



Original Contribution

THE MECHANISM OF ACTION OF A FRACTION ISOLATED FROM *GALEGA OFFICINALIS* L., A STRONG INHIBITOR OF PLATELET AGGREGATION

***Atanas Atanasov¹, Veselina Petrova¹, Bojidar Tchobanov², Boriana Jakimova², Vania Ivanova²**

¹Faculty of Medicine, Thracian University, Stara Zagora, 6000; ²Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1113.

ABSTRACT

The presence of elastin-derived peptides (EDP) and the corresponding antibodies in circulation leads to formation of circulating immune CIC. The aim was to determine if serum level of free-EDP (unbound in CIC) correlate with the development of microvascular complications. To this end we used a method for detecting immune complexes (CIF-ELISA) in combination with an ELISA for detection of EDP. The levels of free EDP were studied in sera of 71 diabetic children (mean age-13.46±3.51 years, diabetes duration 5.17±4 years). Forty of the children had vascular complications (group 1) and 34 were without vascular complications (group 2). Diabetics showed significantly higher levels of free EDP (68.1±25 vs. 51±12.5 ng/ml; p=0.003) than controls. In group 1, free EDP showed significantly higher levels than controls (78.9±25.6 vs. 51 ± 12.5 ng/ml; p=0.0001). Thirty eight of the patients were positive for free EDP in group 1 and 8 - in group 2). Patients who had vascular pathology showed a correlation of free EDP with microalbuminuria (r=0.41; p=0.0081), retinopathy (r=0.32; p=0.041), insulin dose (r=0.37; p=0.02), HbA1c (r=0.35; p=0.03), systolic blood pressure (r=0.30; p=0.045) and total cholesterol (r=0.36; p=0.02). These data suggest elevated levels of free EDP are associated with the diabetic vascular complications.

Key words: Захарен диабет, CIF-ELISA, Несвързани еластинови деградационни пептиди, Микроалбуминурия, Ретинопатия

INTRODUCTION

Galega officinalis L. is a plant used in the traditional medicine system of Bulgaria and Italy in the treatment of *diabetes mellitus*. A preliminary analysis has been shown, that the plant consists of lipids, proteins and cellulose [1]. The recent experimental results by Atanasov [2, 3] show that the water extract of this plant suppresses *in vitro* and *in vivo* platelet aggregation induced by aggregating agents such as adenosine 5-diphosphate, epinephrine, thrombin and collagen. The isolation of a fraction from *Galega officinalis* L. with anti-aggregating effect on platelets is described in a previous work [4]. The aim of this publication is to study the mechanism of action of a fraction isolated from *Galega officinalis* L., using flow cytometric assays with monoclonal antibodies CD62P-FITC.

Correspondence to: A. Atanasov, Medical Faculty, Thracian University, 11 Armejska Str., St. Zagora, e-mail: atanastod@abv.bg

MATERIALS AND METHODS

Plant material. The aerial parts of *Galega officinalis* at flowering stage were collected between May and August 2005 in different parts of Thrace, region of Bulgaria. Plant was verified in Department of Botany, Faculty of Pharmacy (University of Sofia, Bulgaria).

Preparation of the extract. 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 at temperature below 35° C to dry matter about 165-170 mg / ml.

Isolation of the fraction. Concentrated crude extract (10 ml; 75mg/ml dry matter) was applied to a column equilibrated with NH₃-H₂O 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 fractions were collected in glass-tubes. The active fraction (from 400 ml to 550 ml) was concentrated and lyophilized.

Platelet aggregation and preparing of platelet-rich plasma. Blood samples were collected by disposable syringes at a ratio of 1 part 3.8% trisodium citrate and 9 parts venous blood [12]. Platelet-rich plasma (PRP) was prepared by centrifugation (180 x g for 10 min) and diluted to 300×10^6 platelets per ml with autologous platelet-poor plasma (1800 x 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 [5] at the final concentration of 5 μ M ADP and 25 μ M 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 maximal aggregation (%max).

Flow Cytometric Assays. Probes and agonists. Monoclonal antibody (MAbs) used was: anti-CD62P-FITC (Serotec, UK), a murine MAb directed against P-selectin expressed on platelet surface.

Platelet P- selectin expression. The flow cytometric assays were performed in platelet-rich plasma (PRP) on a Coulter EPICS XL flow cytometer (Coulter Corp., Hialeah, EL). The method was previously described [9, 10, 11]. In brief, the citrated PRP was diluted with the PPP 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 were added. The samples were incubated without stirring at 26 °C and the reaction stopped after 30 min by adding 1% formaldehyde. The samples were analyzed within the following hour. The percentages of FITC-fluorescence positive platelets (%PL) were obtained.

