Ca²⁺-DEPENDENT REGULATION OF FIBRINOLYTIC SYSTEM ACTIVATION ON FIBRIN(OGEN) D-DOMAINS

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In the present study, we investigated whether calcium content modulation in D-domains of fibrin(ogen) was involved in fibrinolytic process activation. To investigate the effect of Ca²⁺-dependent changes in D-domains two types of fibrinogen fragments D and cross-linked fibrin fragments DD were obtained from plasmin hydrolysate of human fibrin(ogen): chelator-treated and without chelating agents. The study of plasminogen activation by tissue-type plasminogen activator on D- and DD-fragments had shown the intensification of plasmin formation in case of EDTA pretreatment of fragments. The proenzyme activation rate on DD also increased in the presence of EGTA in concentration-dependent manner. Potentiating effect of EGTA-pretreated DD-fragment on plasminogen activation by tPA was decreased in the presence of Ca²⁺. Activation rate reduction was observed according to the increase of CaCl₂ concentration in the reaction medium. The intensification of plasminogen activation potentiation by chelator-treated fibrin(ogen) D-domain containing fragments and subsequent potentiation decrease in the presence of Ca²⁺ indicated the requirement of Ca²⁺-dependent changes in D-domains for plasminogen activation sites exposure and initiation of fibrinolysis.

Key words: Ca²⁺-dependent regulation, fibrinogen, fibrin, plasminogen, tissue-type plasminogen activator, fibrinolysis.

Fibrinogen plays a key role in blood coagulation by forming the polymer fibrin lattice of blood clot in case of blood vessels trauma. Plasminogen activation in response to fibrin formation promotes plasmin generation and clot dissolution. The impairing of clotting/fibrinolytic balance causes a wide range of cardiovascular diseases which cure requires the understanding of mechanisms of clotting and fibrinolytic system interactions.

Fibrinogen is a 340 kDa symmetrical dimeric protein with three pairs of polypeptide chains – (A α , B β , γ)₂ – linked together by disulfide bonds. The fibrinogen molecule contains two distal C-terminal D regions (each consists of independently folded β - and γ -nodules) linked by coiled-coils to central E region, which is formed by amino termini of all six polypeptide chains. Two C-terminal α C-domains link to coiled-coils beyond D regions by α -chain connector [1]. Under polymerization, fibrinogen molecule undergoes structural changes: thrombin cleavage of fibrinopeptides from E-domain leads to formation there of A and B polymerization sites, which inter-

act with complement *a* and *b* polymerization sites (knob-to-hole interaction) in D-domains of two other fibrin molecules providing protofibril formation. Fibrin structure is stabilized when C-terminal parts of A α -chains and two adjacent fibrin molecules D-domains are covalently cross-linked by factor XIIIa.

The fibrinogen molecule has several important binding sites relating to its function. Two sets of high-affinity binding sites for both tissue-type plasminogen activator and plasminogen, which play an important role in the initiation of fibrinolysis, are located on the α C-domain and the peripheral D-domain [2]. These sites are hidden in the intact fibrinogen and exposed in fibrin during polymerization. In D-domain knob-to-hole interactions provide conformational changes and only known A α 148-160 plasminogen binding region and γ 312-324 tPA-binding region become uncovered and bind proenzyme and its activator initiating fibrinolytic system activation and plasmin formation [3].

Fibrinogen has both strong and weak binding sites for calcium ions, which are important for its

structural stability and functions. Calcium binding sites are integral parts of the fibrinogen molecule. There are three high-affinity binding sites, two of which are associated with the D-domain and one located at the E-domain [2]. In the E-domain Ca^{2+} -binding site is not clearly identified [4]. Structural data from X-ray crystallography has identified two calcium-binding sites located in each of the γ ($\gamma 1$ and $\gamma 2$) and β modules ($\beta 1$ and $\beta 2$) of the D region [5, 6]. Other low-affinity binding sites are less well defined. There is evidence that some of these sites may be associated with the sialic acid residues on the carbohydrate chains [7].

