled rabbit anti-human fibrinogen as described previously. [4] The cells were then fixed in 0.2% (v/v) formyl saline and analysed by counting 5000 events within the gated population using flow cytometry. For analysis of VASP phosphorylation on serine 239, platelets were permeabilised using 0.1% Triton and stained using a phosphospecific antibody (Cell Signaling technology, Watford, UK) following the methodology of Spurgeon *et al.*[6]. Flow cytometric acquisition was performed using Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). Data were analysed by calculating median fluorescence intensity (MFI).

Assessment of haemostasis

C57BL/6 mice (~16 weeks old) (The Jackson Laboratory, USA) were anesthetized using ketamine (125 mg/kg), xylazine (12.5 mg/kg) and atropine (0.25 mg/kg) administered via the intraperitoneal route 20 minutes prior to the experiment and placed on a heated mat. FXR agonist, GW4064 (10 μM) or vehicle control (DMSO) was injected via femoral vein 10 minutes before 1mm of tail tip was removed using a scalpel blade and the tail tip was placed in sterile saline at 37°C. The time to cessation of bleeding was measured up to 20 minutes. Data were analysed by comparing the bleeding time obtained with vehicle (containing DMSO (0.1% (v/v)) or GW4064 treated mice.

Statistical analysis

Median fluorescence intensity values obtained in fibrinogen binding and granule secretion assays were converted into percentage for comparison. Data were analysed using ANOVA with Bonferroni post test as indicated, or where appropriate by t-test. Tail bleeding assay data were analyzed using the Mann-Whitney test.

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