STATISTICAL ANALYSES

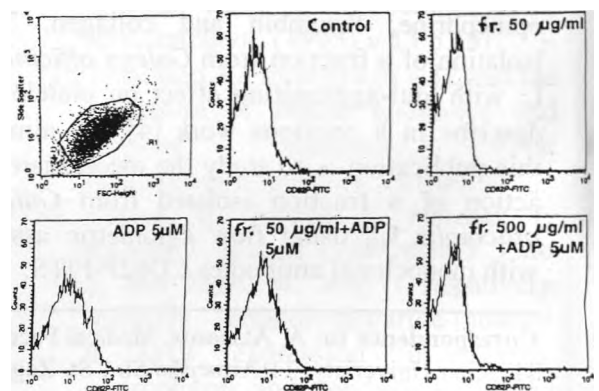
All laboratory and data analyses were performed blind to treatment allocation. The mean of duplicate measurements obtained before and after treatment with fraction were compared by the ANOVA tests for repeated measures. The significance of the correlation coefficients were evaluated by t-tests. Results are expressed as means $\pm$ standard deviation. A p value <0.05 was retained for statistical significance.

RESULTS

Anti-platelet aggregating activity of the fraction. The fraction inhibited 50% platelet aggregation initiated by 25 μ M ADP with IC_{50} 35 pg/ml PRP. This dose was about 30 timeslower than the dose of the crude extract (IC_{50} 1100130.6 pg/ml). The inhibiting effect of the fraction was 7 times higher than the activity of theophylline (IC_{50} 250110 pg/ml). In doses of 100 pg/ml the fraction inhibited 100% (IC_{100}) platelet aggregation induced by 25 μ M ADP, 0.7 U/ml thrombin and 0.1 mg/ml collagen. The activity of the fraction was 50 times higher than activity of the aspirin (IC_{50} 1800163 pg/ml). The fraction had a strong dis-aggregating effect on a PRP as previously aggregated by ADP and collagen, but not by thrombin

Flow cytometric Assays. This study clearly documented an influence of the fraction, as strong inhibitor of the P-selectin expression in activated by 5 μ M ADP platelets-Fig.1. On control in Fig. 1 the fluorescence is mean 4.90 and median is 3.80. The addition of 50 pg/ml fraction on platelet-rich plasma reduce the fluorescence to mean 4.10 and median to 3.40. This show, that the fraction has a slight modifying effect on P-selectin expression. After addition of 5 μ M ADP to platelet-rich plasma, the fluorescence increased about 2.5 fold (to mean 12.70 and median 8.0). The addition of 50 μ g/ml and 5 μ M ADP to platelet-rich plasma reduced the fluorescence to mean 10.40, and median 6.70. The addition of 500 μ g/ml fraction and 5 μ M ADP to platelet-rich plasma reduced the fluorescence near to control- mean 4.70, and median 3.60.

Fig. 1. Representative histogram of the flow cytometry setting for detection of platelet-fraction binding.



DISCUSSION

The P-selectin receptor antagonists, including the monoclonal antibody CD62 against this receptors block platelet-platelet and platelet-subendothelium adhesion and this block platelet aggregation [9, 10, 11]. The GPIIb/IIIa receptor antagonists, including the monoclonal antibody against the receptor and synthetic compounds containing the Arg- Gly- Asp (RGD) sequence, block fibrinogen binding to the receptor and platelet aggregation too [6].

Amino-acid analysis of the studied fraction shows, that about 50% of fraction's protein consisted amino acids composition, that composed biologically-active (RGD) peptides: arginine-glycine-aspartic acid and (RGDS, KRDS, RGDS) peptides: arginine-glycine-aspartic acid-serine, lysine-arginine-aspartic acid-serine, arginine-glycine-aspartic acid-serine. For these peptides is established that inhibit in low concentration platelet aggregation [7]. Such amino acids composition: RGD (arginine-glycine-aspartic

acid) and AGDV (alanin-glycine-aspartic acid-valine) sequence in fibrinogen lead to recognition and binding to the glycoprotein IIb/IIIa platelet receptor during aggregation [8]. Possible, the high activity of the fraction due to the amino-acids with same consequence. This study clearly documented an influence of the fraction, as strong inhibitor of the P-selectin expression in activated by ADP platelets. On Fig.1 the control fluorescence is mean 4.90. The addition of 50 µg/ml fraction on platelet-rich plasma reduce the fluorescence to mean 4.10 (against 4.90 control fluorescence) This show, that the fraction has a modifying effect on P-selectin expression. The addition of 500µg/ml fraction and 5µM ADP to platelet-rich plasma fully reduced the fluorescence near to control- mean 4.70. The conclusion is that the fraction from *Galega officinalis* inhibit platelet adhesion and aggregation, acting on P-selectin receptor for adhesion and GPIIb/IIIa receptor for aggregation.

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