Ca^{2+} is important for stabilizing fibrinogen structure and function such as promoting lateral aggregation during fibrin polymerization, protecting against denaturation by heat and pH, and limiting the extent of disulfide bond reduction by reducing agents [1]. There is a conformational change in fibrin associated with fibrinopeptide B cleavage that is calcium-dependent [8, 9]. Covalent cross-linkage by factor XIIIa also needs calcium ions. During fibrin digestion, Ca^{2+} limits the extent of plasmin and trypsin action [2].

Despite the dimensional nearness of Ca^{2+} -binding sites and fibrinolytic system activation sites there is no information whether their function is related and how the sites affect each other. Here we describe the effect of Ca^{2+} -dependent changes in human fibrinogen fragment D and cross-linked fibrin fragments DD on plasminogen activation by tPA.

Materials and Methods

Protein preparation and purification. Plasminogen with an amino-terminal glutamic acid residue (Glu-plasminogen) was prepared from citrate donor plasma by affinity chromatography using the Lysine-sepharose 4B (Sigma, USA) [10].

Plasmin was prepared by activation of Glu-plasminogen with urokinase (HS Medac, Germany), immobilized to BrCN-activated Sepharose 4B. Proenzyme in the amount of 1 mg was incubated with 0.5 ml of urokinase-sepharose gel (1250 IU/ml) during 1 h at 37 °C in 50 mM sodium-phosphate buffer solution (pH 7.4) with 25% glycerol. Plasmin was stored in 0.05 M sodium-phosphate buffer solution (pH 7.4) with 50% glycerol at 20 °C. Activation efficiency was evaluated by plasmin caseinolytic and amidolytic activity and PAGE with SDS in the presence of 2% β -mercaptoethanol.

Fibrinogen was purified from human plasma by fractionation with sodium sulfate [11].

desAB-fibrin was obtained by dissolving of thrombin fibrin clot formed in the presence of 50 mM ϵ -aminocaproic acid (ϵ -ACA) and sodium parahydroxy mercury benzoate (0.35 mg/ml) in 20 mM acetic acid as described elsewhere [12].

Cross-linked fibrin was obtained by thrombin-induced fibrinogen polymerization (2 NIH units thrombin per 1 mg fibrinogen) in the presence of calcium ions (25 mM) at 25 °C during 4 h. Concentration of fibrinogen was 16 mg/ml.

Two types of fibrin fragments DD and fibrinogen fragments D were obtained by different approaches. Fibrin fragments DD were prepared from plasmin digest of human cross-linked fibrin. Digestion was performed in 50 mM tris buffer solution with 150 mM NaCl (pH 7.4) during 16 h at 25 °C, concentration of plasmin was 0.2 CU/ml (caseinolytic units/ml). For the first type fibrin degradation product fragment DD was prepared from plasmin digest inhibited by 1000 KIU (kallikrein-inhibiting units) aprotinin (Merckle GmbH) per 1 ml of reactive solution [13]. Fragment DD was purified by size-exclusive chromatography on Sephadex G-100 (Amersham, Sweden). Fragment DD was eluted by 50 mM tris buffer with 150 mM NaCl (pH 7.4).

For the second type fragment DD the reaction was inhibited by 1000 KIU/ml aprotinin with ϵ -ACA and EDTA (Sigma, USA) in final concentrations 20 mM in accordance to preparation method [14]. Digest dialysis was performed in 100 mM sodium-phosphate buffer solution (pH 6.0) with 10 KIU/ml aprotinin, 20 mM ϵ -ACA and 20 mM EDTA at 4 °C. Fragment DD was purified by ion-exchange chromatography on CM-Sephadex G-50 (Amersham, Sweden). Fragment DD was eluted by 200 mM sodium phosphate buffer solution with 300 mM NaCl (pH 7.6) and then dialyzed in 50 mM tris buffer solution with 150 mM NaCl (pH 7.4) at 4 °C.

Fibrinogen fragment D was prepared from plasmin digest of human fibrinogen. The digestion was performed at 37 °C in 50 mM tris buffer solution with 150 mM NaCl (pH 7.4) during 6 h, fibrinogen concentration was 10 mg/ml, plasmin – 0.2 CU/ml, calcium ion – 5 mM. First type fragment D was obtained by reaction inhibition by 1000 KIU/ml aprotinin with subsequent size-exclusion chromatography on Sephadex G-100. Fragment D was eluted by 50 mM tris buffer solution with 150 mM

NaCl (pH 7.4). Second type fragment D was purified by ion-exchange chromatography on CM-Sephadex G-50 from digest inhibited by 1000 KIU aprotinin/ml with ϵ -ACA and EDTA in final concentrations 20 mM as described elsewhere [14].

