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Influence of Cysteine Proteinase Inhibitors on Platelet and Plasma Components of Blood Coagulation System[1]

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Abstract—Factor XIIIa plays an important role in stabilization of formed fibrin clot during blood coagulation. Recent studies proved that factor XIIIa affects formation of coated platelets, which are highly procoagulant and characterized by a high level of alpha-granular proteins on their surface and expose surface phosphatidylserine after platelet activation. The ability of newly found cysteine proteinase inhibitors (CPIs) from plants to affect thiol group of the factor XIIIa active centre was recently discovered. Here, the effect of CPIs on the formation of coated platelets and activity of plasma components during blood coagulation process was investigated. It was found that CPIs dose-dependently decreased the fraction of coated platelets in the total platelet population during platelet activation and decreased endogenous thrombin potential (ETP) by 40% for thrombin generation in platelet-rich as well as in platelet-poor plasma. Such decrease of ETP could not be explained by the CPIs influence on factor XIIIa. Investigation of the effects of these inhibitors on factor Xa and thrombin activity has shown that CPIs dose-dependently inhibited their activity and might cause an ETP decrease. Thus, the obtained data indicated that CPIs affected both platelet and plasma components of blood coagulation system.

Key words: platelets, plasma, blood coagulation, cysteine proteinase inhibitors.

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The blood coagulation process is a complicated system of biochemical reactions with the function of prevention of blood loss due to the formation of fibrin-containing clot located in the place of blood vessel damage.

The tissue surrounding the vessel, the wall of the vessel, coagulation factors, and platelets are all involved into the hemostasic reactions in human organism. Upon the platelet activation, the asymmetry of the cell membrane bilayer is usually lost and phosphatidylserine appears on the membrane surface. The membrane surface becomes procoagulant and supports the formation of procoagulant complexes.

It was found that platelet activation leads to an appearance of subpopulations of platelets different in their ability to stimulate coagulation. Activation by thrombin with collagen or thrombin with convulxin (an agonist of collagen receptor glycoprotein VI) causes a formation of so-called "coated" platelets that possess a high level of alpha-granular proteins on their surface, express phosphotydilserine and support prothrombinase activity [2, 3]. Plasma factor XIII (or fibrin-stabilizing factor) and transglutaminase, which catalyze the synthesis of ε-(γ-glutamil)-lysine bond between fibrin fibrils, are involved in the coagulation process. Activated factor XIII (or factor XIIIa) stabilizes the clot and prevents its lysis [4].

It was demonstrated that approximately 50% of factor XIIIa circulating in the blood is accumulated in the cytoplasm of platelets and is expressed during the cell activation. Platelet factor XIIIa catalyses the formation of crosslinks and increases fibrin resistance to degradation by plasmin [5, 6]. Dale et al. [3] showed that factor XIIIa influences coated platelet formation. This supports the assumption that transglutaminases might increase the procoagulant surfaces in the injured place and accelerate blood coagulation.

There are some pathologies of coagulation that require the involvement of coagulation inhibitors. Recently, new inhibitors of CPIs which decrease enzymatic activity of factor XIIIa were isolated from potato. These inhibitors modify monomeric fibrin molecules by affecting polymerization centers and the active site of factor XIIIa, finally leading to the lack of cross-links between fibrin threads [7].

[1] The article was translated by the authors.

The role of platelet factor XIIIa during platelet activation and coagulation is not clear. There are little specific inhibitors of factor XIIIa [8]. This encourages attempts to develop new inhibitors of factor XIIIa and to study their influence on the whole coagulation process.

The aim of our study was to investigate the influence of recently found CPIs isolated from potato on the heterogeneity of platelet population during platelet activation and blood coagulation.

