The influence of *Rubus idaeus* and *Rubus caesius* leaf extracts on platelet aggregation in whole blood. Cross-talk of platelets and neutrophils

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SUPPLEMENTARY DATA

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), adenosine 5'-diphosphate sodium salt (ADP), Trizma base, calcium chloride, sodium dodecyl sulphate (SDS), adenosine 3',5'-diphosphate sodium salt (A3P5), Sepharose 2B, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), formyl-methionyl-leucyl-phenylalanine (fMLP), luminol, and apyrase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), sodium chloride, and magnesium chloride were from Chempur (Piekary Śląskie, Poland). Mouse anti-human monoclonal antibodies: anti-CD61 (PerCP), anti-CD62P (PE) (P-selectin), PAC-1 (FITC), anti-CD11b (PE), anti-CD45 (FITC), anti-CD15 (FITC), anti-CD62L (PerCyP5) (L-selectin), anti-CD162 (PE) (PSGL-1), Cellfix, BD FACS Lysing Solution containing azide, azide-free BD Pharm Lyse Lysing Buffer, and buffered sodium citrate were purchased from Becton Dickinson (San Diego, CA, USA). Cangrelor (AR-C69931MX) was obtained from The Medical Company (Parsippany, NJ, USA) and ASA (aspirin P) was a gift from Bayer (Leverkusen, Germany). PLT VASP/P₂Y₁₂ kit was from BioCytex (Marseille, France).

Potassium chloride, glucose, sodium phosphate, and potassium phosphate were from POCH (Gliwice, Poland). Phosphate buffered saline (PBS) was from Biomed (Lublin, Poland). Physiological saline (0.9%) was from Polpharma (Starogard Gdanski, Poland). Hirudin (Refludan) was from Schering (Berlin, Germany). Polymorphprep was from Axis-Shield (Dundee, UK). Magnesium sulphate was from Lach-Ner (Prague, Czech Republic). Dextran T500 was from Pharmacosmos A/S (Holbaek, Denmark). Dulbecco's phosphate buffered saline (D-PBS) and Hanks' balanced salt solution (HBSS) were purchased from Gibco (Paisley, Scotland). Calcein AM was obtained from Molecular Probes (Eugene, OR, USA). Columns for gel filtration were from HTL (Warsaw, Poland). The reagent for counting leukocytes was from AquaMed (Lodz, Poland). Magnetic microbeads conjugated with mouse anti-human monoclonal antibodies to CD15 were from Miltenyi Biotec (Bergisch Gladbach, Germany). The water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Dubuque, IA, USA).

Plant materials

Plant collection and the procedure of extract preparation from raspberry (*R. idaeus* L.) and dewberry (*R. caesius* L.) was described previously [1].

Lyophilized extracts were dissolved in 10% DMSO in PBS (7.5 mg/ml). The PBS-diluted extracts (1.5 mg/ml) were stored in -20°C until used. All experiments presented in this study were controlled with appropriate DMSO dilution in PBS (DMSO concentration was 0.01% in control sample vs. 7.5 µg/ml extract treated sample, and 0.02% in control sample vs. 15 µg/ml extract treated sample). It was

shown that DMSO at the concentrations of 0.01% or 0.02% had no impact on platelet aggregation and neutrophil functions (expression of receptors and ROS production). The concentrations of the extracts corresponded to those used in our earlier study of endothelial function (1-15 µg/ml) [1].

Blood samples and donors

The study was performed according to the guidelines of the Helsinki Declaration for Human Research and approved by the committee of the Ethics of Research on Human Experimentation at the Medical University of Lodz, Poland. No individuals enrolled in the platelet functional study had taken aspirin or other drugs affecting platelet function within the previous 2 weeks. Blood samples were obtained from healthy donors (82 individuals; 34 men and 48 women, mean age 32.5 ± 7.8 years). Blood samples were collected from a peripheral vein into a tube containing 0.105 M buffered sodium citrate (1:9) for light transmission aggregometry (LTA) and flow cytometric analysis or hirudin (final concentration 25 µg/ml) for whole blood aggregometry (WBA).

Isolation of neutrophils

Neutrophils were isolated as described previously from heparinized whole blood layered onto Polymorphprep (1:1) and centrifuged (500 x g, 30 min). Gradient centrifugation allows the separation of mononuclear and polymorphonuclear cells by density. The neutrophils were harvested and washed with PBS without calcium and magnesium (400 x g, 10 min). Residual erythrocytes were removed by lysis with

lysing buffer (BD Pharm Lyse). The isolated neutrophils were suspended in an appropriate amount of a buffer or platelet-poor plasma (PPP). In some experiments, neutrophil granulocytes were isolated from blood by the sedimentation method with the use of 3% dextran D-500 in PBS. The cells were counted in a haemocytometer. The viability of cells was assessed with the use of the trypan-blue method.