All the proteins were tested for homogeneity by 8% SDS-PAGE. D- and DD-fragments, obtained by different methods, have the same molecular weight (Fig. 1).

Calcium chelation. EGTA treatment of D- and DD-fragments was performed by equilibrium dialysis against 25 mM EGTA (Sigma, USA) in 50 mM tris buffer with 150 mM NaCl (pH 7.4) during 18 h. Proteins were then dialyzed in 50 mM tris buffer with 150 mM NaCl (pH 7.4) to dispose of chelation agent in solution.

Amidolytic activity assay. Effect of desAB fibrin, fibrin fragment DD and fibrinogen fragment D on t-PA-mediated plasminogen activation was evaluated by amidolytic activity assay of newly formed plasmin, which cleaves chromogenic substrate S_{2251} (H-D-valyl-L-leucil-L-lysyl-p-nitroanilide, Chromogenix, Sweden). The reaction mixture contained 0.22 μ M Glu-plasminogen, 0.09 nM t-PA (Actylise, Boehringer Ingelheim, Germany), 0.3 mM S_{2251} and 0.22 μ M desAB fibrin or fibrin fragment DD in 50 mM tris buffer solution (pH 7.4) with 150 mM NaCl. The assay was performed in 96-wells plate at 37 °C. The amidolytic activity was determined by the absorbance measurement at 405 nm using Titertek Multiscan MC 96-well plate reader.

Plasmin amount calculation. The reaction rate was calculated as gain of reaction medium absorbance for a minute on linear curve section by formula:

$$v = \frac{\Delta A_{405}}{\Delta t}.$$

The amount of plasmin formed during tPA-mediated Glu-plasminogen activation was calculated by the reaction rate in accordance to the rate of chromogenic substrate cleavage by purified plasmin (1 nM Pm $\rightarrow$ 0.0036 o.u./min).

Statistics. Kinetic curves are typical of a series of experiments ($n \geq 3$). Plasmin amount is expressed as mean $\pm$ SEM. ANOVA Dunnett test was used for P-value assessment. $P < 0.05$ was considered as a level of significance.

Fig. 1. SDS-PAGE (8%) analysis of fibrinogen and fibrin fragments obtained by different methods. 1 – Type I fibrin fragment DD (fibrin digestion was inhibited by aprotinin). 2 – Type II fibrin fragment DD (fibrin digestion was inhibited by aprotinin in the presence of EDTA and ϵ -ACA). 3 – Type I fibrinogen fragment D (fibrinogen digestion was inhibited by aprotinin). 4 – Type II fibrinogen fragment D (fibrinogen digestion was inhibited by aprotinin in the presence of EDTA and ϵ -ACA)

Results and Discussion

The previously published works contain opposed findings about fibrin fragment DD ability to stimulate plasminogen activation [15, 16]. Also there is similar controversies related to fibrinogen fragment D [14, 15].

To study out whether different fibrinogen/fibrin fragments purification approach affects ability of carboxy-terminal D-domain-containing fibrinogen and fibrin fragments to interact with plasminogen and tissue-type activator we tested Glu-plasminogen activation by tPA on fibrinogen fragments D and fibrin fragments DD, obtained by two methods. Type I fragments were purified from plasmin hydrolysate of fibrinogen or fibrin, inhibited by aprotinin, whereas during type II fragments isolation plasmin was inhibited by aprotinin in the presence of ϵ -ACA and EDTA. Type I fibrinogen fragment D has no stimulation ability – activation kinetics is similar to control without stimulators (Fig. 2). Both of type II fibrinogen and fibrin fragments have four-fold higher level of tPA-mediated plasminogen activation than their type I analogs. EDTA and ϵ -ACA using in plasmin inhibition during D-domain-containing fragments isolation results in acceleration of stimulation ability of these fragments.