EXPERIMENTAL

Materials. The following substances were used in the experiments: thrombin (Haemotologic Technologies, USA; Roche, France), convulxin (Pentapharm, Switzerland), prostaglandin E1 (MP Biochemicals, USA), R-Phycoerythrin(PE)- and fluorescein(FITC)-conjugated annexin V (Molecular Probes, USA), papain (Merck, Germany), thromboplastin (RENAM, Russia), S_{2765} (Chromogenix, Italy). Thrombin-specific fluorogenic substrate (BOC-Ile-Gly-Arg-AMC) was provided by Institute of medical and biological chemistry, Russian Academy of Science. All other used reagents were from Sigma (USA).

Factor XIIIa. Factor XIII was isolated from bovine blood using the Lorand method [9]. Factor XIII was activated by adding thrombin with activity of 15 NIH/ml until concentration reached 7.5 NIH/ml. The obtained solution was incubated for 10 min at room temperature. Thrombin was removed by adding antithrombin-III-heparin mixture.

CPIs extraction. CPIs were extracted from potato (sort Istrinskiy, harvest of 2007) according to the method described by Kostranova et al. [7]. CPIs activity was measured with respect to papain, a cysteine-contained proteinase. The mixtures containing the constant amount of papain and various concentrations of CPIs with the total volume 1 ml were incubated for 10 min at 37°C in 50mM of tris-HCl-buffer (pH 8.0) with *L*-cysteine and EDTA in the final concentrations of 5 and 2 mM, respectively. Then the samples were each supplemented with 1 ml of 1% casein as the substrate and residual proteolitic activity of papain with regard to initial papain activity in control using modified Kunitz method [10].

CPI activity measurements. The activity of isolated CPIs was measured by the level of covalent cross-linking of casein in the presence of factor XIIIa. Casein was obtained from non-fat milk using method of Davieva et al. [11]. The reaction was carried out for 20 min at 37°C in 50 mM of tris-HCl-buffer containing 150 mM NaCl and 50 mM $CaCl_2$ (pH 7.4). An aliquot of 10 μl of initially activated factor XIIIa (1.6 unit/ml) or inactivated factor obtained by the incubation at 37°C for 30 min with CPIs at different concentrations (wt/wt ratio for factor XIIIa : CPI was 1 : 0.3; 1 : 0.7; 1 : 1, or 1 : 1.5) was added to 50 μl of 1% casein solution. The reaction was stopped by adding a mixture of 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. The degree of cross-linking of polypeptide chains of casein was determined in reduced samples by SDS-electrophoresis in 12.5% polyacrylamide gel [12].

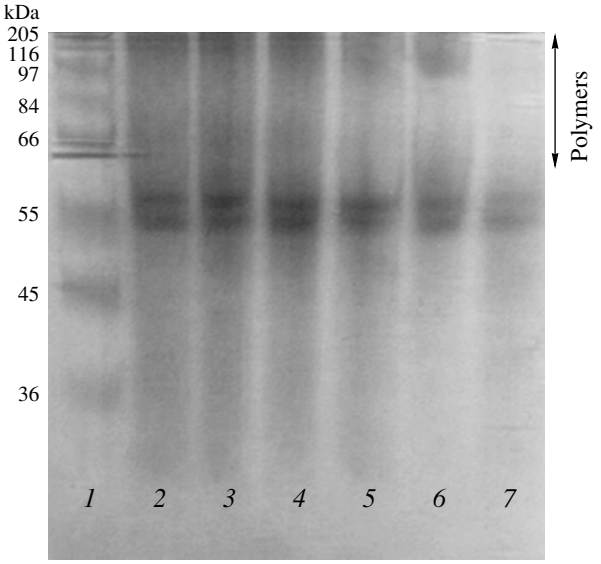

Fig. 1. Electrophoresis results of reduced samples of reaction products after casein coupling under the influence of factor XIIIa in the presence of CPIs. *1*, Protein markers; *2*, casein; *3*, casein with factor XIIa; *4, 5, 6, 7*, casein with factor XIIIa (1.6 units/ml) and CPIs at the enzyme/inhibitor ratio of 1 : 0.3, 1 : 0.7, 1 : 1, and 1 : 1.5, respectively.

