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Flavonolignans reduce the response of blood platelet to collagen

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ABSTRACT

The primary biological function of platelets is to form hemostatic thrombi that prevent blood loss and maintain vascular integrity. These multi-responding cells are activated by different endogenous, physiological agonists due to the vast number of receptors present on the surface of the platelets. Collagen represents up to 40% of the total protein presented in the vessel wall and is the major activator of the platelets' response after tissue injury, and is the only matrix protein which supports both platelet adhesion and complete activation.

The aim of our study was to determine the effects of three major flavonolignans (silybin, silychristin and silydianin) on collagen-induced blood platelets' activation, adhesion, aggregation and secretion of PF-4.

We observed that depending on the dose, silychristin and silybin have anti-platelet properties observed as inhibition of collagen-induced activation (formation of blood platelet aggregates and microparticles, as well as decreased expression of P-selectin and activation of integrin $\alpha_{IIb}\beta_3$), aggregation, adhesion and secretion of PF-4.

These effects highlight the potential of silybin and silychristin as supplementation to prevent primary and secondary thrombotic events wherein excessive blood platelet response to a physiological agonist is observed.

1. Introduction

The primary biological function of platelets is to form hemostatic thrombi that prevent blood loss and maintain vascular integrity. Thanks to their shape and small size, platelets take part in the initial stages of the blood coagulation process, resulting in the arrest of bleeding at sites of vascular injury [1]. Platelets in the circulation occur in a resting state and become activated at sites of vascular injury by adhesion to adhesive proteins, such as von Willebrand factor (vWf) and collagen, or by soluble platelets agonists such as thrombin, ADP, or thromboxane A_2 (formed from arachidonic acid in stimulated platelets). The expression of multiple membrane receptors, both constitutive and activation-dependent mediate platelet adhesion, the secretory process and aggregation at sites of vascular injury. These multi-responding cells are activated by different endogenous, physiological agonists due to the vast number of receptors present on the surface of platelets. The platelet activation process involves multiple platelet receptor-ligand interactions. The most important adhesion molecules on platelet surface are integrins, heterodimeric transmembrane proteins, which mediate interactions with extracellular matrix molecules containing Arg-Gly-Asp (RGD) sequences, and also play an important role in platelet signalling [2–5].

Upon vessel wall injury, circulating platelets are immediately immobilized by interactions with vWF binding to collagen and platelet membranes' glycoprotein complex GPIb-V-IX [6], which initiates adhesion of flowing platelets to the subendothelial extracellular matrix (ECM). This enables interaction between collagen and its receptors GPIa/IIa ($\alpha_2\beta_1$ integrin) and glycoprotein VI (GPVI), which take part in platelet signalling and facilitate platelet activation. All these events promote the shift of β_1 and β_3 integrins from a low to a high affinity state for their ligand through 'inside-out' signalling, allowing formation of platelet adhesion and aggregation. Activation of blood platelets is crucial for their function in both physiological and pathological processes. Binding of fibrinogen, vWf and other matrix proteins mediates stable platelet adhesion, aggregation and thrombus formation [7,8].

Numerous endogenous stimuli induce platelet signal transduction via their respective receptors, inducing cascade of platelet activation, among them mainly thrombin, collagen, ADP, or thromboxaneA2 formed from arachidonic acid. Platelet activation is a dynamic process and involves multiple feedback loops and cross-talk between different pathways in the cell. Its final stage is the

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activation of the main glycoprotein receptors GPIIb/IIIa (α2bβ3) for fibrinogen, whose surface expression is significantly increased by the platelet activation, due to its release from intracellular granules [9]. Activation of the receptor is based on a change in conformation of GPIIb/IIIa complex which leads to binding of fibrinogen, and in consequence, to platelet aggregation [10,11].

Although integrin IIb/IIIa functions as a constituent antigen, which is present even on non-stimulated platelets, the increase in its number of copies and the change in conformation make it a good marker of platelet activation. Similarly, the biomarker of platelet activation is P-selectin, a glycoprotein presented in the α-granules of resting platelets. Platelet activation leads to exocytosis of granule constituents, release of stored mediators and discharge of membrane-bound transcellular signalling molecules [12]. During the activation of blood platelets, P-selectin immediately appears on their surface and is responsible for platelet-platelet or platelet-leukocytes interaction, via P-selectin glycoprotein ligand-1 (PSGL-1) occurring on the surface of monocytes and neutrophils. The expression of P-selectin on cell surfaces reflects platelet degranulation, therefore the measurement of its level is a useful tool in monitoring the activation status of platelets [13,14].

Blood platelets release numerous biologically active agents that contribute to regulation of both primary and secondary hemostasis, as pro- or anticoagulants. Moreover, platelets can release many potent inflammatory signalling molecules, including inflammatory cytokines and chemokines, such as PF-4 (Plate Factor 4, CXCL4 chemokine) [15,16]. PF-4 participates in many other biological processes, including megakaryopoiesis, tumour metastasis, immune response and clot formation. After platelet activation, PF-4 concentrations rise about 1,000 fold at the site of vascular injury and clot formation, relative to plasma concentrations. Due to the fact that PF-4 is synthesized exclusively in megakaryocytes and stored in α plaque granules, this protein is a highly selective marker for platelet activation [17].