Fig. 2. Glu-plasminogen activation by tPA on fibrinogen and fibrin fragments. D-domain-containing fragments were prepared by two different methods: using only aprotinin (fragment type I) or aprotinin in the presence of EDTA and ϵ -ACA (fragment type II) for inhibition of plasmin digestion of fibrinogen/fibrin. Activation was evaluated as optical density gain through S_{2251} chromogenic substrate cleavage by newly formed plasmin. **A.** Kinetic curves demonstrate activation on fibrin fragment DD type II (1), fibrinogen fragment D type II (2), fibrin fragment DD type I (3), fibrinogen fragment D type I (4), and control (5). Activation was evaluated as amidolytic activity of newly formed plasmin against chromogenic substrate S_{2251} . Plasminogen activation by tPA without stimulators was a control. **B.** Amount of plasmin generated during the activation stimulated by types I and II of D- and DD-fragments, * $P < 0.05$, $n \geq 3$

We suggest that the presence of chelation agent in inhibition medium leads to plasminogen activation sites exposition due to Ca^{2+} removing. To confirm the suggestion tPA-mediated Glu-plasminogen activation on fragments D and DD (both type I) was carried out in the presence of more calcium specific chelator – ethylene glycol tetraacetic acid (EGTA) – since previous studies provided the data about EDTA ability to bind with fibrinogen [17]. EGTA was added to activation medium as a component of working buffer solution (50 mM tris buffer solution (pH 7.4) with 150 mM NaCl) in concentration 0–20 mM and preincubated with the fragments during 60 min before other components addition. Control probe contained Glu-plasminogen and tPA in the presence of maximal EGTA concentration without fibrinogen/fibrin fragments and did not show any plasmin activity during the assay time. DD- and D-fragments ability to stimulate plasmin formation increased in dose-dependent manner in the presence of EGTA. Addition of 20 mM EGTA results in four-fold increase of the activation level compared to probes without chelator (Fig. 3, A, B). It affirms the chelation-induced plasminogen activation sites exposition. Maximal effect of EGTA on the activation rate was observed at 10 mM for fibrin fragment DD and at 20 mM for fibrinogen fragment D (Fig. 3, C). Pre-

sumably, conformational changes in D domains under cross-linked fibrin formation cause more removability of bound Ca^{2+} , and fibrin fragment DD reaches the maximal stimulation effect at a lower concentration, than fibrinogen fragment D.

Since the effect of chelators is likely related with D-domains Ca^{2+} -binding sites occupation, we have tested the reversibility of Ca^{2+} removing action. The fibrin fragment DD from previous experiment was treated by 25 mM EGTA using equilibrium dialysis. Investigation of calcium effect on the plasminogen activation stimulation ability of this fragment was carried out by 60 min preincubation of various CaCl_2 concentrations with EGTA-treated DD-fragment and subsequent plasminogen activation assay as described above. It was demonstrated that tPA-mediated plasmin formation is reduced by Ca^{2+} in a dose-dependent way (Fig. 4, A). The rate of activation in the presence of 10 mM CaCl_2 on EGTA-treated fragment DD has no significant difference as compared to initial non-treated fragment DD (Fig. 4, B). The 10 mM CaCl_2 presence in the reaction medium with Glu-plasminogen and tPA without stimulators did not accelerate the activation.

To examine whether Ca^{2+} specifically affects the plasminogen activation by tPA on EGTA-treated fibrin fragment DD we have conducted the reaction

Fig. 3. Effect of EGTA on Glu-plasminogen activation by tPA. The activation kinetic on fibrin fragment DD (A) and fibrinogen fragment D (B) in the presence of EGTA (0–20 mM) assessed by chromogenic substrate assay. tPA-mediated Glu-plasminogen activation without stimulators in the presence of 20 mM EGTA was a control. C – Plasmin amount generated during the activation stimulated by fibrinogen fragment D and fibrin fragment DD depending on EGTA concentration

in the presence of other two-valent cations (Fig. 5). It was demonstrated that only CaCl_2 lowers the activation level, while MnCl_2 and MgCl_2 do not influence the process.