Whole blood aggregometry (WBA)

Blood was incubated with extracts for 15 min (37°C), either alone or in combination with purinergic receptor inhibitors, A3P5 (35 µM) or cangrelor (6.5 nM), added to the blood for the final 3 min or 5 min of incubation (37°C). The concentration of platelet inhibitors, A3P5 or cangrelor, and the time of blood incubation with platelet inhibitors were established experimentally [2,3,4]. Blood incubated with 0.02% DMSO was used as the reference (control). The samples of blood were diluted 1:1 with 0.9% NaCl prior to the measurements. Platelet aggregation was initiated by the addition of ADP (6.4 µM) and measured for 15 min at a temperature of 37°C in an impedance aggregometer (Multiplate Platelet Function Analyzer, Dynabyte Medical, Munich, Germany). The results were expressed as maximal aggregation values (arbitrary units, AU).

The effect of the extract on platelet aggregation was measured also in the blood samples with a reduced neutrophil number. In the neutrophil depletion experiments, 1 ml aliquots of whole blood were preincubated with magnetic microbeads conjugated with anti-CD15 antibodies for 15 min at an ambient temperature. The mixture of blood and beads was placed on a magnet (MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany) and neutrophils coated with beads were removed

from the blood. The blood samples were carefully collected and measurements of aggregation were performed as described above. The efficiency of neutrophil depletion was assessed by counting the cells before and after the exposure of blood samples to the magnetic field. This technique allowed the number of neutrophils to be reduced by approximately 90%. The results were calculated as the inhibition vs. the relevant paired control as follows: for the samples, treated with either extract or cangrelor, the inhibition was calculated in relation to the untreated sample (control), for the samples depleted with neutrophils ("beads +"), treated with the extracts or cangrelor, the inhibition was calculated in relation to the untreated samples depleted with neutrophils (control "beads +").

Light transmission aggregometry (LTA)

Measurement of platelet aggregation by LTA was performed in platelet-rich plasma (PRP). After withdrawal of blood, whole blood samples were immediately centrifuged for 12 min at 190 x *g* to obtain PRP. The PRP was then removed and the remaining sample was subsequently centrifuged (12 min, 2000 x *g*) to get PPP to adjust the platelet count in PRP to $2-3 \times 10^8/\text{ml}$. Platelet aggregation was monitored for 10 min in Chrono-Log 490-2D (Chrono-Log, Havertown, PA, USA) after stimulation with 6.4 μM ADP. In some experiments, platelets were isolated by gel filtration, as described previously [5].

Flow cytometry measurements

Measurements of platelet viability

Platelet viability in whole blood preincubated with the examined plant extracts was determined by flow cytometry as described in detail elsewhere [6].

Determination of VASP Phosphorylation

The phosphorylation status of vasodilator-stimulated phosphoprotein (VASP) has been widely used in a literature to determine the blood platelet response to stimulation by ADP via the P_2Y_{12} receptor [7,8]. In our study, the effect of plant extracts on the VASP phosphorylation in blood platelets was determined in whole blood by the use of PLT VASP/ P_2Y_{12} assay. Prior to the activation with ADP, blood samples were incubated with either raspberry or dewberry leaf extract (15 μ g/ml, 15 min at an ambient temperature) and in some experiments, with cangrelor (50 nM), a selective inhibitor of the P_2Y_{12} receptor. After fixation, permeabilization and staining with fluorescently labelled antibodies, the samples were measured according to the manufacturer's instructions.

The magnitude of platelet activation was expressed as the platelet reactivity index (PRI) as described by the manufacturer. The index of platelet reactivity was calculated from the mean fluorescence intensity (MFI) of samples incubated with PGE_1 and $PGE_1 + ADP$ with the following formula: $PRI (\%) = [MFI(PGE_1) - MFI(PGE_1 + ADP)] / MFI(PGE_1) \times 100$.

Expression of P-selectin and activated form of the GPIIb/IIIa receptor on platelets

The samples of whole blood were incubated with the extracts at concentrations of 7.5 µg/ml and 15 µg/ml for 15 min at 37°C prior to addition of ADP (20 µM, 5 min at ambient temperature). Next, the blood samples were stained with anti-CD62P (PE) (P-selectin), PAC-1 (FITC) (activated GPIIb/IIIa receptor) and anti-CD61 (PerCP) (platelet-gating) antibodies for 30 min in the dark at ambient temperature, and then fixed in 1% Cellfix for 2h. The fluorescence of 5000 platelets was measured with a LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA). The specific fluorescence of antigen-positive platelets was obtained after subtraction of nonspecific fluorescence of the control samples labelled with corresponding isotype antibodies. All flow cytometry measurements were fluorescence-compensated on a daily basis, for each set of measured samples, using calibration beads. The results were analysed with FACS DIVA v6.1.3 software (San Diego, CA, USA) and expressed as percentages of CD62P- or PAC-1-positive cells.

