11-09

Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 69, No 11, 2016

BIOLOGIE

Biotechnologie

MECHANISM OF ACTION OF A FRACTION ISOLATED FROM textitGALEGA OFFICINALIS L., STUDIED BY FLOW CYTOMETRY ASSAYS WITH MONOCLONAL ANTIBODIES AGAINST P-SELECTIN EXPRESSION

Atanas Atanasov

(*Submitted by Academician A. Atanassov on May 4, 2016*)

Abstract

Galega officinalis L. is a plant used for the treatment of *diabetes mellitus.* A fraction from crude extract of the plant has been purified by chromatography on Sephadex G-25. The preliminary analyses of the fraction show that it consists of about 23% protein and 74% polysaccharides. The fraction is a strong inhibitor of platelet aggregation induced by adenosine 5'-diphosphate. The mechanism of action of fraction was studied using flow cytometry assays with monoclonal antibodies CD62P-FITC, specific for P-selectin. The experimental results clearly documented action of fraction like inhibitor of the P-selectin expression molecules on platelet surface-a support of platelet adhesion. Possibly the fraction is antagonist of GPIIb/IIIa receptors for fibrinogen, since it has a strong disaggregating effect on aggregated by ADP platelet-rich plasma.

Key words: *Galega officinalis* L., fraction, ADP, platelet aggregation, flow cytometry

Introduction. *Galega officinalis* L. is a plant used in the traditional medicine system of Bulgaria and Italy for the treatment of *diabetes mellitus.* A preliminary analysis has shown that the plant contains lipids, proteins and polysaccharides [1]. The recent experimental results of Atanasov [2, 3] show that the water extract of this plant suppresses in vitro and in vivo the platelet aggregation induced by adenosine 5'-diphosphate (ADP), epinephrine, thrombin and collagen.

The isolation of a fraction from *Galega officinalis* with anti-aggregating effect on platelets is described in a previous work [4].

The aim of the study is to clear the mechanism of anti-aggregation action of a fraction from *G. officnalis*, using flow cytometry assays with specific for P-selectin monoclonal antibodies CD62P-FITC. P-selectin functions as a cell adhesion molecule on the surface of activated endothelial cells and platelets [5].

Materials and methods. The aerial parts of *Galega officinalis* at flowering stage were collected between May and August 2014 in different parts of Thrace, region of Bulgaria. Plant was verified in Department of Botany, Faculty of Pharmacy (University of Sofia, Bulgaria).

Crude aqueous extracts were obtained by maceration of 200 g dry matter in 2000 ml distilled water (pH 8 with sodium hydrogen carbonate) for 20-24 hours at 18–20 °C. Fresh extracts were filtered and concentrated by vacuum evaporation at temperature below 35 °C to dry matter about 165–170 mg/ml.

Concentrated crude extract (10 ml, 75 mg/ml dry matter) was applied to a column equilibrated with NH_3-H_2O solution, pH 7.3. The column was eluted at 16 °C by the starting solution with a flow-rate 720 ml/h and 20 ml fraction were collected in glass-tubes. The active fractions (from 400 ml to 550 ml) was concentrated and lyophilized.

Blood samples were collected by disposable syringes at a ratio of 1 part 3.8% trisodium citrate and 9 parts venous blood. Platelet-rich plasma (PRP) was prepared by centrifugation (180× g for 10 min) and diluted to 300×10^6 platelets per 1ml with autologous platelet-poor plasma (1800× g for 15 min). Adenosine 5′-diphosphate (ADP) from Reanal (Hungary) was used as aggregating agent. Platelet aggregation was measured in platelet-rich plasma (PRP) using a spectrophotometer set to operate at 600 nm by Born method [6] at the final concentration of 5 μM ADP and 25 μM ADP. The agonists were diluted in Tyrode's buffer solution (TBS). Platelet aggregation was measured for 10 min after the addition of an agonist and was evaluated as maximum aggregation (% max).

The used monoclonal antibody was: CD62P-FITC (Serotec, UK), a murine monoclonal antibody directed against P-selectin expresses on platelet surface.

The flow cytometry assays were performed in platelet-rich plasma on a Coulter EPICS XL flow cytometer (Coutler Corp., Hialeah, EL). The method was previously described [7–9]. In brief, the citrated PRP was diluted with platelet poor plasma prepared from the same blood samples to a ratio of 1:4. Saturating concentration of anti-CD62P-FITC, the platelet agonists and fraction or an equal volume of TBS was added. The samples were incubated without stirring at 26 °C and the reaction stopped after 30 min by adding of 1% formaldehyde. The samples were analyzed within the following hour. The percentages of FITC-fluorescence positive platelets (% PL) were obtained.

All laboratory and data analyses were performed blind to treatment allocation. The mean of duplicate measurements obtained before and after treatment

with fraction were compared by the ANOVA tests for repeated measures. The significance of the correlation coefficients were evaluated by t-test. Results are expressed as Mean ± Standard Deviation. A p-value ≤ 0.05 was retained for statistical significance.