Our previous studies, performed on human blood platelets [18], have shown that flavonolignans – active chemical compounds presented in a silymarin (extract from Milk thistle (*Silybum marianum*) fruits) – inhibit ADP-induced blood platelets' activation. In accordance with this observation, the aim of our study was to determine the effects of three major flavonolignans (silybin, silychristin and silydianin) on collagen-induced blood platelets' activation, adhesion, aggregation and secretion of PF-4.

2. Methods

2.1. Reagents

Dimethyl sulfoxide (DMSO), bovine serum albumin, collagen type I (for adhesion), Tris, BCA kit, acetic acid and flavonolignans (silybin, silychristin and silydianin – United States Pharmacopeia [USP] Reference Standards) were all obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Flow cytometry reagents: anti-CD61/PerCp, anti-CD61/PE, anti-CD61/FITC, anti-CD62/PE, PAC-1/FITC, isotype controls and CellFix were all obtained from Becton Dickinson (San Diego, CA, USA). Collagen, aggregation cuvettes and strips were purchased from Chrono-Log (Havertown, PA USA). All other chemicals were reagent grade or the highest-quality available.

2.2. Blood samples

Blood samples collected from healthy volunteers were purchased from the Regional Centre for Transfusion Medicine in Lodz (Poland). All samples were collected in the morning, from fasting donors. All donors were checked by a doctor and found to have no cardiovascular disorders, allergy, lipid or carbohydrate metabolism disorders, nor any traces of medication [19]. Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (Resolution No. 16/KBBN-UŁ/II/2016).

2.3. Flow cytometry measurements of platelet activation in whole blood samples

The fresh whole blood samples (control or pre-incubated with flavonolignans) were activated by collagen: 2 μg/ml (10 min at 37 °C). An additional sample was not activated. Next, samples were fixed in 1% Cellfix at 37 °C (10 μl of blood + 90 μl of Cellfix solution). After 1 hour of fixation, the samples were stained with specific antibodies: anti-CD61/PerCP, anti-CD62P/PE and PAC-1/FITC (3 μl of each antibodies + 10 μl of sample), and left for 30 minutes in the dark, in RT. For the compensation procedure, a set of probes with used fluorochromes was prepared (10 μl of un-activated sample stained with 3 μl of anti-CD61/PerCP, anti-CD61/PE or anti-CD61/FITC). An additional unstained sample was also prepared. Blood platelets in whole blood samples were detected according to PerCP fluorescence. A fluorescence of 10,000 platelets (CD61/PerCP-positive objects) was measured using an LSR II Flow Cytometer (Becton Dickinson, San Diego, CA, USA). The specific fluorescence fractions from the platelets were obtained after subtraction of nonspecific fluorescence in the control samples (labelled with anti-CD61/PerCP and proper isotype control). In the CD61/PerCP-positive objects, PE and FITC fluorescences were detected and reported values were presented as fractions of CD62P or PAC-1 positive platelets, representing the expression of a given target antigen. Additionally, the fraction of platelet-derived microparticles as well as platelet aggregates in whole blood samples were measured in experiments on P-selectin expression and PAC-1 binding to platelets. Based on size and granularity, we determined forward light scatter (FSC) vs. side light scatter plots (SSC) in CD61/PerCP positive objects, formation of platelet aggregates, as well as release of platelet-derived microparticles. Using reference beads, we estimated FSC gates. CD61PerCP positive objects with an FSC lower than 10^3 were characterized as microparticles, while objects with FSC higher than $10^{4.4}$ were considered aggregates. Gates for PE and FITC fluorescences were estimated based on the fluorescence of unstained probes. In the case of PE, the objects with fluorescences greater than $10^{3.2}$ were characterised as platelets with surface expression of P-selectin, while FITC with fluorescences greater than $10^{2.2}$ were characterized as platelets with bound PAC-1 antibodies. The percentage values of CD62P positive platelets and PAC-1 positive platelets were calculated relative to the total number of platelets (CD61 positive cells) present in each sample [18]. All data analysis was performed in FACSDiva Version 6.1.2.

2.4. Isolation of platelet-rich-plasma and blood platelets

The blood was centrifuged (200 × *g*, 10 min, RT) to isolate the platelet-rich plasma (PRP). The obtained PRP was then used to measure aggregation. Blood platelets were isolated by differential centrifugation, as described above [20,21]. The final concentration of platelet suspension was about 4×10^8 platelets/ml. The platelets were counted using a photometric method according to Walkowiak et al. [22]. The platelet washing procedure was performed in plastic tubes, at room temperature. Washed human platelets were suspended in a modified Tyrode's Ca^{2+}/Mg^{2+} free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4).

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