Although casein is not a specific substrate for factor XIIIa, it was found that β-casein and some of its derivatives are good substrates for factor XIIIa.

The results of electrophoresis analysis are shown in Fig. 1. The ability of CPIs, as the mix of proteins extracted from potato using affinity chromatography, to inhibit the activity of factor XIIIa is quite discernible. It is clear that the process of covalent coupling of casein polypeptide chains occurs more slowly under the influence of factor XIIIa inhibited by CPIs (in Fig. 1, trace *3* is control; traces *4*–7, CPIs effect). Formation of polymers decreased or disappeared with the increase of CPIs concentrations (Fig. 1, traces *4–7*). This could be caused by specific interactions between inhibitors and the active site of factor XIIIa that led to a decrease of the enzymatic activity.

Plasma preparation. Human blood plasma was obtained from the blood of healthy donors and treated with 3.8% sodium citrate solution (111 mM of sodium citrate, pH 5.5) to prevent coagulation. The blood/citrate ratio was 9/1. In order to obtain platelet-rich plasma (PRP), blood was centrifuged at 100 *g* and room temperature for 8 min. To obtain platelet-poor plasma (PPP), whole blood was centrifuged at 1300 *g* for 15 min at room temperature.

Platelet preparation. To obtain platelets, citrate-treated whole blood was supplemented with prostag-

landin E1 (1 μM) and apyrase (0.1 unit/ml) and centrifuged to obtain PRP. The PRP supernatant was supplemented with 3.8% sodium citrate at 3:1 ratio to prevent platelet aggregation. PRP was then centrifuged at 400 *g* for 5 min to sediment platelets. The platelet pellet was resuspended in 300 μl of buffer A containing 20 mM HEPES, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5 mM glucose, 0.5% bovine serum albumin, pH 7. The obtained suspension was purified from plasma proteins using gel chromatography with a column 1 cm in diameter and 6 cm in height loaded with sepharose CL-2B and equilibrated with buffer A.

Inhibition of platelet activation by CPIs. Gel-filtered platelets at 20000/μl were activated for 15 min at room temperature in the absence (control) or in the presence of 0.05; 0.1; 0.2, or 0.4 mg/ml CPI. The cells were activated in buffer A with 2.5 $CaCl_2$ and 0.2% fluorescently labeled annexin V by adding either 10 nM thrombin with 10 ng/ml convulxin, or 100 nM thrombin, or 100 ng/ml convuxin. The activation level was analyzed using a Becton Dickinson flow cytometer (USA) and WinMDI 2.8 software developed by Joseph Trotter from Scripps Research Institute, La Jolla, USA. The activation level was evaluated according to the quantity of bound annexin V.

Thrombin generation test. The influence of CPI on the plasma component of coagulation was studied using thrombin generation test, which allows measurements of concentration of total thrombin generated in response to standard recalcification and activation. The kinetics of thrombin generation in plasma was determined by continuous registration of the fluorogenic substrate (BOC-Ile-Gly-Arg-AMC) cleavage by thrombin formed during coagulation [17, 18].

The measurements were performed as follows. To the wells of 96-well flat bottom plate, 90 μl of platelet-poor (PPP), or platelet-rich (PRP) plasma with citrate, 10 μl of cysteine proteinase inhibitors (CPIs) or buffer (20 mM HEPES, 140 mM NaCl, pH 7.5) as well as 10 μl of slow fluorogenic substrate BOC-Ile-Gly-Arg-AMC (5 mM) were added and incubated at 37°C for 10 min. Coagulation was triggered simultaneously in all wells by addition of 25 μl of activator in the same buffer, containing additionally 90 mM $CaCl_2$ (pH 7.5). As activators, 250-fold diluted rabbit thromboplastin solution (PT reagent, Renam, Moscow, Russia) was used. Final concentrations of tissue factor were 4 nm as determined using Actichrome® TF chromogenic activity assay (American Diagnostica, Stamford, CT, USA). AMC fluorescence was monitored continuously for 125–130 min using fluorometric Fluoroscan II reader (LabSystem, Finland) at $\lambda_{ex} = 380$ nm and $\lambda_m = 440$ nm. The obtained results were averaged over two similar probes. The standard error of these measurements did not exceed 2–5 %. The speed of the product accumulation at every time moment was proportional to the thrombin concentration in plasma. Thus, the time-dependence of thrombin concentration in the system was obtained by differentiation of AMC accumulation curve. The units of fluorescence for each sample were converted into AMC concentration using a calibration curve for standard AMC concentrations. The total thrombin quantity in the probe formed over the course of 120 min or endogenous thrombin potential (ETP) was calculated as the square under the curve of dependence of thrombin concentration on time. In addition, maximal thrombin concentration (A_{max}), time of maximal concentration (t_{max}), and lag-period (t_{lag}), required to reach thrombin concentration of 5 nM, were determined.