Results and discussion. The fraction inhibited 50% platelet aggregation initiated by 25 μM ADP with IC_{50} 35 μg/ml PRP. This dose was about 30 times lower than the dose of the crude extract with IC_{50} 1100 ± 30.6 μg/ml. The inhibiting effect of the fraction was 7 times higher than the activity of theophylline (IC_{50} 250 ± 10 μg/ml). In dose of 100 μg/ml the fraction inhibited 100% platelet aggregation (IC_{100}) induced by 25 μM ADP, 0.7 U/ml thrombin and 0.1 mg/ml collagen. The activity of the fraction was 50 times higher than the activity of the aspirin (IC_{50} 1800 ± 63 μg/ml). The fraction has a strong dis-aggregating effect on a PRP as previously aggregated by ADP and collagen, but not by thrombin.

The flow cytometry study clearly documented an influence of the fraction, as strong inhibitor of the P-selectin expression on surface of the activated by 5 μM ADP platelets (Fig. 1*a*). On control platelet-rich plasma (Fig. 1*b* – without fraction and aggregating agent) the fluorescence is mean 4.90 and median is 3.80. The addition of 50 μg/ml fraction to platelet-rich plasma (Fig. 1*c* – without ADP), reduce the fluorescence to mean 4.10 and median to 3.40. This shows the modifying effect of a fraction on platelet P-selectin expression. After addition of 5 μM ADP as aggregating agent to platelet-rich plasma (Fig. 1*d* – without fraction), the fluorescence increased about 2.5 fold (from mean 4.90 and median 3.80 to mean 12.70 and median 8.0). The simultaneous addition of 50 μg/ml fraction and 5 μM ADP as aggregating agent to platelet-rich plasma (Fig. 1*e*) reduces the fluorescence to mean 10.40 and median 6.70. The addition of 10 folds more fraction to platelet-rich plasma (500 μg/ml) with the same concentration of aggregating agent (5 μM ADP) reduce the fluorescence near to control fluorescence – mean 4.70 and median 3.60 (Fig. 1*f*).

Discussion. The P-selectin receptors antagonist, including monoclonal antibody CD62P-FITC against these receptors blocks platelet-platelet and platelet-subendothelium adhesion and as a result this block platelet aggregation [5, 10–12]. The GPIIb/IIIa receptor antagonists, including the monoclonal antibodies against the receptors and synthetic compounds containing the RGD sequence block the fibrinogen binding to receptors and platelet-aggregation. The previous amino-acid analyses of the fraction [13] shows that protein in fraction consisted of amino acids, that composed biologically-active RGD peptides (arginine-glycine-aspartic acid), RGDS, KRDS and RGDS peptides (arginine-glycine-aspartic acid-serine and lysine-arginine-aspartic acid-serine, and arginine-glycine-aspartic acid-serine).

For these sequences is established that they inhibit the platelet aggregation in relatively low concentrations. These sequences lead to recognition and binding to the glycoprotein GPIIb/IIIa platelet receptors during platelet aggregation.