A decrease of Ca^{2+} effect on plasmin formation was observed in desAB fibrin-stimulated plasminogen activation as well (Fig. 6). In contrast to fibrin fragment DD, 0–0.25 mM CaCl_2 does not affect the activation process on desAB fibrin, but concentration near 0.5 mM is effective enough to lower the plasminogen conversion level by 30%. Possible reason of such differences is involvement of other plasminogen-binding sites in the activation process; the sites are located in αC domains of fibrin molecule [18] besides those located in D-domains and affected by calcium ions.

The results have clearly demonstrated that the exposure of plasminogen and tissue-type plasminogen activator binding sites in fibrinogen/fibrin D-domains are related to Ca^{2+} interaction with these domains. According to X-ray crystal structures of plasma fragment D, plasma cross-linked fragment DD, and recombinant fragment D four possible calcium sites in the D-domain of fibrinogen are identified: two of them – one high-affinity and one low-affinity – are located in γ -module and other two with lower affinity – in β -module [6, 19, 20]. The $\gamma 1$ site is a high affinity calcium-binding site adjacent to hole “a” (Fig. 7). It includes residues γAsp 318 and γAsp 320 and backbone carbonyls of γPhe 322 and γGly 324, whereas the presumably lower affinity $\gamma 2$ calcium-binding site may exist only in the crystal. The $\gamma 1$ site is involved in the “A-a” bond formation [21] and located near γ 312–324 tPA-binding sequence. The $\beta 1$ calcium-binding site is similar to the $\gamma 1$ site, includes the side chains of residues βAsp 381 and βAsp 383 and the backbone carbonyl oxygen of βTrp 385 and maintains the module structure. The $\beta 2$ site is located near the “b” hole, composed of the side chains of residues βGlu 397, βAsp 398, βAsp 261, and γGlu 132 and the carbonyl oxygen of βGly 263 [19]. The $\beta 2$ site links the β module to the coiled-coil connector containing A α 148–160 sequence [22]. Since only two of calcium-binding sites of D-domain, $\gamma 1$ and $\beta 2$, are involved in interaction with fibrinolytic system components binding sites we propose that the chelating agents effect on plasminogen activation be related to calcium occupation of these sites.

Everse et al. have demonstrated a significant rearrangement of the amino acid side chains involved in calcium ion binding during the interaction with synthetic peptide GHRPam, which mimics the fibrin polymerization “B”-site. In the absence of the peptide, the side chains of residues βGlu 397 and βAsp

Fig. 4. Glu-plasminogen activation by tPA on EGTA-pretreated fibrin fragment DD in the presence of Ca^{2+} . **A** – Effect of CaCl_2 (0–10 mM) on the activation kinetics. Plasminogen activation by tPA on fibrin fragment DD without EGTA pretreatment was a control. **B** – Level of plasmin generation effected by CaCl_2 . Control was the plasmin amount formed on fibrin fragment DD non-treated by chelator, * $P < 0.05$, $n \geq 3$

398 are involved in formation of calcium ion bridge with γ -chain and directed to coiled-coil. Formation of the polymerization “b”-site deforms the calcium-bridge between the coiled coil and the β -chain carboxyl domain, βGlu 397 and βAsp 398 flip 180° to form binding pocket for the GHRPam and the $\beta 2$ -site loses its calcium ion [24]. Disruption of the $\beta 2$ -site neighboring the “b” polymerization site positively influences fibrin polymerization *in vitro* [23]. Yakovlev et al. have shown that the coiled-coil region $\text{A}\alpha$ 148–160 is opened in the DDE complex but not in fragments D and cross-linked DD [15]. It was suggested that under polymerization the β -module moves away from coiled-coil region of fibrin molecule to make plasminogen-binding site accessible.

Fig. 5. Effect of two-valent cations on Glu-plasminogen activation by tPA on EGTA-pretreated fibrin fragment DD. Level of plasminogen activation evaluated as newly formed plasmin amidolytic activity at 90 min of reaction. Concentration of CaCl_2 , MgCl_2 , MnCl_2 was 10 mM, * $P < 0.05$, $n \geq 3$

It was proposed that calcium removing from $\beta 2$ site and loss of calcium $\rightarrow$ coiled-coil anchor by β -module is a necessary stage of “B”-“b” interaction in polymerization process [23].