Effect of CPIs on thrombin and factor Xa. Thrombin (10 nM) or factor Xa (5 nM) was incubated in buffer A (150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20 mM HEPES, 5 mM glucose, 0.5% bovine serum albumin, pH 7.4) with 2.5 mM $CaCl_2$ and added indicated concentrations of CPIs (0; 0.05; 0.1; 0.2; 0.4 mg/ml) and incubated for 10 min at 37°C. After specific chromogenic substrate for thrombin Chromozym íç or S_{2765} for factor Xa and was measured initial rate of a reaction of a hydrolysis of the substrate at 405nm for 30 min at 37°C using Thermomax reader (Molecular Devices, USA). The degree of inhibition each enzymes were estimated so decrease of the rate of the hydrolysis (in %) as regards the rate of the hydrolysis corresponding substrate in absence CPIs, which was taken for 100%.

Statistics. To determine the effects of specific receptor inhibition or stimulation on coated platelet production, the data were compared using the paired Student's *t*-test. Statistical significance was set as $P < 0.05$. Values are reported as mean SE. Statistical analysis was carried out using OriginPro 7.5 software.

RESULTS AND DISCUSSION

CPIs influence on coated platelets formation. The mechanism of factor XIIIa influence on coated platelets appearance during platelet activation is not yet clear. Some works suggested a role for factor XIIIa in coated platelets formations [3]. However, factor XIIIa-deficient mice did not have decrease or defect in coated platelets [19]. In the present study, we supposed that if factor XIIIa can regulate the amount of coated platelets, then its inhibitors would influence platelet population heterogeneity during activation process by decreasing amount of coated platelets. In our work, we used CPIs extracted from potato, which decrease the factor XIIIa enzymatic activity ([7] and Fig. 1 in this work). It should be mentioned that the inhibitor preparations used in our work are a mixture of proteins not completely separated yet. The coated platelets were determined as the cell population with high phosphatidylserine location on their surface. These cells were able to efficiently bind fluorescently labeled annexin V that allowed a quantitative evaluation of the coated platelet amount in the population using flow cytometry. It was found that at different CPIs concentrations the

amount of coated platelets dose-dependently decreased (Fig. 2). At maximal concentration of the inhibitor (0.4 mg CPIs/ml), the amount of coated platelets was 2–10-fold lower even in the presence of various activators. For platelet activation by thrombin with convulxin, the half-maximal concentration of CPI was equal to 0.1 mg/ml. During separate activation of platelets with thrombin or convulxin the effect was reached at the CPIs concentration of 0.05 mg/ml. Thus, the inhibitory effect of CPIs led to a decrease of coated platelet amount and hence to a decrease of procoagulant surfaces during coagulation process. As washed platelets (without plasma proteins) were used for these experiments, the CPIs effect might be explained only by its inhibitory effect on factor XIIIa.