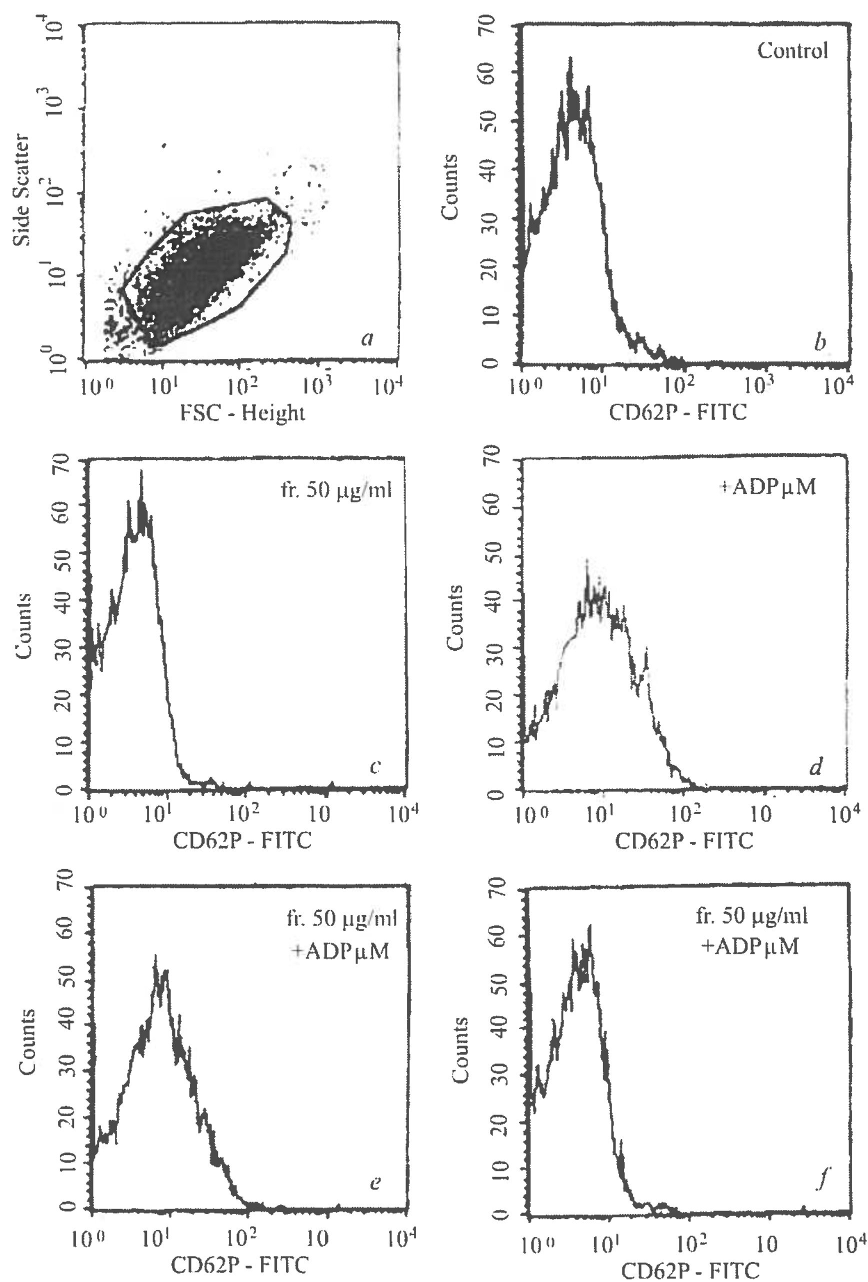

Fig. 1. *a*) Flow cytometry setting for detection of platelet-fraction binding; *b*) Control PRP – without fraction and aggregating agent; *c*) PRP with 50 µg/ml added fraction; *d*) PRP with 5 µM added ADP as aggregating agent; *e*) PRP with added 50 µg/ml fraction and 5 µM ADP; *f*) PRP with added 500 µg/ml fraction and 5 µM ADP as aggregating agent

Possibly, the high anti-aggregating activity of the fraction is due to the same consequences in protein of the fraction. However, this study clearly documented an influence of the fraction as strong inhibitor of the P-selectin expression in the activated by ADP platelets.

In Fig. 1 the control fluorescence is mean 4.90. The addition of 50 µg/ml fraction to platelet-rich plasma reduced the fluorescence to mean 4.10 (against 4.90 control fluorescence). This shows the modifying effect of the fraction on P-selectin expression. The additional of 500 µg/ml fraction on platelet-rich plasma fully reduced the fluorescence to control level (mean 4.70), instead of the presence of the (5 µM ADP) as aggregating agent.

REFERENCES

[1] Aprikyan S. V., V. S. Karapetyan (1987) *Galega officnalis* L., Izv. S-kh Nauk (Armenian), **21**, 24–30.

[2] Atanasov A. T. (1994) Effect of the water extract of Galega officnalis L. on human platelet aggregation in vitro, Phytotherapy Research, **8**, 314–316.

[3] Atanasov A. T. (1995) An effect of Galega officnalis L. extract on platelet aggregation in rats, Journal of Herbs, Spices & Medicinal Plants, **3**, 71.

[4] Atanasov A. T., B. Tchorbanov (2002) Anti-platelet fraction from *Galega officinalis* L. inhibits platelet aggregation, Journal of Medicinal Food, **5**, 229–234.

[5] Shattil S. J., M. Cunningham , J. A. Hoxie (1987) Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry, Blood, **70**, 307–315.

[6] Born G. V. R. (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature, **194**, 927–929.

[7] Coller B. S. (1995) Blockage of platelet GPIIb/IIIa receptors as an antithrombotic strategy, Circulation, **92**, 2373–2380.

[8] Michelson A. D. (1996) Flow cytometry: A clinical test of platelet function, Blood, **87**(12), 4925–4936.

[9] Michelson A. D., S. J. Shattil (1996) The use of flow cytometry to study platelet activation. In: Watson S. P., K. Authi (Eds): Platelet Function and Signal Transduction: A Practical Approach, Oxford, UK.

[10] Nagy B. Jr., I. B. Debreceni, J. Kappelmayer (2012) Flow cytometric investigation of classical and alternative platelet activation markers, Flow Cytometry in the Clinical Laboratory, **23**(4), 18–28.

[11] Theroux P., Z. H. Xiao (1995) Platelet-aggregation and fibrinogen binding following pharmacological inhibiting of the GPIIb/IIIa receptor, Circulation, **92**(8), 986.

[12] **Zucker M. B.** (1989) Platelet aggregation measured photometric method, Methods in Enzymology, **169**, 117–133.

[13] ATANASOV A. T. (2005) Water fraction isolated from *Galega officnalis* L. inhibiting platelet aggregation, PhD Thesis, Stara Zagora (Bulgaria).

Department of Biophysics
Faculty of Medicine
Thracian University
6000 Stara Zagora, Bulgaria
e-mail: atanastod@abv.bg