On the other hand, tPA-binding amino acid sequence γ 312–324 is exposed in D-domain, but does not bind the activator prior to “A”-“a” interaction [15, 19]. “A”-“a” polymerization bond formation is accompanied by rearrangement of calcium-binding site $\gamma 1$, but does not lead to its disruption. Presumably, calcium removing from this site is a reason of such rearrangement and results in acquiring necessary conformation by γ 312–324 for tPA binding.

Our results support the both above-mentioned hypotheses: calcium ion chelation by EDTA or EGTA from fibrinogen fragment D or fibrin frag-

Fig. 6. Level of tPA-mediated Glu-plasminogen activation on desAB fibrin at different Ca^{2+} concentration in the reaction medium. Activation was estimated by amidolytic activity assay on the 40th min of the reaction

Fig. 7. Localization of calcium-binding sites in fibrinogen/fibrin D domain. Calcium ions in $\beta 1$, $\beta 2$ and $\gamma 1$ sites depicted as spheres. β module binds to coiled coil by calcium anchor and masks A α 148-160 region (black segment of coil), which contains plasminogen-binding sequence. tPA-binding site γ 312-324 is marked by black. The picture was drawn with the program PyMol using PDB ID 1FZE

ment DD leads to intensification of plasminogen activation by tPA. Thus, calcium removing from D-domains results in conformational changes related to plasminogen-binding sites exposition and fibrinolytic system activation initiation.

Different approach in fibrinogen/fibrin fragment preparation is a probable reason of controversies in a number of works providing the data about D-domain-containing fibrinogen/fibrin fragments abilities to interact with fibrinolytic system components. Some researchers have shown high plasminogen activation stimulation ability of fibrin cross-linked fragment DD [4, 14, 16, 25], whereas in other publications [15, 26] plasminogen interaction with DD-fragment was not confirmed. The present work is a clear explanation of the data differences – we have shown that the usage of calcium-binding agents (EDTA, sodium citrate etc.) in the fibrinogen/fibrin fragments preparation procedure on plasmin inhibition and fragments purification stages provides the plasminogen activation potentiation ability of the fragments.

Calcium removing from D-domains is a reversible process. The invert saturation of calcium-binding sites of EGTA-treated fibrin fragment DD is reached at 10 mM concentration of CaCl_2 corresponding to

plasminogen activation data (Fig. 4). At this calcium concentration the level of activation stimulated by the chelator-treated fragment is decreased to the initial non-treated fragment level. Previous experiments showed a 2-3-fold increase in the rate of lateral aggregation of the mutant fibrin with impaired $\beta 2$ calcium-binding site due to the “B”-“b” interaction and that polymerization can be returned to normal at 10 mM calcium chloride, presumably by induced occupation of the $\beta 2$ site [23]. When considered with our data it indicates that chelation treatment effect on D-domain-containing fibrinogen and fibrin fragments ability to stimulate plasminogen activation is related to calcium-mediated structure changes in β -module but not to possible influence of chelators on protein structure per se. Furthermore, reverse occupation of $\beta 2$ calcium-binding sites induces backward conformational reorganization of D-domains, which leads to plasminogen-binding sites closing down. This process is calcium-specific (Fig. 5).

In further evidence of the calcium-binding sites role, we have shown that the presence of calcium partly suppresses plasminogen activation by tissue-type activator on non-crosslinked desAB fibrin. Thus, calcium ions can re-associate not only with isolated DD-fragment but with full fibrin molecule

Fig. 8. Model of the fibrinolytic system activation sites exposition in fibrinogen/fibrin D-domains as a result of calcium ions removing from β - and γ -modules. **A** – Intact D-domain contains 4 calcium ions (depicted as black circles) which maintain β - and γ -modules structure. **B** – Chelation of calcium by EGTA (or calcium-binding sites rearrangement during polymerization interactions) leads to conformational changes in D-domain: β -module moves from coiled coil region; γ -module undergoes reorganization. **C** – Amino acid sequences A α 148-160 and γ 312-324 become available to plasminogen and tPA binding after conformational rearrangements in β - and γ -modules. **D** – tPA bound to γ -module activates plasminogen leading to active plasmin formation

as well and affect fibrinolytic system activation. It is a possible way for fibrinolysis regulation and a direction in search for therapeutic agents.