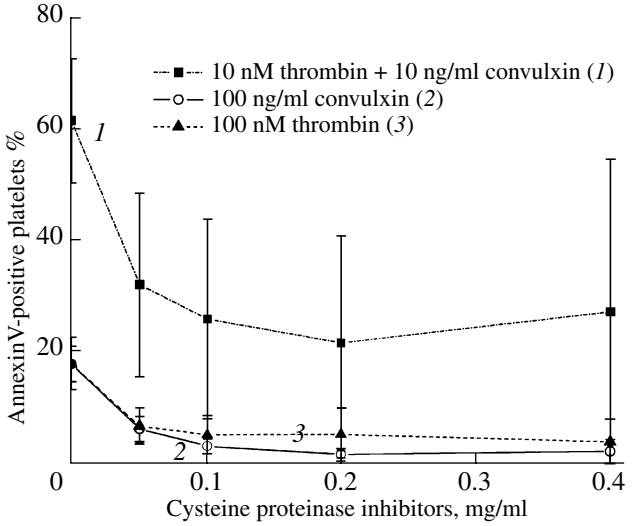

Fig. 2. Influence of CPIs on the formation of coated platelets. Platelets at 20.000/μl were stimulated with 10 nM thrombin with 10ng/ml convulxin (*1*); 100 ng/ml convulxin (*2*), or 100 nM thrombin (*3*). Mean values SEM for $n = 4$ experiments with platelets from four donors are shown. $P < 0.05$ for all data.

Influence of CPIs on plasma coagulation system. Factor XIIIa plays an important role in plasma coagulation by stabilizing clot with crosslinks between fibers of polymerized fibrin. In this case, the presence of specific inhibitors of factor XIIIa activity should not affect thrombin formation during coagulation. Thus, in order to characterize the CPIs specificity, effects of these inhibitors on coagulation in plasma was studied.

We used thrombin generation assay (endogenous thrombin potential (ETP)) for description of an integral state of the system. The kinetics of thrombin generation and measurements of its total amount formed in plasma sample in response to standard activation was evaluated using this test. Thrombin generation was measured in the presence and in the absence of CPIs in PRP and PPP. It was found that the presence of CPIs at a concentration of 0.15 mg/ml decreased thrombin generation by about 40% in both types of plasma. At the same time, the maximal thrombin concentration (A_{max}) decreased, while the time (t_{max}) taken to reach A_{max} as well as lag-period (t_{lag}) increased (see Table). Thus, all measured parameters during thrombin generation test testify to inhibition of coagulation process by CPIs. As the influence of inhibitors on thrombin potential was almost similar in plasma containing various quantities of platelets, it is possible to suppose that inhibition of coagulation was related to their direct influence on the enzymes of plasma coagulation.

In order to understand possible mechanisms of the CPIs effect on plasma components of coagulation, we tested the influence of CPIs on thrombin and factor Xa activity in the purified buffer system with specific chromogenic substrates. The results are shown in Fig. 4. In both cases, CPIs dose-dependently inhibited activity of these enzymes. Inhibition of thrombin was 10% of the control at the maximal CPIs concentration (0.4 mg/ml) and was not statistically significant. The inhibition of factor Xa at the same CPIs concentration reached approximately 30% and was statistically significant (Fig. 4). As the activated factor Xa is an important element of the prothrombinase complex, the decrease of its activity and inhibition of thrombin production might cause the thrombin generation decrease in the presence of CPIs.

The significant effect of CPIs on the thrombin generation suggests that this inhibitor may affect not only factor Xa and thrombin but also other coagulation factors in plasma, for instance, extrinsic tenase, which is the complex of factor VIIa and tissue factor. These options require additional studies.

Thus, we have shown that preparations of CPIs extracted from potato (a) decrease platelet factor XIIIa

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Образец	ЭТП, нМ × мин	t_{max}, мин	A_{max}, нМ	$t_{5нМ}$, мин	Снижение ЭТП, %
БТП	2027.6	7.93	71.88	0.39	–
БТП + ИЦП (0.154 мг/мл)	1165.7	8.88	34.33	0.78	42.5
ОТП	2355.3	6.97	106.05	0.33	–
ОТП + ИЦП (0.150 мг/мл)	1437.2	10.75	51.07	0.78	39.0

Примечание. БТП – бедная тромбоцитами плазма, ОТП – обогащенная тромбоцитами плазма, ЭТП – эндогенный тромбиновый потенциал.