Platelet–neutrophil aggregates

The fraction of platelet-neutrophil aggregates was analyzed by three-color flow cytometry in the whole blood samples used in the earlier performed whole blood impedance aggregometry experiments. After WBA measurements, the 100 µl aliquot of blood sample was directly transferred from the aggregometry cuvette to a separate tube. Erythrocytes were removed by the use of the BD FACS Lysing Solution (1% formaldehyde). The sample was then centrifuged for 7 min at 2000 x g at ambient temperature and the cell pellet was rinsed with PBS enriched with 1 mM CaCl₂, 1 mM MgCl₂ and 0.1% BSA.

The cells in the samples were supplemented with a mixture of antibodies, either anti-CD45 (FITC) for leukocyte gating, anti-CD11b (PE) for neutrophil gating or anti-CD61 (PerCP) for platelet gating, and left in the dark at an ambient temperature for 30 minutes. After the staining procedure, the cells were washed twice with PBS (enriched with 0.1% BSA). The cells were then fixed with 1% CellFix (1h at 37°C) and the fluorescence of 50000 CD45 FITC-positive cells was measured with a FACSCanto Flow Cytometer (Becton Dickinson, San Diego, CA, USA).

Neutrophils were gated by size (forward scatter of light, FSC) versus granularity (side scatter of light, SSC), and next by the fluorescence of CD45 (FITC) antibodies. The neutrophil-platelet aggregates were identified as CD61 PerCp-positive cells in the region of neutrophils (FSC vs. SSC and CD45 FITC-positive fluorescence). In addition, the fluorescence intensity of CD11b (PE) (MFI, median fluorescence intensity) was measured as a marker of neutrophil activation in the analysed blood samples. The results were expressed as arbitrary fluorescence units (AFU).

Expression of CD11b, PSGL-1 and L-selectin on neutrophils

The expression of CD11b, PSGL-1 and L-selectin on the neutrophils was measured in whole blood samples incubated with the extract (15 µg/ml for 15 min), before and after activation with ADP (20 µM, 30 min, 37°C) or fMLP (0.5 µM, 15 min, 37°C), the latter being used as a reference activator.

Blood samples were stained with the antibodies for 30 min in the dark at a temperature of 4°C to prevent further activation of neutrophils. Erythrocytes were then removed by BD FACS Lysing Solution (1% formaldehyde). The fluorescence intensity (MFI, median fluorescence intensity) of 8000 cells per sample was examined

using a LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA). The results were expressed as arbitrary fluorescence units (AFU). Neutrophils were identified based on the forward and side light scatter (FSC vs. SSC parameters) and CD15-positive staining. The results were analyzed with FACS DIVA v6.1.3 software (San Diego, CA, USA).

Oxidative burst of neutrophils

The production of ROS by neutrophils in the response to the exogenous stimulation (oxidative burst) was measured in whole blood samples and in isolated neutrophil suspensions, using the luminol-enhanced chemiluminescence (L-CL) method described elsewhere [9,10]. The samples of whole blood or neutrophil suspension in HBSS were incubated without or with the extracts (15 µg/ml, 15 min). Following this, 1 mM luminol in HBSS was added to the cell suspension to enhance chemiluminescence and samples were immediately distributed onto 96-well white plates. Then, the aliquots of fMLP (1 µM) or ADP (100 µM) were added to the cells in order to initiate ROS production. The luminol-enhanced chemiluminescence was recorded over 60 min (1.0 – 1.5 µl of whole blood samples) or 30 min (isolated cells, 10^5 cells/well) in a Fluoroscan Ascent FL fluorometer (Labsystems, Finland). The results were expressed as the total values of the relative light units (RLU), calculated as the area under the curve of CL over time of the assay (the integral signals of L-CL values). The RLU values for the blood samples were normalized to 5×10^3 neutrophils per well, accordingly to the neutrophil counts in the blood. To further verify the antioxidant activities of the extracts, in some experiments the extracts were

added to cell suspensions in luminol-HBSS buffer, immediately before the stimulation and L-CL measurements.

Statistical analysis

The Shapiro-Wilk test was used to verify whether the data was normally distributed. Levene's test was used to verify the homogeneity of variances. Depending on data distribution, mean $\pm$ SE or median and interquartile ranges (IQR): from lower (LQ, 25%) to upper quartile (UQ, 75%), are given for all parameters. In the case of normally-distributed data, the significance of the differences between samples and controls was determined with one-way or two-way ANOVA for repeated measures, followed by either the analysis of contrasts (two way analyses) or the *post-hoc* multiple comparison test (Scheffe's test, Dunnett's test or the paired Student t-test with the Bonferroni's correction for multiple comparisons). For the comparing a few extract concentrations the dose-dependent effect was verified with the linear trend testing in the analysis of contrasts. In the case of non-normally-distributed data, the significance of differences between samples and controls was determined with the rank Friedman test followed by the Wilcoxon signed-rank test with the Bonferroni's correction for multiple comparisons. The Pearson's linear correlation or Spearman rank correlation was used to assess associations between the measured parameters (maximal platelet aggregation vs. neutrophil-platelet aggregate fraction in whole blood; ROS production vs. CD11b expression).

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