Considering the foregoing results and discussion, we suggest a possible scenario of calcium-dependent events in the D-domains (Fig. 8).

During the chelator agent treatment calcium ions are removed from calcium-binding sites. Calcium ion removing from β 1-site by chelators as well as “B”-“b” interaction leads to destruction of calcium salt bridge between β -module and coiled-coil. As a result, plasminogen-binding region A α

148-160 hidden in intact fibrinogen becomes uncovered. Chelation of calcium from γ 1 causes structure changes in γ -module and γ 312-324 region gets tPA-accessible conformation as in fibrin at DDE-interaction. The exposed sites bind plasminogen and tissue-activator thereby induce plasmin formation.

In summary, we have demonstrated the role of calcium-dependent conformational changes in fibrinolytic system activation on fibrin D-domains and reversibility of these changes. Thus, the calcium content modulation in fibrin may reveal the way to affect the rate of fibrinolytic process.

**Ca²⁺-ЗАЛЕЖНА
РЕГУЛЯЦІЯ АКТИВАЦІЇ
ФІБРИНОЛІТИЧНОЇ СИСТЕМИ
НА D-ДОМЕНАХ ФІБРИН(ОГЕН)У**

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У роботі досліджено роль іонів кальцію D-доменів фібрин(оген)у в активації фібринолітичного процесу. Для встановлення значення Ca²⁺-залежних змін у D-доменах використано два типи D-фрагментів фібриногену та DD-фрагментів фібрину, оброблених кальційхелатуючими агентами та одержаних у присутності іонів кальцію. Дослідження активації плазміногену тканинним активатором на D- і DD-фрагментах показало інтенсифікацію утворення плазміну після обробки фрагментів EDTA. Швидкість активації проензиму на DD-фрагментах також дозозалежно збільшувалась у присутності EGTA. Стимулюючий ефект DD-фрагмента, попередньо обробленого хелаторами, на активацію плазміногену дозозалежно знижувався в присутності Ca²⁺. Зміна інтенсивності стимуляції D-доменвмісними фрагментами фібрин(оген)у залежно від вмісту Ca²⁺ свідчить про необхідність кальційзалежних змін у D-доменах для експонування центрів взаємодії із плазміногеном і тканинним активатором та ініціації процесу фібринолізу.

Ключові слова: Ca²⁺-залежна регуляція, фібриноген, фібрин, плазміноген, тканинний активатор, фібриноліз.

**Ca²⁺-ЗАВИСИМАЯ
РЕГУЛЯЦИЯ АКТИВАЦИИ
ФИБРИНОЛИТИЧЕСКОЙ
СИСТЕМЫ НА D-ДОМЕНАХ
ФИБРИН(ОГЕН)А**

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В работе исследована роль ионов кальция D-доменов фибрин(оген)а в активации фибринолитического процесса. Для установления значения Ca²⁺-зависимых изменений в D-доменах использовано два типа D-фрагментов фибриногена и DD-фрагментов фибрина, обработанных кальцийхелатирующими агентами и полученных в присутствии ионов кальция. Исследование активации плазминогена тканевым активатором на D- и DD-фрагментах показало интенсификацию образования плазмина после обработки фрагментов EDTA. Скорость активации проэнзима на DD-фрагментах также дозозависимо увеличивалась в присутствии EGTA. Стимулирующий эффект DD-фрагмента, предварительно обработанного хелаторами, на активацию плазминогена дозозависимо снижался в присутствии Ca²⁺. Изменение интенсивности стимуляции D-доменсодержащими фрагментами фибрин(оген)а в зависимости от содержания Ca²⁺ свидетельствует о необходимости кальцийзависимых изменений в D-доменах для экспонирования центров взаимодействия с плазминогеном и тканевым активатором и инициации процесса фибринолиза.

Ключевые слова: Ca²⁺-зависимая регуляция, фибриноген, фибрин, плазминоген, тканевой активатор, фибринолиз.

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