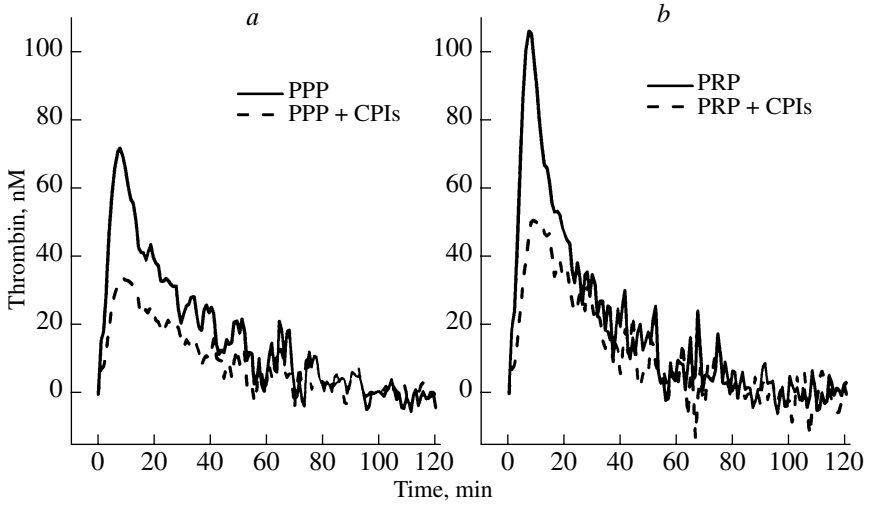

Fig. 3. CPIs effect to the endogenous thrombin potential in platelet-rich and platelet-poor plasma. Represented curves from one of experiments.

activity and (b) decrease the number of coated platelets. The obtained data can be of practical importance, as there are only a few known of inhibitors factor XIIIa by now and hardly any that could be used in clinical practice. At the same time, activity of factor XIIIa can be increased in pathology. Such processes are observed after surgical procedures, during thromboembolic syndrome, atherosclerosis, diabetes, and after injections of adrenalin or glucocorticoids [20, 21]. The higher activity of factor XIIIa might be considered as the risk factor for thrombosis. In these cases the activity of this factor can be decreased using the proper inhibitor. However, inhibitors used in this study are still not well purified and their selectiveness and toxicity have not been determined. Thus, the following step should include obtaining of purified preparations and studies of their effects on various components of coagulation.

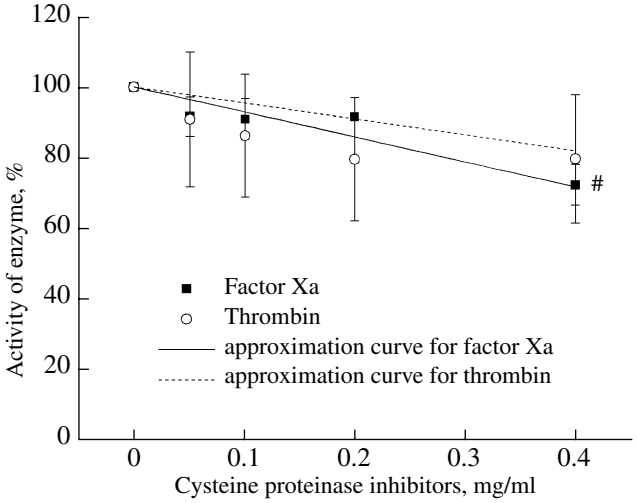

Fig. 4. Thrombin and factor XIIIa activity reduction in the presence of various concentrations of CPIs. Reactions in buffer systems with specific chromogenic substrates. Average data from three independed experiments. Data for factor Xa significant at $P < 0.05$.

ACKNOWLEDGMENTS

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